SUPPLEMENTARY MATERIAL

Non-alkaloids extract from *Stemona sessilifolia* enhances the activity of chemotherapeutic agents though P-glycoprotein mediated multidrug-resistant cancer cells

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Abstract

One of the major impediments to the successful treatment of cancer is the development of resistant cancer cells, which could cause multidrug resistance (MDR), and overexpression of ABCB1/P-glycoprotein (P-gp) is one of the most common causes of MDR in cancer cells. Recently, natural products or plant-derived chemicals have been investigated more and more widely as potential multidrug resistance (MDR) reversing agents. We current study demonstrated for the first time that non-alkaloids extract from *Stemona sessilifolia* significantly reversed the resistance of chemotherapeutic agents, adriamycin, paclitaxel and vincristine to MCF-7/ADR cells compared with MCF-7/S cells in a dose-dependent manner. The results obtained from these studies indicated that the non-alkaloids extract from *Stemona sessilifolia* plays an important role in reversing MDR of cancer as a P-gp modulator in vitro and may be effective in the treatment of multidrug-resistant cancers.

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Keywords

Stemona sessilifolia, non-alkaloids extract, multidrug resistance, P-gp, MCF-7/ADR cells, cancer

Experimental

Preparation of non-alkaloids extract from S. Sessilifolia

The roots of *S. Sessilifolia* were collected in WanShou Medicinal materials market of Shenyang, Liaoning Province, China, and were authenticated by Professor Jin-cai Lu (School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University), voucher number is SS-201303. The roots of *S. Sessilifolia* (1kg) was powdered and then extracted with 60% ethanol. The ethanol extract was concentrated under vacuum to yield crude extract (146g) , which was suspended in water and then successively extracted with petroleum ether, dichloromethane, respectively. Each fraction was dried under reduced pressure and then submitted for bioassays. From the bioassay-guided fractionation, the dichloromethane extract (48g) was extracted with 2% dilute hydrochloric acid to remove alkaloids, and then non-alkaloids extract (NAE, 25g) was yield (Bournine et al., 2013). The extract was standardised by the determination of little alkaloids components content using Dragendorff reagent (Figure.S1)(Bournine et al., 2013).

Cell lines and culture conditions

Human breast cancer cell lines MCF-7/S and the Corresponding P-gp-overexpressing drug-resistant cell line MCF-7/ADR were donated by professor Zhong-gui He from Shenyang Pharmaceutical University, Shenyang, China. These cells were cultured in RPMI-1640 medium supplemented with 10% FBS at 37℃ in a humidified atmosphere of 5% CO_2. MCF-7/ADR cells were maintained in the medium with 1μg/mL adriamycin, and were cultured in drug-free medium for two weeks before formal experiments.

Cell cytotoxicity and drug sensitivity assay

Cytotoxicity of NAE was estimated in MCF-7/S and MCF-7/ADR cells by tetrazolium-based colorimetric MTT assay (Limtrakul et al., 2005). MCF-7/S (7.5×10^4 cells/mL) and MCF-7/ADR (1×10^5 cells/mL) were seeded in 96-well plates. After over night incubation, the cells were treated with various concentrations of NAE for 24 h. 20μL of MTT solution (5mg/mL) was added to each well, and the plate was further incubated for 4 h, the medium was discarded, and 150μL of dimethylsulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The optical density (OD) of each well was measured using a microtiter plate reader at a wavelength of 490 nm. The concentrations required to inhibit growth by 5% (IC_5) was adopted in the experiments (Xuan et al., 2013).

Drug sensitivity was analysed with the same methods (Limtrakul et al., 2005). The cells were treated with or without various concentrations of NAE combined with different anticancer drugs (adriamycin, paclitaxel and vincristine). The concentrations required to inhibit growth by 50% (IC_{50}) were calculated from survival curves. Resistance index (RI) was calculated using the following formula: RI = IC_{50} (MCF-7/ADR cells) / IC_{50} (MCF-7/S cells)

The reversal fold (RF) values, as potency of reversal, were calculated from fitting the data to RF = IC_{50} of adriamycin alone / IC_{50} of adriamycin incubated with modulators. In all the experiments, verapamil (VP, 10μmol/L) was used as a positive control. Experiments were repeated at least three times.

Accumulation of fluorescent substrates

To measure accumulation of Rhodamine123 (Rh123) (Anucha preeda et al., 2002), MCF-7/S and MCF-7/ADR cells were incubated with 5 mg/mL of Rh123 for 30 min at 37°C in RPMI 1640 without phenol red medium either with or without the reversal agents (NAE and verapamil). The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in 0.1% bovine serum albumin (BSA)–PBS. Samples were analysed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA,USA).

Western bloting

The procedure of Western blot analysis was carried out following the protocol as previously described(Limtrakul et al., 2005) with some modifications. Equal amounts of total cell lysates (50μg of protein) were resolved by 12% sodium dodecyl sulfate polycrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto PVDF membranes. After being incubated in blocking solution (5% skim milk in TBST) to block nonspecific binding for 1h at room temperature, the membranes were immunoblotted overnight with primary antibodies anti-P-gp (1:500 dilution) and anti-β-actin (1:500 dilution) at 4℃. The next day, the membranes were washed three times with TBST buffer (0.3% Tris, 0.8% NaCl, 0.02% KCl, 0.05% Tween20) and then incubated for 3 h with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit IgG for P-gp (1:500 dilution). Protein-antibody complexes were detected by enhanced chemoluminescence detection system (Amersham, NJ). β-actin was used to confirm that the cell lysates were equally loaded in each well.

Statistical analysis

Data were shown in bar graph form, expressed as mean ± standard deviation (SD). of three independent experiments and statistically determined by Student's *t*-test, with $p < 0.01$ considered significant.

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Table Legends

Table S1. Determination of multidrug resistance of MCF-7/ADR cells toward anticancer drugs.

Table S2. Effect of NAE on the cytotoxicity of adriamycin, paclitaxel and vincristine in multidrug resistance (MDR) cancer cells.

Figure captions

Figure S1. Thin-layer chromatographic analysis of non-alkaloids extract (NAE) and total dichloromethane extract by Dragendorff reagent

Figure S2. Effect of NAE on Rh123 accumulation in MCF-7/ADR cells.

Figure S3. The effect of NAE on P-glycoprotein (P-gp) expression.

Table S1. Determination of multidrug resistance of MCF-7/ADR cells toward anticancer drugs

Treatment	IC_{50}±SD[a](μM)		RI[b] (Resistance index)
	MCF-7/ADR	MCF-7/S	
ADR	47.3±0.117	0.849±0.015	55.71
Taxol	5.43±0.053	0.126±0.011	43.09
VCR	12.4±0.122	0.306±0.008	40.52

Cell survival was determined using MTT assay as described in methods. [a]Each value represents the mean ± S.D. of three independent experiments. [b]Resistance index (RI) was calculated using the following formula: RI = IC_{50} (MCF-7/ADR cells) / IC_{50} (MCF-7/S cells)

Table S2. Effect of NAE on the cytotoxicity of adriamycin, paclitaxel and vincristine in multidrug resistance (MDR) cancer cells.

Treatment	MCF-7/ADM		MCF-7/S	
	IC_{50}±SD[a](μM)	RF[b]	IC_{50}±SD[a](μM)	RF[b]
ADR	47.3±0.117	—	0.849±0.015	1.00
ADR + NAE 100μg/mL	16.3±0.090*	2.90	0.850±0.015	1.00
ADR + NAE 150 μg/mL	8.17±0.055*	5.79	0.833±0.012	0.98
ADR + NAE 200 μg/mL	5.51±0.049*	8.58	0.842±0.007	0.99
ADR + VP[c] 10 μM	16.2±0.081*	2.91	0.843±0.007	0.99
Taxel	5.43±0.053	—	0.126±0.011	1.00
Taxel + NAE 100 μg/mL	2.53±0.086*	2.15	0.130±0.015	1.03
Taxel + NAE 150 μg/mL	1.24±0.049*	4.38	0.130±0.010	1.03
Taxel + NAE 200 μg/mL	1.01±0.082*	5.34	0.140±0.006	1.11
Taxel + VP 10 μM	2.20±0.031*	2.45	0.127±0.008	1.01

VCR	12.4±0.122	—	0.306±0.008	1.00
VCR + NAE 100 μg/mL	5.06±0.083*	2.45	0.291±0.008	0.95
VCR + NAE 150 μg/mL	3.14±0.046*	3.95	0.303±0.015	0.99
VCR + NAE 200 μg/mL	2.50 ±0.095*	4.96	0.301±0.009	0.98
VCR + VP 10 μM	4.39±0.075*	2.82	0.307±0.012	1.00

[a]Cell survival was determined by MTT assay. Data are means ± SD of at least three independent experiments performed in triplicate.

[b]The fold reversal (RF) of MDR was calculated by dividing the IC_{50} for cells with the anticancer drug alone by that obtained in the presence of NAE.

[c]verapamil (VP, 10μmol/L) was used as a positive control.

*Values that were significantly different from the control untreated cells ($p< 0.01$).

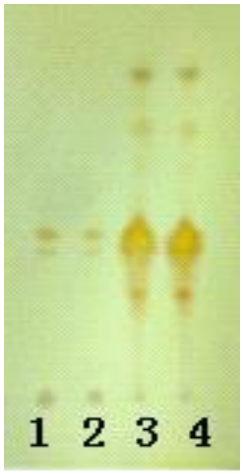

Figure S1. Thin-layer chromatographic analysis of non-alkaloids extract (NAE) and total dichloromethane extract by Dragendorff reagent. TLC was analysed on a Merck silica gel 60 F254 aluminum sheet with a mobile phase system including dichloromethane/methanol/formic acid (15:1:0.3) at ambient temperature. The same amount (1ml

of 10 mg/mL) of samples was loaded on lane 1 ~ 2: non-alkaloids extract and lane 3 ~ 4: total dichloromethane extract.

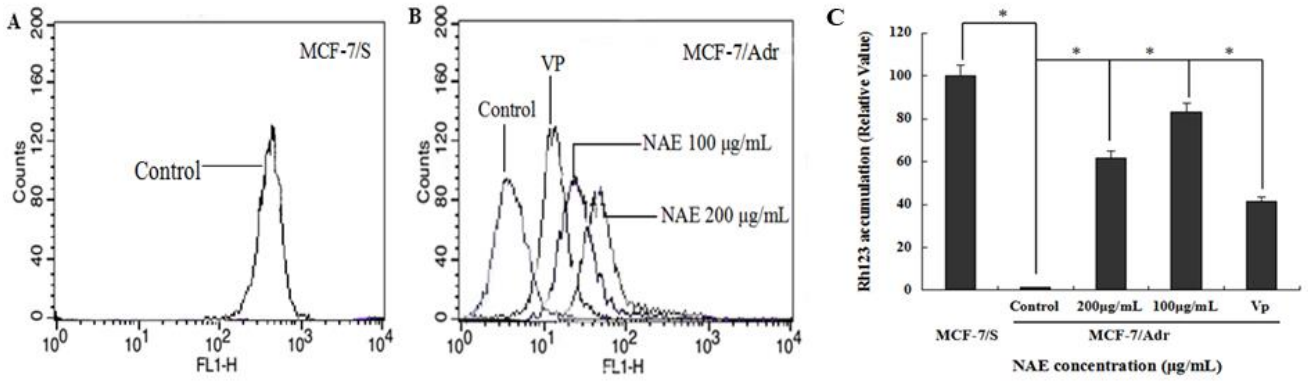

Figure S2. Effect of NAE on Rh123 accumulation in MCF-7/ADR cells. MCF-7/S (A) and MCF-7/ADR (B) cells were treated with 100 μg/mL and 200 μg/mL NAE for 3 h at 37℃, 5 mg/mL Rhodamine 123 (Rho123) was added for another 0.5 h incubation. (C) The relative value of Rho123 accumulation in the presence of various concentrations of NAE and the positive control. The intracellular fluorescence was analysed by flow cytometry. Control cells were treated with DMSO and cells treated with 10μM VP were used as positive control. Columns represent means of triplicate determinations figure legends with bars (S.D.). *$p < 0.01$, MCF-7/S vs MCF-7/Adr control group and MCF-7/Adr control group vs treatment group.

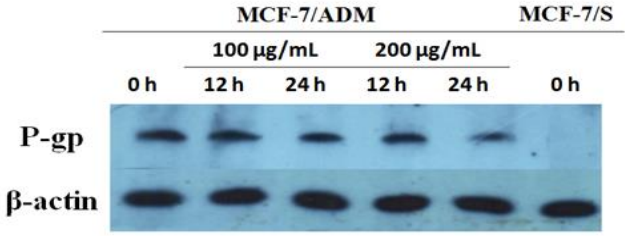

Figure S3. The effect of NAE on P-glycoprotein (P-gp) expression. Effect of NAE at 10 mM on the expression level of P-gp in MCF-7/ADM cells for 12 h and 24 h. Equal amounts (50 mg protein) of total cell lysate of each sample was loaded and separated using sodium dodecyl sulfate-polycrylamide gel electrophoresis. Then proteins were then transferred onto polyvinylidene fluoride membrane. The membranes were immunobloted overnight with primary antibodies against P-gp (1:500 dilution) and β-actin (1:500 dilution) at 4℃, then incubated for 3 h with horseradish peroxidase-conjugated secondary antibody at 1:1000 dilution. Protein-antibody complexes were detected by enhanced chemoluminescence detection system. β-actin was used as an internal control for equal loading.