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CHEMOTHERAPEUTIC ACTIVITIES OF CARTHAMI FLOS AND ITS REVERSAL EFFECT ON MULTIDRUG RESISTANCE IN CANCER CELLS

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Abstract

Multidrug-resistance (MDR) represents a major cause of failure in cancer chemotherapy. The need for a reduction in MDR by natural-product-based drugs of low toxicity led to the current investigation of applying medicinal herbs in future cancer adjuvant therapy. Carthami Flos (CF), the dried flower of safflower (*Carthamus tinctorius* L.), is one of the most popular traditional Chinese medicinal herbs used to alleviate pain, increase circulation, and reduce blood-stasis syndrome. The drug resistance index of the total extract of CF in MDR KB-V1 cells and its synergistic effects with other chemotherapeutic agents were studied. SRB cell viability assays were used to quantify growth inhibition after exposure to single drug and in combinations with other chemotherapeutic agents using the median effect principle. The combination indexes were then calculated according to the classic isobologram equation. The results revealed that CF showed a drug resistance index of 0.096. In combination with other chemotherapeutic agents, it enhanced their chemo-sensitivities by 2.8 to 4.0 folds and gave a general synergism in cytotoxic effect. These results indicate that CF could be a potential alternative adjuvant antitumour herbal medicine representing a promising approach to the treatment of some malignant and MDR cancers in the future.

Keywords: Carthami Flos, Safflower, Honghua, *Carthamus tinctorius* L, KB cells, MDR

Introduction

Multidrug-resistance (MDR) represents a major cause of failure in cancer chemotherapy. The most common form of MDR is the result of over-expression of P-glycoprotein (P-gp), a protein that pumps the anti-cancer drug out of the cell, in the cancer cell membrane. To overcome MDR, enormous efforts have been exerted to identify novel compounds that may specifically reverse P-gp-mediated MDR in cancer cells. Although hundreds of compounds, such as verapamil, cyclosporin, quinidine, tamoxifen, progesterone, reserpine, and others, have been found to be able to modulate the MDR phenotype, their clinical applications were limited due to their unacceptable side effects or toxicity at the doses required for effectiveness (Ford, J.M. and Hait, W.N., 1990). For instance, some possible side effects of verapamil are headaches, facial flushing, dizziness, light-headedness, swelling, increased urination, fatigue, nausea, ecchymosis, galactorrhoea, and constipation. Nowadays, many researchers have been working on screening compounds with low toxicity and high reversal activity to reverse MDR phenotype.

Carthami Flos (CF) is the dried flower of safflower (*Carthamus tinctorius* L.), which has been grown for centuries in Asia for the orange-red dye (carthamin) and for its quality oil rich in polyunsaturated fatty acids. CF is known to have many medicinal properties for curing several chronic diseases, and they are widely used in Chinese herbal preparations (Li and Mundel, 1996). In traditional Chinese medicine, CF is known as Honghua, which has been widely used to alleviate pain, increase circulation, and reduce blood-stasis syndrome with dysmenorrhoeal, amenorrhoea, postpartum abdominal pain and mass, trauma and pain of joints, etc. In addition, it can also be used for angina pectoris of coronary heart diseases, thromboangiitis obliterans and erythema multiiforme, etc. (Chen Songyu, 2006). CF also has been studied for its antitumour activity in certain cancers. A compound (Zhu-xiang) from herbal extracts containing ginseng and carthamus tinctorius was used to treat the MDA-MB-231 breast cancer cells and normal human mammary gland cell lines and they exhibited the ability to inhibit proliferation in solid tumour (Loo WT et al., 2004). The water extract of CF could activate dendritic cells in cancer treatment, which might promote the recognition of antigens and facilitate antigen presentation to Th1 immune responses in an animal model (Chang JM et al., 2011). However, no *in vitro* experimental study for the antitumour activities and MDR reversal effect of CF was reported, and its function as a folk medicine in cancer adjuvant therapy has not been proven.

The objective of this study was to provide an *in vitro* experimental study to determine the antitumour activities of CF in human cavity cancer MDR KB-V1 cell line. The results may provide a foundation for future researches on CF as an anti-cancer adjuvant agent.

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Materials and methods

Herb collection

CF was collected from Henan Province of China and the herbarium voucher specimen was deposited in Hong Kong, Baptist University.

Extraction method

CF was air-dried at room temperature and pulverised. The ethanol (50% v/v) extract was prepared by maceration of the powder for 72 h at room temperature. The total extract was filtered using filter paper No.2 and the rotary evaporator was used to remove the ethanol. The total extract was finally freeze-dried to yield a red powder and weighed. The total extract of CF was re-dissolved in DMSO making up a stock concentration of 150 mg/ml calculated on the dry mass of concentrated total extract.

Chemicals

All chemicals and chemotherapeutic agents used in this study were purchased from Sigma-Aldrich. Chemicals were dissolved in DMSO for experiments.

Cell culture

The KB-V1 line was derived from human oral epidermoid carcinoma cell line KB-3-1 cells by a series of stepwise selections for vinblastine resistance (Shen et al., 1986). KB-3-1 cells were maintained in MEM (Gibco, MD, USA) containing 10% foetal bovine serum. KB-V1 cells, the P-gp over-expressing cell line, were grown in the same medium, with the addition of 150ng/ml of vinblastine to maintain the MDR phenotype.

Cell viability assay

Sulfo-Rhodamine B (SRB) assay (Skehen et al., 1990) was used to determine cell cytotoxicity. SRB stained the protein of cells and the colour intensity can be positively correlated to number of cells. The solvent concentrations of drugs in all of the samples were ≤ 0.1% DMSO. Solvent controls were run with the assays to compare the cytotoxicity. Cells ($5x10^3$ cells in 100µl/well) were incubated in 96-well plate overnight. The drug treatments lasted for 72 h and cells were fixed by 20% trichloroacetic acid for 2 h at 4°C. Cells were rinsed with water, and then stained with 0.4% SRB in 1% acetic acid for 30 min. Unbound dye was removed by 1% acetic acid and the protein-bound dye was dissolved in un-buffered 10mM Tris base and measured by a microtiter plate reader. Cell number was estimated by correlating to OD at 515nm. Cytotoxicity was expressed as the percentage of viable cells at different concentrations of drugs. IC_{50} was calculated as the dose at which 50% cell death occurred relative to the untreated cells.

Determination of drug resistance index of CF alone in comparison with other chemotherapeutic agents

The effect of a drug in a sample was calculated according to the median effect principle as described by Chou and Talalay (1984). The median effect principle of mass action states that

$$\frac{fa}{fu} = (\frac{D}{Dm})^m$$

where Dm shared the same meaning of the IC_{50}, D is the required drug dose, fa is the % of cell affected, fu is the % of cell unaffected, and m is the coefficient of sigmoidicity.

$$\log(\frac{fa}{fu}) = m\log(D) - m\log(Dm)$$

This equation can be rearranged to give a linear equation by taking logarithms of both sides so that y=log (fa/fu) is plotted against x=log (D). From this, m and Dm can be derived from the slope and the y-intercept of the line respectively. Note that the linear correlation coefficient r ($0 \leq r \leq 1$) describes the conformity of the data to the median effect principle.

Drug resistance index is defined as the ratio of Dm in MDR KB-V1 cells over Dm in drug sensitive KB-3-1 cells in fold. The Dm of CF and a panel of other chemotherapeutic agents including doxorubicin (Dox), vinblastin (Vin) and colchicine (Col) in KB-3-1 and KB-V1 cells were compared and their respective drug resistance indexes were determined.

Analysis of the drug sensitivities and combination indexes of CF with other chemotherapeutic agents

For assessing drug cytotoxicity effect of CF in combination with other chemotherapeutic agents in MDR KB-V1 cells, Combination Index (CI) for two-drug combination at a particular fractional killing rate (fa) of cells were used to construct a classic isobologram equation as described below (Chou TC & Talalay P, 1997, 1981 & 1984):

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$$CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2} + \frac{\alpha(D)_1(D)_2}{(Dx)_1(Dx)_2}$$

where $(D)_1$ and $(D)_2$ are doses of drug 1 and drug 2 in combination to have a certain fa of cells. $(Dx)_1$ and $(Dx)_2$ are the doses of each drug alone that achieves the same fa of cells. If CI <1, =1 or >1, then synergism, additive effect or antagonism is respectively indicated. So, if the combined observed effect is greater than the calculated additive effect, synergism is indicated. When the effects of two drugs are mutually exclusive, that is, having same modes of action, the combined effect is the sum of the first two terms ($\alpha = 0$), and when the effects of two drugs are mutually nonexclusive, that is, having different or independent modes of action, the combined effect is the sum of the three terms ($\alpha = 1$). For a conservative determination of CI value, it was assumed that the effects of two drugs are mutually nonexclusive, that is, having different or independent modes of action, where $\alpha = 1$.

From the median effect equation of CF in MDR KB-V1 cells, a concentration of less than IC_{10} is determined and combined with other chemotherapeutic agents to form different mass ratio of combined drugs. As the combined drugs were serially diluted in the experiment, their mass ratio remained constant. For comparison purposes, the cytotoxicity of CF is neglected and the IC_{50} of the combined drugs is calculated in terms of the mass of chemotherapeutic agent used only. When the IC_{50} for combined drug is compared with the IC_{50} of their corresponding single drug, it gives an increase of chemo-sensitivity in folds.

Statistical analysis

Experimental result values were expressed as mean ± standard error of the mean (SEM) of at least 3 independent experiments. The difference between two groups was analysed using the paired t-test, with $P<0.05$ considered statistically significant.

Results and Discussion

The cytotoxicity of the total extract of CF and other chemotherapeutic agents as reference drugs were evaluated *in vitro* against the drug sensitive KB-3-1 cells and MDR KB-V1 cells. Their respective median effect equations were determined (Table 2) and IC_{50} was obtained (Table 1). The IC_{50} of CF in MDR KB-V1 cells is 695± 47 µg/ml, which is about 10 folds higher than its drug sensitive KB-3-1 cells of 7237± 1455µg/ml. The drug resistance index of CF is 0.096 in comparison with 317.4 to 625.0 for reference chemotherapeutic agents including doxorubicin, colchicine and vinblastin (Table 1). The results suggest that MDR KB-V1 cells are more susceptible to CF which in turn induces a low cytotoxicity to drug sensitive KB-3-1 cells.

The mass ratios of CF in combination with other chemotherapeutic agents were formulated by combining with a low cytotoxicity concentration of CF against MDR KB-V1 cells. Based on the median effect equation of CF in MDR KB-V1 cells, 100µg/ml of CF was selected as it would induce a very low cytotoxicity with over 90% of cell viability in MDR KB-V1 (calculation not shown). The mass ratio of the combined drug were then set at 20µg/ml: 100µg/ml (1:5) for Dox with CF, 2µg/ml: 100µg/ml (1:50) for Col with CF, and 2µg/ml: 100µg/ml (1:50) for Vin with CF. Their respective median effect equations were determined (Table 2) and IC_{50} was obtained (Table 1). For comparison purposes, the IC_{50} for combined drug was compared with the IC_{50} of their corresponding single drug *in vitro* against the MDR KB-V1 cells. Results indicated that CF enhanced the chemo-sensitivity of the chemotherapeutic agents including doxorubicin, colchicine and vinblastin by 4 folds to 2.8 folds respectively, such that the dose of chemotherapeutic agents used could be reduced by 4 folds to 2.8 accordingly (Table 1).

Table 1: Drug resistance indexes and chemo-sensitivities increased when CF combined with different chemotherapeutic agents in MDR KB-V1 cells

Drug Treatment (Mass ratio)	IC_{50} of single drugs in KB-3-1 cells for comparison (µg/ml)	IC_{50} of single and combined drugs in KB-V1 cells (µg/ml)	Drug Resistance Index (fold)	Chemo-sensitivity of emotherapeutic agents Increased (fold)
CF alone	7237± 1455	695± 47	0.096[a]	
Dox alone	0.46± 0.01(0.79± 0.002µM)	146± 34(252±58.7µM)	317.4 [a]	
Col alone	0.009± 0.001(0.023± 0.003µM)	3± 0.1(7.51± 0.25µM)	333.3 [a]	
Vin alone	0.008± 0.001(0.009± 0.001µM)	5± 0.1(5.5± 0.11µM)	625.0 [a]	
Dox with CF (1:5)		31.17±6.17(53.83±10.66µM)		4.0 [b]
Col with CF (1:50)		1.02± 0.07(2.55±0.18µM)		3.0[b]
Vin with CF (1:50)		1.78± 0.09(1.96±0.1µM)		2.8[b]

Legend: CF: Carthami Flos, Dox: Doxorubicin, Col: Colchicine, Vin: Vinblastin, Drug Resistance Index: Ratio of IC_{50} in KB-V1 cells / IC_{50} in KB-3-1 cells in respect of different treatments in terms of fold. Chemo-sensitivity of chemotherapeutic agents increased: Ratio of IC_{50} in KB-V1 cells in respect of corresponding single drug and combined drug treatments in terms of fold. ^aP <0.05 vs KB-3-1; ^bP <0.05 vs. corresponding single drug treatment

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Table 2: Median effect equations of CF in combination with different chemotherapeutic agents in MDR KB-V1 cells

Drug Treatment (Mass ratio)	Median Effect Equation in KB-3-1cells (r)	Median Effect Equation in KB-V1cells (r)
CF alone	y=0.37x-1.43 (0.97)	y=1.36x-3.88 (0.92)
Dox alone	y=1.06x+0.36 (0.98)	y=0.65x-1.41 (0.94)
Col alone	y=2.61x-+5.36 (0.92)	y=0.65x-0.3 (0.96)
Vin alone	y=1.34x+2.8 (0.90)	y=3.28x-2.30 (0.96)
Dox with CF (1:5)		y=0.54x-1.22 (0.90)
Col with CF (1:50)		y=1.48x-2.53 (0.98)
Vin with CF (1:50)		y=1.93x-3.78 (0.99)

Legend: CF: Carthami Flos, Dox: Doxorubicin, Col: Colchicine, Vin: Vinblastin, r: Coefficient of determination provides a measure of how well future outcomes are likely to be predicted by the model ($0 \leq r \leq 1$).

From their respective median effect equations, the combination indexes of combined drugs were also evaluated *in vitro* against the MDR KB-V1 cells. Positive control is the combined drug of Dox with Ver at a mass ratio of 20µg/ml: 10µg/ml (1:0.5). Their respective CI values at fa=25, 50, 75 & 90 were determined. Combined drugs of Col with CF and Vin with CF gave a general synergistic effect between $25 \leq fa \leq 90$. Combined drug of Dox with CF gave a synergistic effect only at $fa \leq 50$, additive effect at fa=75 and antagonistic effect at $fa \geq 90$ (Table 3). In contrast, combined drug of Dox with Ver gave a general synergistic effect between $25 \leq fa \leq 90$ indicating that doxorubicin has an antagonistic effect with CF at a higher fa.

Table 3: Combination indexes of CF in combination with different chemotherapeutic agents in MDR KB-V1 cells

	CI value for combined drugs in KB-V1cells			
Drug Treatment (Mass ratio)	**Fa=25**	**fa=50**	**fa=75**	**fa=90**
Dox with CF (1:5)	0.22	0.49	1.32	4.37
Col with CF (1:50)	0.97	0.45	0.21	0.12
Vin with CF (1:50)	0.49	0.53	0.60	0.69
Dox with Ver (1:0.5)	0.89	0.76	0.78	0.89

Legend: CF: Carthami Flos, Dox: Doxorubicin, Col: Colchicine, Vin: Vinblastin, Ver: Verapamil, If CI <1, =1 or >1, then synergism, additive effect or antagonism is respectively indicated. Dox with Ver was used as positive control.

Vinblastine and colchicine are microtubule destabilising agents, which bind to tubulin and inhibit the microtubule polymerisation in mitosis. Doxorubicin interacts with DNA by intercalation and inhibition of macromolecular biosynthesis. Verapamil is a calcium channel blocker and an inhibitor of drug efflux pump proteins such as P-glycoprotein. Although the mode of action of the MDR reversal activity of CF needs further investigation, it can be suggested from the present experiment that CF is a mixture of compounds which have a preferential cytotoxic effect targeted at P-gp over-expressing cancer cells. In combination with other chemotherapeutic agents, CF showed synergistic effects with microtubule destabilising agents and DNA intercalating agents respectively. However, the overall synergistic effect of CF is not as effective as the calcium channel blocker in combination with DNA intercalating agents. It may be due to the fact that there are some active compounds in CF that may cause antagonistic effect with DNA intercalating agents or the mass ratio of CF with doxorubicin needs further optimisation.

Conclusion

The total extract of CF alone did show a preferential cytotoxic effect in MDR cancer cells. In combination with other chemotherapeutic agents, CF did enhance their chemo-sensitivities in MDR cancer cells and gave a synergistic cytotoxic effect. Therefore, these results indicate that CF may be a potential alternative adjuvant antitumour herbal medicine representing a promising approach to the treatment of a variety of malignant and MDR cancers in the future. Future work, however, should focus on the identification of active compound(s) of CF to gain a better perspective of its antitumour activities as well as its mode of action in the MDR reversal activity.

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References

1. Chang JM, Hung LM, Chyan YJ, Cheng CM, Wu RY (2011). Carthamus tinctorius Enhances the Antitumor Activity of Dendritic Cell Vaccines via Polarization toward Th1 Cytokines and Increase of Cytotoxic T Lymphocytes. Evid Based Complement Alternat Med. 2011; 2011:274858. Epub 2011 Jan 3.
2. Chen Songyu (2006). Science of Chinese Materia Medica. Nanjing University of TCM. Pages 227-228.
3. Chou TC, Talalay P (1977). A simple generalized equation for the analysis of multiple inhibitions of Michaelis-Menten Kinetic Systems. J Biol Chem, 252:6438.
4. Chou TC, Talalay P (l981). Generalized equations for the analysis of inhibitors of Michaelis-Menten and higher order kinetic systems with two or more mutually exclusive and nonexclusive inhibitors. Eur J Biochem, 115:207.
5. Chou TC, Talalay P (1984). Quantitative analysis of dose– effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul, 22: 27–55.
6. Ford, J.M. and Hait, W.N. (1990). Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol. Rev., 42: 155-199.
7. Li, D. and H.H. Mundel (1996). Safflower. *Carthamus tinctorius* L. promoting the Conservation and Use of Underutilized and Neglected Crops. 7. Institute of Plant Genetics and Crop Plant Research, Gatersleben/International Plant Genetic Resources Institute, Rome, Pages 83.
8. Loo WT, Cheung MN, Chow LW (2004). The inhibitory effect of a herbal formula comprising ginseng and carthamus tinctorius on breast cancer. Life Sci. 2004 Nov 26;76(2):191-200.
9. Shen, D.W., Cardarelli, C., Hwang, J., Cornwell, M., Richert, N., Ishii. S., Pastan, I., Gottesman, M.M. (1986). Multiple drug-resistant human KB carcinoma cells independently selected from high-level resistance to colchicines, doxorubicin, or vinblastine show changes in expression of specific proteins. J. Biol. Chem., 261: 7762-7770.
10. Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon., J, Vistica, D., Warren, J.T., Bokesch, H., Kenney, S., Boyd, M.R. (1990). New colorimeteric cytotoxicity assay for anti-cancer-drug screening. J Natl Cancer Inst., 82: 1107-1112.