

Inhibition of P-Glycoprotein Mediated Multidrug Resistance by Stemofoline Derivatives

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Resistance to chemotherapy in cancer patients has been correlated to the overexpression of the ATP-binding cassette (ABC) drug transporters including P-glycoprotein (P-gp) that actively efflux chemotherapeutic drugs from cancer cells. We examined the multidrug resistance reversing property of stemofoline derivatives in drug-resistance human cervical carcinoma (KB-V1) and human leukemic (K562/Adr) cell lines that overexpress P-gp. Didehydrostemofoline and eleven of its derivatives were synthesized and the cytotoxicity and their effect on doxorubicin, vinblastine and paclitaxel sensitivity in drug resistant (KB-V1 and K562/Adr) and drug sensitive (KB-3-1 and K562) cell lines by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay were determined. We found that three out of the twelve stemofoline derivatives including OH-A1, NH-B6 and NH-D6 showed commitment efficiency to increase sensitivity to doxorubicin, vinblastine and paclitaxel in KB-V1 cells and increase sensitivity to doxorubicin, and paclitaxel in K562/Adr cells whereas the effects have not been seen in their parental sensitive cancer cell lines (KB-3-1 and K562). These results indicate that stemofoline derivatives reversed P-gp-mediated multidrug resistance *in vitro*, and thus could be developed as effective chemosensitizers to treat multidrug-resistant cancers. The molecular mechanism of modulation of P-gp would be further determined.

Key words ATP-binding cassette transporter; chemosensitizer; multidrug resistance; P-glycoprotein; stemofoline derivative

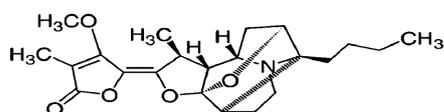
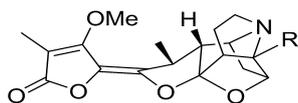
The development and strategic use of anticancer drugs has become one of the most important ways of controlling malignant disease. However, the emergence of drug resistance has made many of the currently available chemotherapeutic agents ineffective. Drug resistance is a major impediment to the treatment of cancer patients receiving single or multiple drugs. Efforts to reverse the drug resistance of tumor cells have been largely unsuccessful.¹⁾ In recent years, considerable research has been directed toward understanding the underlying mechanisms that confer drug resistance. Many studies using tumor cell lines as model systems have demonstrated that exposure of cells to one drug often results in cross-resistance to many other structurally, chemically, and functionally distinct agents. This phenomenon is broadly known as the multidrug resistant (MDR) phenotype.^{2–5)} The mechanism of MDR now has been shown that some of the ATP-binding cassette (ABC) transporter proteins especially ABCB1, or as it is more commonly referred to in the literature as P-glycoprotein (P-gp), which is normally expressed in tumors derived from epithelial tissues, including cancers of the kidney, liver, colon and brain, has been associated with intrinsic drug resistance of these cancers.⁶⁾ Some other tumors (for example breast, ovarian and small cell lung cancers) exhibit generally low levels of P-gp expression at diagnosis. However, the P-gp expression can be induced during the course of treatment, causing the cancer to become resistant to anticancer drugs.⁶⁾ P-gp has been proven to be responsible for resistance to a variety of structurally and functionally unrelated antitumor drugs, including, vinblastine,

vincristine, doxorubicin, daunorubicin, etoposide, teniposide and paclitaxel.^{4,7,8)} At present, due in part to the disappointing results associated with the many side effects of P-gp modulators that have been used in clinical trials, current research efforts are directed towards the identification of novel compounds with attention to dietary natural products or dietary herbs such as curcumin,^{9–11)} stemofoline¹²⁾ and kuguacin J.¹³⁾ The advantage is that these dietary herbs might exhibit little or virtually no side effect and further, do not increase the patient's medication burden. The investigation of natural product compounds to modulate the function of this transporter will be useful for treating cancer patients in combination with the conventional chemotherapy.

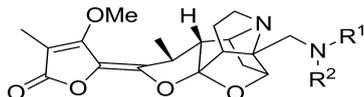
Stemona (non-tai-yak) has been used as an ingredient in Thai folk medicines. Recent study demonstrated that *Stemona collinsiae* root extract exerted anticancer effect against cell proliferation in cancer cell lines, including the human hepatocellular carcinomas cell line (HepG2) and the human breast cancer cell line (MCF-7)¹⁴⁾ and antiviral property in human herpes virus.¹⁵⁾ In our previous study, Stemona alkaloids including stemofoline from *Stemona burkillii*, stemocurtisine and oxystemocurrine from *Stemona aphylla* have been isolated and evaluated for synergistic growth inhibitory effect with cancer chemotherapeutic agents.¹⁶⁾ We found that stemofoline had the ability to reverse the MDR phenotype, increased the intracellular accumulation of P-gp fluorescent substrates, decreased the [³H]-vinblastine efflux in multidrug-resistant human cervical carcinoma KB-V1 cells and increased their sensitivity to vinblastine, paclitaxel and doxorubicin.^{12,16)} In this study, twelve stemofoline derivatives were prepared

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(a) Stemofoline**(b) Stemofoline derivatives**

- R = (1E)-butenyl** **OH-A1**
R = (1E,3E)-5-hydroxy-1,3-pentadien-1-yl **OH-C3**
R = (1R)-1-hydroxy-3-butenyl **OH-C4**
R = hydroxymethyl **OH-C5**
R = (1E)-3-hydroxy-1-propen-1-yl **OH-E3**
R = (1S,2S)-dihydroxybutyl **OH-E4**



- R¹, R² = morpholinyl** **NH-A3**
R¹ = 2S-phenylethyl; R² = H **NH-B6**
R¹ = N,N-dimethylaminoethyl; R² = H **NH-C1**
R¹, R² = 4-ethoxycarbonylpiperazyl **NH-C3**
R¹ = c-C₃H₅; R² = CO₂Et **NH-D6**

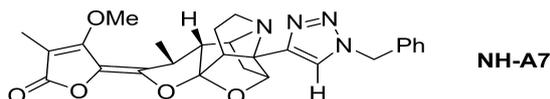


Fig. 1. Structure of Stemofoline (a) and Its Derivatives (b)

from didehydrostemofoline according to the literature^{17,18}) and investigated for their MDR phenotype reversing properties in human MDR cell lines, KB-V1 cells and leukemic K562/Adr cells. Our results revealed that three of the 12 stemofoline derivatives including, OH-A1, NH-B6 and NH-D6 increased the intracellular accumulation and cytotoxicity of chemotherapeutic drugs in drug-resistant human cervical carcinoma and leukemic cell lines *in vitro*.

Experimental

Chemicals Doxorubicin (Dox), verapamil (Ver), vinblastine (Vin), paclitaxel (PTX), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute 1640 medium (RPMI1640) were purchased from Gibco BRL (Grand Island, NY, U.S.A.).

The twelve stemofoline derivatives comprised didehydrostemofoline (OH-A1), five alcohol derivatives (OH-C3, OH-C4, OH-C5, OH-E3 and OH-E4) and six of amine derivatives (NH-A3, NH-A7, NH-B6, NH-C1, NH-C3 and NH-D6). These derivatives were prepared from didehydrostemofoline according to the literature.^{17,18})

Cells and Cell Culture A MDR cervical carcinoma cell line (KB-V1) and a drug-sensitive cervical carcinoma cell line (KB-3-1) were generous gifts from Dr. Michael M. Gottesman (National Cancer Institute, Bethesda, MD, U.S.A.). Both cell

lines were cultured in DMEM with 4.5 g of glucose/L plus 10% fetal bovine serum (FBS), 5 mM L-glutamine, 50 IU/mL penicillin and 50 mg/mL streptomycin; 1 μ M of Vin was added only to the KB-V1 culture medium.

A MDR leukemic cell line (K562/Adr) was purchased from RIKEN Cell Bank (Tsukuba, Ibaraki, Japan). A drug-sensitive leukemic cell line (K562) was purchased from The American type Culture Collection (ATCC, Manassas, VA, U.S.A.). Both cell lines were cultured in RPMI1640 with 10% fetal bovine serum (FBS), 5 mM L-glutamine, 50 IU/mL penicillin and 50 g/mL streptomycin; 700 nM of Dox was added only to the K562/Adr culture medium.

These cell lines were maintained in a humidified incubator with an atmosphere comprising 95% air and 5% CO₂ at 37°C. When the cells reached 70–80% confluence, they were harvested and plated either for subsequent passages or for drug treatments.

Cytotoxicity Assay KB-V1 and KB-3-1 cells were plated at 1.0×10^3 cells per well in 96-well plates. Twenty four hours after plating (these cell lines are adherent cell that require time period for culture-plate surface adhesion), the cells were incubated with stemofoline derivatives (2.5, 5, 10, 20, 40 μ M) for 48 h at 37°C.

K562 and K562/Adr cells were plated at 9.0×10^3 cells per well in 96-well plates. Two hours after plating (these cell lines are non-adherent cell which not require time period for the adhesion), increasing concentrations of stemofoline derivatives (5, 10, 20, 40, 50 μ M) were added and the cells were then further incubated for 48 h at 37°C.

Overall cell number/viability was assessed by MTT assay.¹⁹) In each experiment, determinations were carried out in triplicate.

Chemosensitivity Testing For measurement of Dox, Vin and PTX cytotoxicity, KB-V1 and KB-3-1 cells were plated at 1.0×10^3 cells per well in 96-well plates. After 24 h, stemofoline derivatives and various concentrations of Dox or Vin or PTX were added. The cells were incubated for 48 h at 37°C, and then cell growth was assessed by means of an MTT colorimetric assay.²⁰) In each experiment, determinations were carried out in triplicate. Relative resistance was calculated as the ratio of the IC₅₀ value of the KB-V1 cells to the IC₅₀ value of the KB-3-1 cells.

For measurement of Dox and PTX cytotoxicity, K562/Adr and K562 cells were plated at 9.0×10^3 cells per well in 96-well plates. After 2 h, stemofoline derivatives and various concentrations of Dox or PTX were added. The cells were incubated for 48 h at 37°C, and then cell growth was assessed by means of an MTT colorimetric assay.²⁰) In each experiment, determinations were carried out in triplicate. Relative resistance was calculated as the ratio of the IC₅₀ value of the K562/Adr cells to the IC₅₀ value of the K562 cells.

Statistical Analysis The results are presented as means \pm S.D. from triplicate samples of three independent experiments. Differences between the means were analyzed by one-way ANOVA. Statistical significance was considered when $p < 0.05$, or $p < 0.01$, or $p < 0.001$. All statistical analyses were performed using Prism 5.0 software.

Results

Effects of Stemofoline Derivatives on the Cytotoxicity of KB-V1, KB-3-1, K562/Adr and K562 Cells Cytotoxicity

Table 1. Modulation of Resistance to Dox in KB Cells by Stemofoline Derivatives

	IC ₅₀ ^{a)}	Relative resistance ^{b)}
Doxorubicin treatment		
KB-3-1	0.23±0.03 nM	1.00±0.00
KB-V1	3.40±0.36 μM	17±0.00
Stemofoline derivative		
KB-V1+5 μM of OH-A1	0.72±0.26 μM***	4±1.30***
KB-V1+5 μM of OH-C3	2.63±1.88 μM	13±5.90
KB-V1+5 μM of OH-C4	2.80±0.20 μM	14±1.00
KB-V1+5 μM of OH-C5	3.43±0.12 μM	17±0.60
KB-V1+5 μM of OH-E3	3.07±0.46 μM	15±2.30
KB-V1+5 μM of OH-E4	3.47±0.50 μM	17±2.50
KB-V1+5 μM of NH-A3	1.58±0.33 μM**	8±1.70**
KB-V1+5 μM of NH-A7	4.27±0.93 μM	21±4.60
KB-V1+5 μM of NH-B6	0.78±0.19 μM***	4±0.90***
KB-V1+5 μM of NH-C1	3.93±0.31 μM	20±1.50
KB-V1+5 μM of NH-C3	3.97±0.12 μM	20±0.60
KB-V1+5 μM of NH-D6	1.48±0.20 μM***	7±1.00***
KB-V1+5 μM of stemofoline	1.33±0.58 μM***	7±2.90 ***

a) Determined by the MTT assay, and the values are means±S.D. of three independent experiments. b) IC₅₀ of KB-V1/IC₅₀ of KB-3-1. Each point represents the mean (±S.D.) of three independent experiments performed in triplicate. ***p*<0.01 and ****p*<0.001, vs. control treated without stemofoline derivatives.

assays showed that the stemofoline derivatives (treatment with 0–40 μM for 48 h) were not cytotoxic to KB-V1, KB-3-1, K562/adr and K562 cells (data not shown). The compounds which were applied in all subsequent experiments are at the final concentration of 5 μM (>90% cell survival).

Effect of Stemofoline Derivatives on Cytotoxicity of Dox, PTX and Vin in KB-3-1 and KB-V1 Cells To examine the MDR reversing property of stemofoline derivatives on Dox, PTX and Vin cytotoxicity, the growth inhibition of cells was investigated in response to increasing concentrations of Dox, PTX or Vin with or without each stemofoline derivative. The results showed that 5 μM of OH-A1, NH-B6 and NH-D6 dramatically increased sensitivity of KB-V1 cells to Dox, PTX and Vin, 5.0- (*p*<0.001), 4.4- (*p*<0.001) and 2.3-fold (*p*<0.001), respectively for Dox, 3.0- (*p*<0.001), 3.2- (*p*<0.001) and 1.5-fold, respectively for PTX, and 5.8- (*p*<0.001), 4.3- (*p*<0.001) and 3.6-fold (*p*<0.001), respectively for Vin. Besides, NH-A3 treatment also significantly increased sensitivity of KB-V1 cells to Dox (2.1-fold, *p*<0.01) and PTX (2.1-fold, *p*<0.01). While similar treatment of KB-3-1 cells provided no modulating effect (Tables 1–3, Figs. 2a–f).

Effect of Stemofoline Derivatives on Cytotoxicity of Dox and PTX in K562 and K562/Adr Cells To examine the MDR reversing property of stemofoline derivatives on Dox, and PTX cytotoxicity, the growth inhibition of cells was investigated in response to increasing concentrations of Dox or PTX with or without each stemofoline derivatives. The results showed that 5 μM of OH-A1, NH-B6 and NH-D6 significantly (*p*<0.001) increased sensitivity of K562/Adr cells to Dox and PTX, 3.7-, 7.6- and 2.3-fold, respectively for Dox, 5.6-, 19.5- and 3.9-fold, respectively for PTX while similar treatment of K562 cells provided no modulating effect (Tables 4, 5, Figs. 3a–d).

Table 2. Modulation of Resistance to PTX in KB Cells by Stemofoline Derivatives

	IC ₅₀ ^{a)}	Relative resistance ^{b)}
Paclitaxel treatment		
KB-3-1	1.52±0.08 nM	1.00±0.00
KB-V1	7.00±0.10 μM	4773±227
Stemofoline derivative		
KB-V1+5 μM of OH-A1	3.50±0.87 μM***	1591±394***
KB-V1+5 μM of OH-C3	9.75±2.05 μM	4432±930
KB-V1+5 μM of OH-C4	10.33±1.89 μM	4697±860
KB-V1+5 μM of OH-C5	9.17±1.04 μM	4167±473
KB-V1+5 μM of OH-E3	10.67±2.02 μM	4848±919
KB-V1+5 μM of OH-E4	11.17±2.25 μM	5076±1,025
KB-V1+5 μM of NH-A3	5.77±1.50 μM**	2621±684**
KB-V1+5 μM of NH-A7	7.17±1.15 μM	3254±525
KB-V1+5 μM of NH-B6	3.27±1.57 μM***	1485±713***
KB-V1+5 μM of NH-C1	9.83±1.76 μM	4470±798
KB-V1+5 μM of NH-C3	12.00±0.87 μM	5455±394
KB-V1+5 μM of NH-D6	6.80±1.74 μM*	3091±793
KB-V1+5 μM of stemofoline	1.43±0.45 μM***	652±205***

a) Determined by the MTT assay, and the values are means±S.D. of three independent experiments. b) IC₅₀ of KB-V1/IC₅₀ of KB-3-1. Each point represents the mean (±S.D.) of three independent experiments performed in triplicate. ***p*<0.01 and ****p*<0.001, vs. control treated without stemofoline derivatives.

Table 3. Modulation of Resistance to Vin in KB Cells by Stemofoline Derivatives

	IC ₅₀ ^{a)}	Relative resistance ^{b)}
Vinblastine treatment		
KB-3-1	0.61±0.01 μM	1.00±0.00
KB-V1	0.60±0.05 μM	984±0.00
Stemofoline derivative		
KB-V1+5 μM of OH-A1	0.11±0.04 μM ***	187±71.50***
KB-V1+5 μM of OH-C3	0.71±0.05 μM	1164±71.10
KB-V1+5 μM of OH-C4	0.73±0.05 μM	1202±78.60
KB-V1+5 μM of OH-C5	0.68±0.09 μM	1121±151.40
KB-V1+5 μM of OH-E3	0.67±0.08 μM	1093±123.10
KB-V1+5 μM of OH-E4	0.74±0.09 μM	1212±131.30
KB-V1+5 μM of NH-A3	0.61±0.14 μM	993±220.50
KB-V1+5 μM of NH-A7	0.65±0.08 μM	1070±108.50
KB-V1+5 μM of NH-B6	0.15±0.04 μM ***	240±64.80***
KB-V1+5 μM of NH-C1	0.61±0.13 μM	1008±235.40
KB-V1+5 μM of NH-C3	0.74±0.05 μM	1214±106.30
KB-V1+5 μM of NH-D6	0.18±0.05 μM ***	290±81.80***
KB-V1+5 μM of stemofoline	0.09±0.01 μM***	142±21.10***

a) Determined by the MTT assay, and the values are means±S.D. of three independent experiments. b) IC₅₀ of KB-V1/IC₅₀ of KB-3-1. Each point represents the mean (±S.D.) of three independent experiments performed in triplicate. ****p*<0.001, vs. control treated without stemofoline derivatives.

Discussion

Resistance to chemotherapy is a major problem in the management of cancer patients and is caused by various molecular mechanisms. One of these mechanisms is the overexpression of MDR1/P-glycoprotein, which is the major cause of multidrug-resistance (MDR) of human cancers. Potent MDR modulators are being investigated in clinical trials. Verapamil, a calcium channel blocker, and cyclosporin A, an immunosuppressive agent are effective P-gp inhibitors *in vitro*, but they

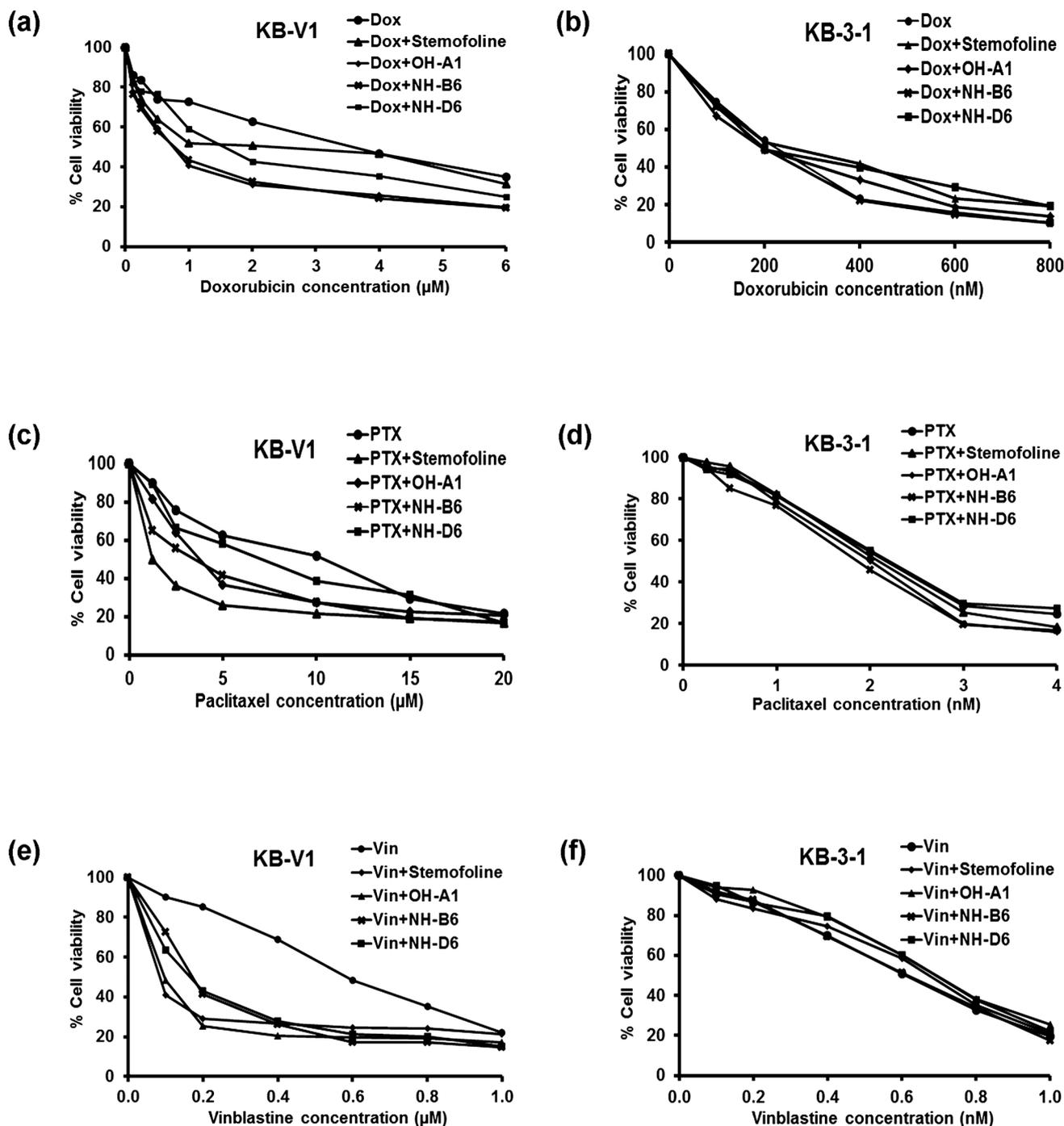


Fig. 2. The Effect of Stemofoline Derivatives (OH-A1, NH-B6 and NH-D6) on Dox, PTX and Vin Cytotoxicity in KB-V1 ((a), (c), (e)) and KB-3-1 ((b), (d), (f)) Cell Lines

Cells were incubated in the presence or absence of stemofoline derivatives (OH-A1, NH-B6 and NH-D6) in combination with Dox or PTX or Vin. The number of viable cells was determined by an MTT assay. The Y-axis shows the percent of cell survival, and the X-axis shows varying concentrations of stemofoline derivatives. Each point represents the mean of three independent experiments performed in triplicate.

have limited clinical use. Many current studies are focused on the use of dietary herbs as alternatives due to the fact that these have been used for centuries without producing any harmful side effects.^{10,11,21-23)}

The present study has determined the MDR reversing property of stemofoline derivatives on the cytotoxicity of Dox, PTX or Vin in KB-V1 and KB-3-1 cell lines. It was found that OH-A1, NH-B6 and NH-D6 markedly increased the sensitivity of KB-V1 cells to Dox, PTX, and Vin, but did not have this effect on KB-3-1 cells (Tables 1-3, Figs. 2a-f). The

similar study in K562/Adr and K562 cell lines also showed that OH-A1, NH-B6 and NH-D6 significantly increased the sensitivity of K562/Adr cells to Dox, and PTX, but did not have the effect on K562 cells (Tables 4, 5, Figs. 3a-d). Our previous report demonstrated that *Stemona* extract did not influence MDR-mediated multidrug resistance protein 1 (MRP-1) but P-gp,²⁴⁾ while PTX is a P-gp specific substrate that differ from Dox and Vin which are the substrates of the MRP-1 as well.^{25,26)} These might be the reason why the reversing property of stemofoline and its derivatives including

Table 4. Modulation of Resistance to Dox in K562 Cells by Stemofoline Derivatives

	IC ₅₀ ^{a)}	Relative resistance ^{b)}
Doxorubicin treatment		
K562	453.33±5.77 nM	1.00±0.00
K562/Adr	17.33±1.15 μM	38.23±2.35
Stemofoline derivative		
K562/Adr+5 μM of OH-A1	4.70±0.26 μM***	10.37±0.64***
K562/Adr+5 μM of NH-B6	2.27±0.49 μM***	5.01±1.14***
K562/Adr+5 μM of NH-D6	7.57±0.40 μM***	16.69±0.95***
K562/Adr+5 μM of stemofoline	4.47±0.55 μM***	9.85±1.22***

a) Determined by the MTT assay, and the values are means±S.D. of three independent experiments. b) IC₅₀ of K562/Adr/IC₅₀ of K562. Each point represents the mean (±S.D.) of three independent experiments performed in triplicate. ***p<0.001, vs. control treated without stemofoline derivatives.

Table 5. Modulation of Resistance to PTX in K562 Cells by Stemofoline Derivatives

	IC ₅₀ ^{a)}	Relative resistance ^{b)}
Paclitaxel treatment		
K562	8.17±0.76 nM	1.00±0.00
K562/Adr	0.78±0.03 μM	96.34±7.44
Stemofoline derivative		
K562/Adr+5 μM of OH-A1	0.14±0.01 μM***	16.90±2.84***
K562/Adr+5 μM of NH-B6	0.04±0.01 μM***	5.30±0.28***
K562/Adr+5 μM of NH-D6	0.20±0.02 μM***	25.17±4.48***
K562/Adr+5 μM of stemofoline	0.10±0.02 μM***	12.30±2.34***

a) Determined by the MTT assay, and the values are means±S.D. of three independent experiments. b) IC₅₀ of K562/Adr/IC₅₀ of K562. Each point represents the mean (±S.D.) of three independent experiments performed in triplicate. ***p<0.001, vs. control treated without stemofoline derivatives.

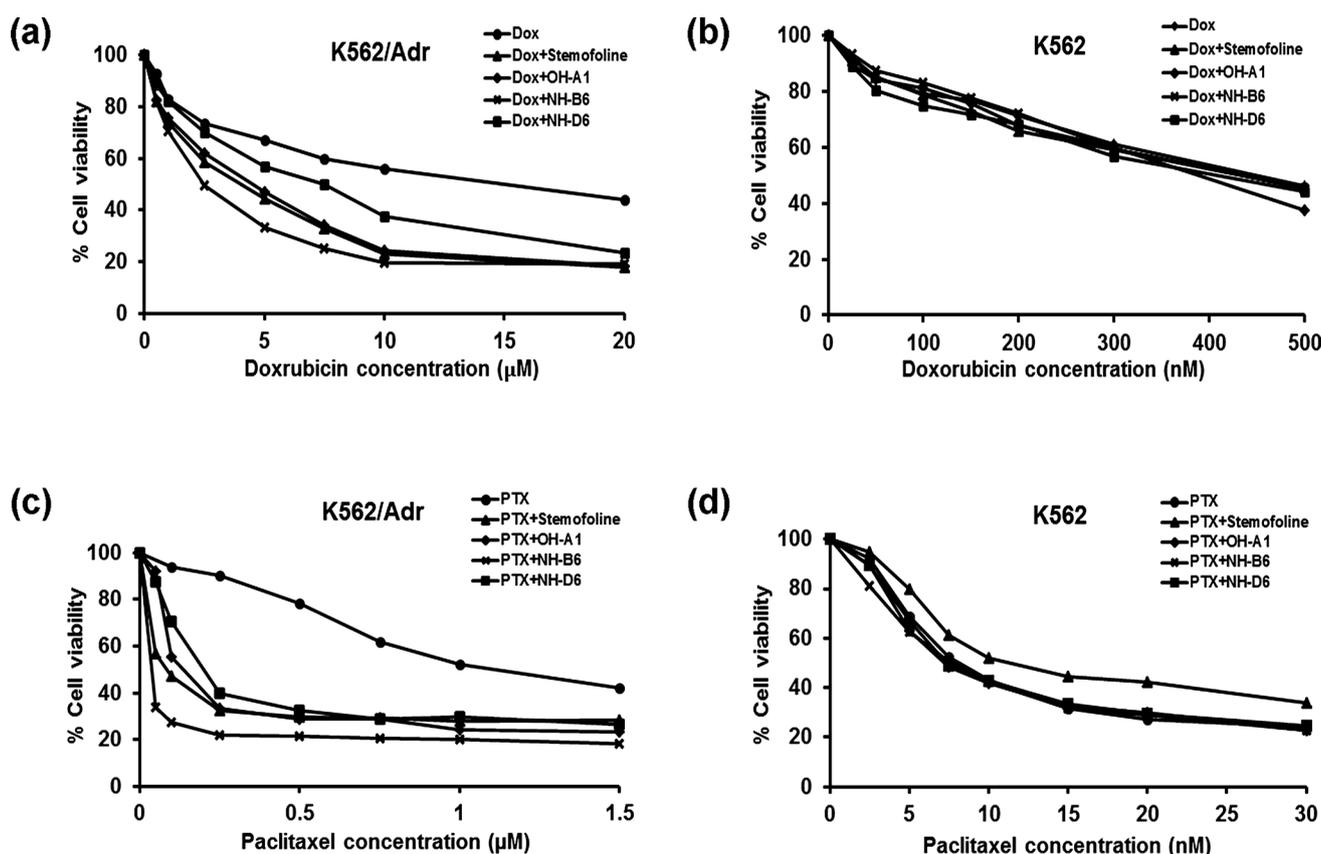


Fig. 3. The Effect of Stemofoline Derivatives (OH-A1, NH-B6 and NH-D6) on Dox and PTX Cytotoxicity in K562/Adr ((a), (c)) and K562 ((b), (d)) Cell Lines

Cells were incubated in the presence or absence of stemofoline derivatives (OH-A1, NH-B6 and NH-D6) in combination with Dox or PTX. The number of viable cells was determined by an MTT assay. The Y-axis shows the percent of cell survival, and the X-axis shows varying concentrations of stemofoline derivatives. Each point represents the mean of three independent experiments performed in triplicate.

OH-A1, NH-B6 and NH-D6 in PTX-treated cells was greater than in Dox-, and Vin-treated cells.

Stemofoline and its derivatives have a common caged structure but different side chain structures. While the number of compounds studied is limited making any structure–activity relationship discussions only tenuous, it is clear that none of the alcohol derivatives tested, specifically compounds OH-C3, OH-C4, OH-C5, OH-E3 and OH-E5, were active. The presence of hydroxyl group (–OH) may reduce the bioactivity. In contrast the primary benzylamino derivative NH-B6 and the

carbamate derivative NH-D6 were active modulators along with didehydrostemofoline (OH-A1), the alkene derivative of stemofoline. In some cases (Tables 1–3) these three derivatives had similar or lower activities than stemofoline. In some cases, especially in the treatment of K562/Adr, NH-B6 showed the most efficacy (Tables 4, 5), while stemofoline were more effective than OH-A1 (Tables 4, 5). The lower activity of OH-A1 compared to stemofoline may be because of the less flexible side chain of OH-A1. The presence of 2S-phenylethyl group in NH-B6 may be significant for enhancement of its

MDR reversing property. In one study, the morpholine derivative NH-A3 (Tables 1, 2) was nearly as effective as stemofoline itself. This latter compound was not very effective in the other studies (Table 3).

Our previous study showed that P-gp function was inhibited, but not its expression in KB-V1 when treating the cells with stemofoline.¹²⁾ The present study provided the reversal of P-gp-mediated MDR by stemofoline derivatives in P-gp over-expressing cancer cell lines, KB-V1 and K562/Adr. The mechanism of MDR reversal by stemofoline derivatives might be via the inhibition of expression and/or function of P-gp. Thus, the modulation of stemofoline derivatives, especially NH-B6, on P-gp function and expression would be further determined to observe their molecular mechanisms.

Conclusion

In conclusion, this study is the first to demonstrate the structure–activity relationships of stemofoline derivatives on MDR reversing property, which could be introduced as candidate molecules for treating cancers exhibiting P-gp-mediated MDR. Animal experiments should be further studied to determine if these compounds have potential as effective chemosensitizers to be used in combination with conventional chemotherapy.

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