Multidrug-resistance reversing activity of the local *Citrus* fruits in Jeju Island, Korea

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Abstract

We examined whether extracts from 14 local *citrus* spp. on Jeju Island (Korea) contained chemosensitizing activity that would increase the cytotoxic effect of vincristine(VCR) in drug-resistant cancer cells. We report that methanol extracts from fruits and flowers of some species had a chemosensitizing effect that reversed P-glycoprotein (Pgp)-mediated multidrug resistance (MDR). Using drug-sensitive AML-2/WT and drug-resistant AML-2/D100 in the absence of VCR in human acute myelogenous leukemia cells we found that fruit or flower extracts alone generally had low cytotoxicity (IC_{50}>200 ㎍/ml). In studies examining the effect of extracts on 120 ng/ml VCR cytotoxicity in drug-resistant AML-2/D100 cells, we found that immature fruit extracts had greater chemosensitizing activity than either extracts from mature fruit or flower. Of the 14 species examined, the immature fruit extract from Inchangkyool (*Citrus ichangiensis*) showed the highest chemosensitizing index (CI) value. Immature fruit extracts of Hongkyool (*C. tachibana),* Byungkyool (*C. platymamma),* Cheongkyool (*C. nippokoreana)* and Jinkyool (*C. sunki)* also strongly potentiated VCR cytotoxicity in AML-2/D100 cells. The chemosensitizing effect of peel extracts was 2-10-fold that of whole fruit extracts

from Hongkyool (*C. tachibana),* Byungkyool (*C. platymamma*) and Inchangkyool (*C. ichangiensis*). The CI values for flower extracts were higher than those for mature fruit extracts, but lower than those for immature fruit extracts. These results indicate that immature citrus fruits contain compounds that do not exert their activity solely through cytotoxicity. In particular, Inchangkyool (*C. ichangiensis*), Byungkyool (*C. platymamma*), Cheongkyool (*C. nippokoreana*) and Hongkyool (*C. tachibana*) may be useful sources of chemosensitizing compounds.

Keywords: Citrus spp.; Chemosensitizing effect; Citrus ichangiensis; Citrus tachibana; Citrus platymamma ; Citrus nippokoreana

1. Introduction

Multidrug resistance (MDR) in tumor cells is a major obstacle for successful cancer chemotherapy. There have been many attempts to identify effective chemosensitizers to overcome cancer cell drug resistance. Much recent work has attempted to identify natural plant products that can inhibit P-glycoprotein (Pgp)-mediated MDR and sensitize cancer cells without undesired toxicity [1-4]. Of 450 plants tested, Kim reported that *Cynanchum wilfordii*, *Torilis japonica*, *Celastrus orbiculatus*, *Melia toosendan* and *Terminalia chebula* plant extracts strongly potentiated vinblastine cytotoxicity in KB-V1 cells [5]. To date, the identified plant-derived chemosensitizing compounds are reserpine, yohimbine and thaliblastine, which are indole alkaloids, and quinine, cinchonine which are quinoline alkaloids[6-7].

Citrus (Rutaceae) fruits are eaten and are also used as a natural source of juice, seasoning and vinegar. Citrus plant crude extracts have been shown to exhibit a diverse range of physiological activities, including antimicrobial [8], hypotensive [9], analgesic, antiphlogsitic, laxative, sedative and anti-tumor activities *in vivo* and *in vitro* [10]. Though not necessarily for medicinal reasons, citrus extracts have also been investigated for use as food additives in

chocolate, wine and cosmetics.

For over 100 years on Jeju Island (Korea), various types of citrus plants have been cultivated for edible fruit, including Byungkyool (*C. platymamma*), Binkyool (*C. leiocarpa*), Hongkyool (*C. tachibana*) and Dongjeongkyool (*C. erythrosa*). However, most of the local citrus plants have no economic value as a source of edible fruit as they have a strong sour taste compared to fruits such as navel oranges, tangerines and grapefruits. As such, the local citrus trees are being investigated as a possible source of medicines. To our knowledge, the present report is the first to describe chemosensitizing activity in local species of Jeju Island citrus plants.

2. Experimental

2.1. Plant material and cell lines

Flowers and fruits from 14 local citrus spp. were collected from the cultivated areas of the Jeju Citrus Research Institute Rural Development Administration (RDA) in May, August and November 2003. The plants were identified as Kamja (*C. benikoju*), Dangjyooja (*C. grandis*), Dongjeongkyool (*C. erythrosa*), Binkyool (*C. leiocarpa*), Byungkyool (*C. platymamma*), Sadookam (*C. pseudogulgul*), Sambokam (*C. sulcata*), Inchangkyool, Jikak (*C. aurantium*), Jinkyool (*C. sunki*), Cheongkyool (*C. nippokoreana*), Pyunkyool (*C. tangerina*), Hngjin (*C. unshu*) and Hongkyool (*C. tachibana*) by Dr. Sung Ku Kang from the Breeding Division, Jeju Citrus Research Institute R.D.A., Korea.

Cells used were wild-type drug-sensitive AML-2/WT cells of the human acute myelogenous leukemia cell line (provided by Dr. C. H. Choi, Chosun University, Korea). From these cells was derived a daunorubicin-resistant AML-2/D100 cell line that was positive for Pgp expression (see below).

2.2. Extract preparation

Extracts from 50 g fresh fruit or flowers were prepared using 500 ml 100% MeOH at 40℃. The extract was filtered (Advantec Toyo: Filter paper No. 1) and the solvent removed under reduced pressure (20 mm Hg). Extracts were

freeze-dried and stored at -20℃ until assayed. All extracts were dissolved in DMSO (dimethyl sulfoxide) on the day of assay.

2.3. Cell culture

The AML-2/WT and AML-2/D100 cells were cultured in α-MEM (Gibco) with 10% (v/v) fetal bovine serum (Gibco) at 37℃ in a humidified chamber containing 5% CO_2. Cells were grown and subcultured as suspension cultures. The daunorubicin-resistant AML-2/D100 subline were selected from the parental cell line AML-2/WT after a chronic exposure to daunorubicin on an intermittent dosage schedule at sufficient time intervals to permit the expression of the resistance phenotypes. Initially, these cells were grown in 50% inhibitory concentration (IC_{50}) of daunorubicin, after which the daunorubicin concentration was increased at a rate of 50%, and the cells were finally maintained in 100 nM daunorubicin. The cells were maintained in suspension cultures and subcultured when confluent [4].

2.4. Cytotoxicity assay

Assays were performed in 96-well flat bottom plates (Corning, 6.4×11 mm). In vitro cytotoxicity of the drugs was determined using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma] assay. Cells (2×10^5 cells/ml) in supplemented α-MEM were seeded in triplicate wells of 96-well plates in the presence of a range of drug concentrations (200, 100, 50, 25, 12.5, 6.25, 3.125 ㎍/ ml) and were incubated at 37 °C for 72 h. AML-2/D100 cell was treated in the presence or absence of 120 ng/ml VCR. Wells containing no drugs were used as controls for cell viability, and wells containing no cells were used for calibrating and zeroing the spectrophotometer. A stock solution of 5 mg/ml MTT was prepared in saline and stored at -20 °C. After cells were incubated at 37 °C for 3 days, 10 μl MTT solution was added to each well, the plates shaken for 1 min, and then incubated for 5 h. Formazan crystals were dissolved by the addition 100 μl 0.04 N HCl-isopropanol alcohol. The 540 nm optical density of the wells was measured using a microplate reader. The IC_{50} of

a particular agent was defined as the drug concentration that caused a 50% reduction in the cell number versus the untreated control. The IC_{50} values were directly determined from semilogarithmic dose-response curves. All experiments were performed at least in triplicate.

2.5. Statistical analysis

Statistical significance was determined using Student's *t* test. P-values less than 0.05 were considered to indicate statistically significant differences. The chemosensitizing index (CI) was calculated by dividing the 'IC_{50} without VCR' by the 'IC_{50} with VCR'.

3. Results and Discussion

While various species of citrus plants have been grown on Jeju Island for hundreds of years, there are few reports regarding their taxonomy, their physiological effects or their biochemical components. To our knowledge there is no study investigating their possible medicinal value.

We screened fruit and flower extracts from 14 Jeju Island citrus plants for their chemosensitizing effects against Pgp-mediated MDR. We examined cytotoxicity on both drug-sensitive 2/WT and drug-resistant 2/D100 cells. We also examined their cytotoxicity on 2/D100 cells in the presence or absence of VCR, and determined CIs.

We found that extracts from immature fruit had a remarkable chemosensitizing effect compared to extracts from mature fruits or flowers. Seven citrus species extracts showed CIs greater than 10, and they were ranked in the following order: Inchangkyool (CI; >50.0) > Hongkyool (CI; >40.0) > Byungkyool (CI; >33.3) > Cheongkyool (CI; >25.0) > Jinkyool (CI; >22.2) > Kamja (CI; >15.4) > Binkyool (CI; >11.1). The mean IC_{50} value for Inchangkyool, Hongkyool and Byungkyool extracts in AML-2/WT (sensitive) and AML-2/D100 (drug-resistant) cells without VCR was found to be greater than 200 μg/ml, while the mean IC_{50} values in AML-2/D100 cells in the presence of VCR were 4, 5 and 6 μg/ ml, respectively. This indicates that the

toxicity of these extracts is minimal at the concentrations required to overcome VCR drug resistance. Pyunkyool (*C. tangerina*), Dangjyooja (*C. grandis*), Sambokam (*C. sulcata*), Jikak (*C. aurantium*), Hngjin (*C. unshu*) and Sadookam (*C. pseudogulgul*) extracts had low chemosensitizing activity, with CI values of 6, 7, 4.8, 2.5, 2.1, 1.1 and 1.0, respectively.

Table 1. Cytotoxic and chemosensitizing effects of immature citrus fruit extracts in the absence or presence of vincristine in AML-2/WT and AML-2/D100 cells.

Local name (Scientific name)	IC_{50} of AML-2/WT	IC_{50} of AML-2/D100(μg/ml)		CI
		Iwithout VCR (μg/ml)	with VCR (μg/ml)	
Kamja (*C. benikoju*)	>200	>200	13	>15.4
Dangyooja (*C. grandis*)	>200	>200	42	>4.8
Dongjeongkyool (*C. erythrosa*)	>200	>200	20	>10.0
Binkyool (*C. leiocarpa*)	>200	>200	18	>11.1
Byungkyool (*C. platymamma*)	>200	>200	6	>33.3
Sadookam (*C. pseudogulgul*)	>200	>200	195	>1.0
Sambokam (*C. sulcata*)	>200	>200	81	>2.5
Inchangkyool (*C. ichangensis*)	>200	>200	4	>50.0
Jikak (*C. aurantium*)	>200	>200	93	>2.1
Jinkyool (*C. sunki*)	>200	>200	9	>22.2
Cheongkyool (*C. nippokoreana*)	>200	>200	8	>25.0
Pyunkyool (*C. tangerina*)	>200	>200	30	>6.7
Hngjin (*C. unshu*)	>200	>200	196	>1.1
Hongkyool (*C. tachibana*)	>200	>200	5	>40.0

Cytotoxicity was determined using MTT assays. Experiments were performed in triplicate. IC_{50} = extract concentration which inhibited 50% growth of cells. VCR = vincristine. CI = chemosensitizing index = IC_{50} (without VCR) / IC_{50} (with VCR).

The cytotoxicity of mature fruit extracts was similar to immature fruit (and

flower) extracts in both sensitive and resistant cells, but their chemosensitizing effect appeared weaker. Although Inchangkyool and Cheongkyool mature fruit and flower extracts had high CI values, they were less than those observed for immature extracts. The chemosensitizing effect of peel extracts was 2-10-fold that of whole fruit extract for mature Hongkyool (*C. tachibana),* Byungkyool (*C. platymamma)* and Inchangkyool (*C. ichangiensis*). Byungkyool peel extract showed higher CI value (12.0) than whole fruit(CI: >5.5) and CI value(18.7) of Hongkyool peel extract also was enhanced as compared with whole fruit(CI: >1.1). In terms of chemosensitizing activity towards VCR, extracts were ranked from highest to lowest in the following order: Jinkyool, Hngin, Dongjeonkyool, Binkyool, Kamja, Dangyooja, Pyunkyool, Jikak and Hongkyool (see Table 2). Sadookam and Sambokam extracts showed very low AML-2/D100 cytotoxicity in the presence of VCR.

The cytotoxic activity of citrus extracts was dependent on the maturity of the fruit. For example, for Inchangkyool extracts, the immature and mature fruit CIs were 50.0 and 10.0, respectively, while for Hongkyool, immature and mature fruit CIs were 40 and 1.1, respectively. Similar fruit age-related decreases in activity were observed for Byungkyool (CIs were 33.3 for immature and 5.5 for mature), Cheongkyool (25.0 and 10.5), Jinkyool (22.2 and 6.2), Kamja (15.4 and 2.8) and Binkyool (11.1 and 3.1). Similarly, the chemosensitizing effect of these extracts on resistant cells treated with VCR decreased with fruit maturity. It appears fruit ripening decreased the amount and/or potency of the chemosensitizing compounds.

Kim *et al.* screened MDR activity of *C. aurantium* and *C. unshiu* for drug sensitive cell (KB-3-1) and resistant cell (KB-V1). As a result, cytotoxicity of *C. aurantium* to KB-3-1 and KB-V1 cell were IC_{50} 150.23, 155.80 μg/ml, respectively, also increased IC_{50} value (58.28 μg/ml) as its extract with vinblastine treated to resistant cell (KB-V1). IC_{50} value of *C. unshiu* on both sensitive and resistant cell were 328.65 and 356.32, respectively, but the treatment of extract in the presence of vinblastine were increased toxicity (IC_{50} : 48.94 μg/ml) to resistant cell. These results suggested that Korean local citrus

with different species citrus have significant cytotoxic activity to drug-resistant cancer cells.

Table 2. Cytotoxic and chemosensitizing effects of mature citrus fruit extracts in the absence or presence of vincristine in AML-2/WT and AML-2/D100 cells.

Local name (Scientific name)	IC_{50} of AML-2/WT	IC_{50} of AML-2/D100(μg/ml)		CI
		without VCR (μg/ml)	with VCR (μg/ml)	
Kamja (*C. benikoju*)	>200	>200	72	>2.8
Dangyooja (*C. grandis*)	>200	>200	103	>1.9
Dongjeongkyool (*C. erythrosa*)	>200	>200	42	>4.8
Binkyool (*C. leiocarpa*)	>200	>200	65	>3.1
Byungkyool (*C. platymamma*)				
whole fruit	>200	>200	36	>5.5
peel	>200	239	20	12.0
Sadookam (*C. pseudogulgul*)	>200	>200	>200	ND
Sambokam (*C. sulcata*)	>200	>200	>200	ND
Inchangkyool (*C. ichangensis*)				
whole fruit	>200	>200	20	>10.0
peel	>200	279	22	12.7
Jikak (*C. aurantium*)	>200	>200	157	>1.3
Jinkyool (*C. sunki*)	>200	>200	32	>6.2
Cheongkyool (*C. nippokoreana*)	>200	>200	19	>10.5
Pyunkyool (*C. tangerina*)	>200	>200	118	>1.7
Hngjin (*C. unshu*)	>200	>200	35	>5.7
Hongkyool (*C. tachibana*)				
whole fruit	>200	199	181	>1.1
peel	>200	429	23	18.7

Cytotoxicity was determined using MTT assays. Experiments were performed in triplicate. IC_{50} = extract concentration which inhibited 50% growth of cells. VCR = vincristine. CI = chemosensitizing index = IC_{50} (without VCR) / IC_{50} (with VCR). ND = not determined.

Like fruits, flower extracts also showed cytotoxic IC_{50} values >200 μg/ml on sensitive and resistant cells. Of the 14 citrus flower extracts, the greatest increased in VCR sensitivity in AML-2/D100 cells was observed in the presence of Byungkyool flower extract (see Table 3). Cheongkyool, Inchangkyool and Hongkyool flower extracts also significantly increased this sensitivity. Of the other eight flower extracts, the highest to lowest CI ranking was as follows: Jinkyool > Binkyool > Dongjeongkyool > Kamja > Pyunkyool > Dangyooja > Sadookam > Sambokam. The results showed that flower extracts provided higher CI values than mature fruit but less than immature fruit extracts.

Table 3. Cytotoxic and chemosensitizing effects of citrus flower extracts in the absence or presence of vincristine in AML-2/WT and AML-2/D100 cells.

Local name (Scientific name)	IC_{50} of AML-2/WT	IC_{50} of AML-2/D100(μg/ml)		CI
		without VCR (μg/ml)	with VCR (μg/ml)	
Kamja (*C. benikoju*)	>200	>200	80	>2.5
Dangyooja (*C. grandis*)	>200	>200	104	>1.9
Dongjeongkyool (*C. erythrosa*)	>200	>200	66	>3.0
Binkyool (*C. leiocarpa*)	>200	>200	38	>5.3
Byungkyool (*C. platymamma*)	>200	>200	18	>11.1
Sadookam (*C. pseudogulgul*)	>200	>200	174	>1.1
Sambokam (*C. sulcata*)	>200	>200	194	>1.0
Inchangkyool (*C. ichangensis*)	>200	>200	24	>8.3
Jikak (*C. aurantium*)	>200	>200	134	>1.5
Jinkyool (*C. sunki*)	>200	>200	30	>6.7
Cheongkyool (*C. nippokoreana*)	>200	>200	22	>9.1
Pyunkyool (*C. tangerina*)	>200	>200	82	>2.4
Hngjin (*C. unshu*)	>200	>200	>200	ND
Hongkyool (*C. tachibana*)	>200	>200	26	>7.7

Cytotoxicity was determined using MTT assays. Experiments were performed in triplicate. IC_{50} = extract concentration which inhibited 50% growth of cells. VCR

= vincristine. CI = chemosensitizing index = IC_{50} (without VCR) / IC_{50} (with VCR). ND = not determined.

In summary, extracts from all local citrus species showed relatively low cytotoxicity (IC_{50} >200 ㎍/ml) on both drug-sensitive AML-2/WT cells and drug-resistant AML-2/D100 cells without VCR. Their cytotoxicity was similar in drug-sensitive cells as that observed when drug-resistant AML-2/D100 cells without VCR were treated concomitantly with citrus extracts and VCR. Similar IC_{50} values for both sensitive and resistant cells without VCR indicates that citrus extracts do not have selective cytotoxicity for drug-resistant cells. However, treatment of drug-resistant AML-2/D100 cells with any of the 14 citrus species extracts strongly increased their sensitivity to VCR in a concentration-dependent manner. Inchangkyool(*C. ichangensis*), Hongkyool (*C. tachibana),* Byungkyool (*C. platymamma),* Cheongkyool (*C. nippokoreana)* and Jinkyool (*C. sunki)* extracts strongly potentiated VCR cytotoxicity in AML-2/D100 cells. This result suggests that these five citrus species contain compounds with chemosensitizing properties that can reverse VCR resistance in AML-2/D100 cells. The chemosensitizing effect of Inchangkyool immature fruit extract was 10-fold that of mature fruit or flower extracts, indicating immature fruit should be the target of future studies. Additional work is required in order to identify the components responsible for the chemosensitizing properties of Jeju Island citrus plant extracts.

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