# Reversal of P-Glycoprotein-Dependent Resistance to Vinblastine by Newly Synthesized Bisbenzylisoquinoline Alkaloids in Mouse Leukemia P388 Cells

Feng-Peng WANG,[a] Li WANG,[b] Jin-Song YANG,[a] Masaaki NOMURA,[c,d] and Ken-ichi MIYAMOTO*,[c]

[a] *Department of Natural Organic Chemistry, School of Pharmacy, West China University of Medical Sciences; 3–17 Ren Ming Nan Road, Chengdu 610041, China:* [b] *The National Chengdu Center for Safety Evaluation of Traditional Chinese Medicine, Sichuan University; 3–17 Ren Ming Nan Road, Chengdu 610041, China:* [c] *Department of Hospital Pharmacy, School of Medicine, Kanazawa University; 13–1 Takara-machi, Kanazawa 920–8641, Japan: and* [d] *Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences, Hokuriku University; Ho-3 Kanagawa-machi, Kanazawa 920–1165, Japan.* Received May 23, 2005; accepted June 22, 2005

**We examined the ability of partially synthesized new compounds from fangchinoline and tetrandrine to reverse P-glycoprotein (P-gp)-dependent multidrug resistance (MDR) *in vitro* and *in vivo*. All compound enhanced the *in vitro* cyctotoxic effect of vinblastin (VBL) at 0.1 $\mu$M as potent as 10 $\mu$M verapamil against the resistant cell line P388/ADR. The combination effect tended to be strong by substitution of bulky group, resulting 5,14-dibromotetrandrine (compound #9) showed the strongest effect. Compound #9 increased intracellular VBL accumulation in P388/ADR cells, much stronger than verapamil, as well as cytotoxic combined effect. This mechanism seems to inhibit the function of P-gp, but not the expression of P-gp. In combination with VBL, this compound also synergistically prolonged the life-span of P388/ADR-bearing mice. Bisbenzylisoquinoline alkaloids and their derivatives are possible to be good candidates as modifier of MDR in cancer chemotherapy.**

**Key words** P-glycoprotein; inhibition; vinblastine; bisbenzylisoquinoline alkaloid; 5,14-dibromotetrandrine; P388/ADR

Multidrug resistance (MDR) presents a serious problem in cancer chemotherapy, because the tumor cells are resistant to antitumor agents which are commonly used in clinical situations.[1,2] The major mechanism for MDR is attributed to the reduced accumulation of antitumor agents in resistant cells.[3] It has been well known that a particular class of transmembrane glycoprotein (P-glycoprotein; P-gp) functions as an energy-dependent drug-efflux pump.[4] P-gp is present in both refractory and recurrent tumors and can be induced during treatment with potent and widely used antitumor drugs such as *Vinca* alkaloids or anthracyclines.

There are several chemical agents such as calcium channel blocking agents, calmodulin inhibitors and immunosuppressive agents which can reverse MDR *in vivo*. We previously reported that *Rauwolfia* alkaloids and staurospoline derivatives were increased intercellular accumulation of vinblastin (VBL) in MDR cells.[5,6] However, because these agents have strong pharmacological effects, their clinical use has been limited by high concentration requirements.[7] It is necessary to establish proper protocols for cancer chemotherapy in combination with appropriate MDR-reversing agents which have low pharmacological potencies and side effects. A bisbenzylisoquinoline alkaloid, cepharanthine, is using for treatment of leukopenia without severe side effects, and this drug has been reported to increase the antitumor effect of substrate drugs of P-gp in MDR tumor cells.[8,9] On the other hand, *Stephania tetrandra*, containing other bisbenzylisoquinoline alkaloids such as fangchinoline and tetrandrine, has been used as anti-inflammatory and analgesic medicine in China, and it has been recently shown that these alkaloids enhanced the cytotoxicity of anticancer drugs in P-gp-dependent tumor cells.[10—12] In this study, to search for potent MDR-reversing agents from *Stephania tetrandra* alkaloids, we partially synthesized new compounds from fangchinoline and tetrandrine and examined the ability to reverse MDR *in vitro* and *in vivo*.

## MATERIALS AND METHODS

**Materials** Vinblastine (VBL), cepharanthine, verapamil and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). [$H^3$]VBL sulfate (5 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, U.S.A.). The alkaloids, fangchinoline and tetrandrine, were isolated from the roots of *Stephania tetrandra*, and compounds tested were synthesized as follows in West China University of Medical Sciences (Chengdu, China). Refluxing fangchilonine with 1% NaOH ethanolic solution followed by addition of corresponding alkylbromide gave compounds #1 to #4. Compounds #5 and #6 were obtained by reacting fangchinoline with propanoic anhydride and isobutylic anhydride, respectively, in pyridine. Compounds #7 to #9 were synthesized by halogenation of tetrandrine followed by Wiriyachitra and Cava.[13] Compounds were used just after solubilizing in dimethylsulfoxide and used the final concentration of dimethylsulfoxide in the culture medium below 0.25%.

**Cells and Culture** Mouse leukemia P388/S cells and the multidrug resistant subclone P388/ADR cells were used (kind gifts from Dr. Inaba, Cancer Chemotherapy Center, Tokyo). Cells were maintained by weekly passage in the abdominal cavity of $CDF_1$ (C57BL/DBA2) mice. Cells were suspended in RPMI1640 medium with 10% fetal calf serum, 100 $\mu$g/ml kanamycin and 20 $\mu$M 2-mercapthoethanol in a $CO_2$ incubator.

**MTT Cell Viability Assay** The effects of test alkaloids on the growth inhibitory effects of VBL were evaluated by the MTT method after 72 h culture. P388 cells ($2\times10^4$) were seeded in each well of 96-well plate, after 24 h, were added a test alkaloid at 1 h prior to varying concentration of VBL, and were cultured for 72 h. Subsequently, 25 $\mu$l of MTT (2 mg/ml) in phosphate-buffered saline was added to each

* To whom correspondence should be addressed. e-mail: miyaken@pharmacy.m.kanazawa-u.ac.jp 

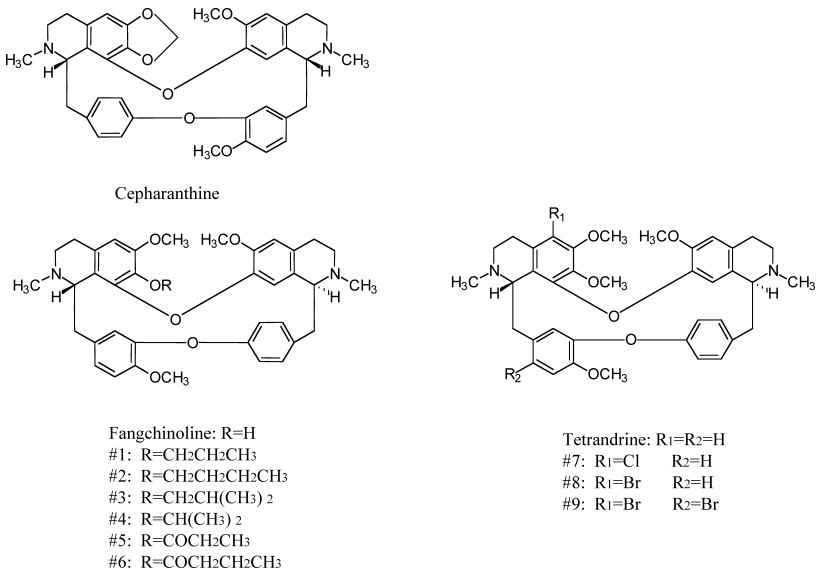


Fig. 1. Structures of Bisbenzylquinoline Alkaloids Used in This Study

well, followed by incubation for 4 h at 37 °C. Formazan crystals formed were dissolved in dimethyl sulfoxide. Absorbance was determined with microplate reader (Multiskan Bichromatic, Labsystems Japan, Tokyo) at 540 nm. Absorbance values were expressed as percentages relative to untreated controls, and the 50% inhibitory concentration of cell growth ($IC_{50}$) was calculated by the least squares method.

**P-gp Immunodetection** P388/ADR cells were cultured for 72 h in the absence or presence of compound #9. The plasma membrane of the cells was prepared by the Percoll sedimentation method.[14] The membrane protein (10 $\mu$g protein) was electrophoresed on 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto nitrocellulose membrane filters (Schleicher & Schuell, Dassel, West Germany). After it was blocked with 5% skim milk, the membrane was incubated overnight with 1 $\mu$g/ml monoclonal antibody against P-gp (C219; Centocor, Inc., Malvern, PA, U.S.A.) and with horseradish peroxidase-conjugated anti-mouse IgG for 1 h. Following each incubation, the membrane was washed extensively with phosphate buffered saline containing 0.1% Tween-20. The immunopositive band was detected by ECL and exposure to a Kodak X-Omat R film (Eastman Kodak Co., Rochester, NY, U.S.A.).

**Drug Accumulation in Cells** Intercellular accumulation of VBL was determined by the following procedures. Cells ($2\times10^6$) suspended in Hanks' solution (pH 7.4) were incubated with various concentrations of compound #9 or verapamil for 10 min, and then incubated in the presence of 2 nM [$H^3$]VBL sulfate for 30 min at 37 °C, and collected by centrifugation. The cells were washed twice with chilled phosphate buffered saline and the radioactivity was determined in scintillation cocktail (ASC II, Amersham Japan, Tokyo) after solubilization.

***In Vivo* Combination Therapy** All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Kanazawa University. Female $CDF_1$ mice (six in a group) were intraperitoneally inoculated with P388/ADR cells ($10^6$), and various doses of compound #9 was intraperitoneally administered with 0.2 mg/kg VBL once a day for day 1 to day 10 after tumor cell inoculation. The survival days of the mice were observed, and the increased life span (ILS) was calculated by subtracting the survival days of the untreated control group from the survival days of the combination treated group.

**Statistical Analysis** All data were expressed as mean$\pm$S.D. Two means were compared by the unpaired Student's *t*-test.

## RESULTS AND DISCUSSION

We examined the ability of *Stephania tetrandra* alkaloids to reverse MDR in P388/ADR cells *in vitro* and *in vivo*. The effects of the alkaloids on the *in vitro* growth-inhibitory activity (50% inhibitory concentration; $IC_{50}$) of VBL against P388 cells are summarized in Table 1. All compounds tested were non-cytotoxic up to 10 $\mu$M. When combined with varying concentrations of VBL, each compound (0.1 $\mu$M) increased the effect of VBL as potent as 10 $\mu$M verapamil against the resistant cell line P388/ADR, although the combination effects of the parent alkaloids were much less than those of synthesized compounds at the same concentration (0.1 $\mu$M). The combination effect tended to be strong by substitution of bulky group, resulting 5,14-dibromotetrandrine (compound #9) showed the strongest effect.

The expression of P-gp in the cell membrane is critical for MDR in P388/ADR cells, and the inhibition of expression of P-gp is an ideal strategy for inhibiting MDR. However, the expression of P-gp in P388/ADR cells was not affected after incubation with compound #9 (1 $\mu$M) for 72 h (Fig. 2).

P-gp-dependent MDR cells are known to actively extrude the substrate drugs and to lower the intracellular concentration. It is also demonstrated that most of MDR-reversing agents inhibit the binding of antitumor drugs to P-gp, resulting lowered efflux and accumulation of antitumor drugs in MDR cells.[15,16] Then, we examined the effect of compound #9 on the intracellular accumulation of VBL in P388/ADR cells and the result was shown in Fig. 3. Compound #9 increased the VBL accumulation in a concentration-dependent manner in MDR cells, but hardly influenced the intracellular

concentration of VBL in sensitive P388 cells (data not shown). The ability of compound #9 on the VBL accumulation was much stronger than that of verapamil, as well as cytotoxic combined effect (Table 1). The potency of antitumor activity of compound #9 may be estimated to be about 100-fold stronger than that of verapamil in concentration.

Finally, the *in vivo* effect of compound #9 was confirmed on P388/ADR-bearing mice. The intraperitoneal administration of VBL (0.2 mg/kg) and compound #9 (10—40 mg/kg) alone had no significant effect on the life-span of P388/ADR-bearing mice (Table 2). When compound #9 was intraperitoneally administered daily at 40 mg/kg with 0.2 mg/kg VBL for 10 d, the life-span of P388/ADR-bearing mice was significantly prolonged up to 25% (Table 2), without any side effects in tumor-bearing mice.

We have searched new agents, which exceed known agents, such as verapamil and cepharanthine, in MDR-reversing activity. Recently, we indicated that 5-bromotetrandrine inhibited P-gp-dependent MDR of several types of tumor, as well as verapamil.[17] Then, in this study, we newly synthesized derivatives of tetrandrine and fangchinoline alkaloids and examined their MDR-reversing activity. In the results, we obtained 5,14-dibromotetrandrine (compound #9), of which *in vitro* MDR-reversing effect was strongest among compounds tested, including 5-bromotetrandrine (compound #8) and verapamil. Compound #9 also synergistically prolonged the life span of the P388/ADR-bearing mice in combination with VBL, but this *in vivo* effect was not so strong. The absorption and distribution may be unsatisfactory due to very high hydrophobicity of this compound. Compound #9 is possible to be a good candidate as modifier of MDR in cancer chemotherapy, although some contrivances in pharmaceutical preparation may be needed.

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Table 1. Effects of Alkaloids on *in Vitro* Sensitivities of P388 Cells to VBL

| Treatment | $IC_{50}$ (nM) | |
|---|---|---|
| | P388/S | P388/ADR |
| VBL alone | 0.78±0.11 (1.0)[a] | 18.12±2.64 (23.2) |
| Combination (μM) | | |
| +Fangchinoline (0.1) | 0.78±0.15 (1.0) | 16.36±1.10* (20.9) |
| +Compound #1 (0.1) | 0.78±0.17 (1.0) | 1.51±0.30** (1.9) |
| + #2 (0.1) | 0.70±0.12 (0.9) | 1.52±0.40** (1.9) |
| + #3 (0.1) | 0.71±0.09 (0.9) | 1.81±0.86* (2.3) |
| + #4 (0.1) | 0.65±0.07 (0.8) | 1.75±0.38** (2.2) |
| + #5 (0.1) | 0.64±0.00 (0.8) | 1.38±0.32** (1.8) |
| + #6 (0.1) | 0.68±0.03 (0.9) | 1.12±0.19** (1.4) |
| +Tetrandrine (0.1) | 0.76±0.11 (1.0) | 12.67±2.92* (16.2) |
| + #7 (0.1) | 0.78±0.17 (1.0) | 1.13±0.32** (1.4) |
| + #8 (0.1) | 0.69±0.13 (0.9) | 2.54±1.56* (3.3) |
| + #9 (0.1) | 0.64±0.13 (0.8) | 0.88±0.28** (1.1) |
| +Cepharanthine (0.1) | 0.85±0.39 (1.1) | 11.54±4.68 (14.8) |
| +Cepharanthine (1.0) | 0.48±0.20 (0.6) | 1.61±0.24** (2.1) |
| +Verapamil (10) | 0.76±0.15 (1.0) | 1.70±0.06** (2.4) |

Cells were added the indicated concentration (in parenthesis) of a compound at 1 h prior to the treatment with varying concentrations of VBL for 72 h. Data are the means $IC_{50}$±S.E. (nM) of at least three experiments done in triplicate. *a*) Number in parenthesis is the relative resistant index *vs.* the $IC_{50}$ value for VBL alone in P388/S cells. *,** Significantly different from the $IC_{50}$ value for VBL alone in P388/ADR cells at $p<0.05$ and 0.01, respectively.

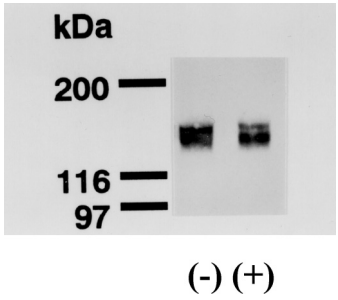


Fig. 2. Expression of P-gp in P388/ADR Cells

Cells were cultured in the absence (−) or presence (+) of 1 μM compound #9 for 72 h.

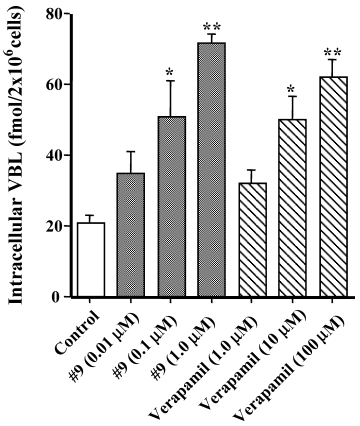


Fig. 3. Effects of Compound #9 on the Intercellular Accumulation of VBL in P388/ADR Cells

Cells were incubated with 2 nM [$H^3$]VBL in the absence (□) or presence of varying concentrations of compound #9 (▩) or verapamil (▨) for 30 min. *, ** Significantly different from the control at $p<0.05$ and 0.01, respectively.

Table 2. Combined Antitumor Effects of Compound #9 with VBL in P388/ADR-Bearing Mice

| Compound | mg/kg | Compound alone | | Combination with VBL (0.2 mg/kg) | |
|---|---|---|---|---|---|
| | | Survival days[a] | % ILS[b] | Survival days | % ILS |
| Control | — | 8.8±0.4 | — | 9.0±0.0 | 2.3 |
| #9 | 10 | 8.0±0.0 | −9.1 | 10.0±1.3 | 13.6 (11.1) |
| | 20 | 8.7±1.8 | −1.1 | 9.5±0.5 | 8.0 (5.3) |
| | 40 | 7.7±1.5 | −12.5 | 11.0±1.3*,† | 25.0 (22.2) |

$CDF_1$ female mice were intraperitoneally inoculated with P388/ADR cells ($10^6$ cells/mouse) on day 0. Compound #9 and VBL were intraperitoneally administrated once a day on days 1—10. *a*) Mean±S.D. ($n=6$). *b*) Percentage increase in life span (ILS) of the experimental group over the control. Number in parentheses; percentage increase in ILS of the combined group over the VBL control group. * Significantly different from the control group at $p<0.01$. † Significantly different from the VBL control group at $p<0.05$.

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