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Full Length Research Paper

The effects of quercetin and kaempferol on multidrug resistance and the expression of related genes in human erythroleukemic K562/A cells

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Leukemia chemotherapy is believed to be impeded by multidrug resistance (MDR). Some compounds of flavonoid molecules were previously shown to inhibit drug transporter Pgp or induce apoptosis to sensitize MDR tumors. In this study, we attempted to investigate the possibility and mechanism of quercetin and kaempferol, flavonoid molecules, in reversing MDR. K562/A cells were cultured *in vitro* with the flavonoids as single and in combination respectively. Cell growth inhibition and adriamycin (ADR) sensitivity were detected by 3-[4,5-dimethylthiazol-2-yl]-2,5 -diphenyltetrazolium bromide (MTT) assay. Cell apoptosis was examined by Annexin V/PI staining method. Moreover, the expression of related genes for drug transporters and apoptosis was also tested after incubation with resveratrol for the first time. Results show a dose dependent manner and synergistic effect of the two compounds on K562/A. Furthermore, some of the genes in drug transporter families such as ATP-binding cassette (ABC) and solute carrier (SLC) and apoptosis related genes such as Bcl-2、tumor necrosis factor (TNF) and tumor necrosis factor receptor (TNFR) families were regulated. The experiment indicates that quercetin and kaempferol may be used as reveratrol in leukemia chemotherapy, but their interaction and difference should be noticed.

Key words: Flavonoids, leukemia, multidrug resistance, polymerase chain reaction (PCR) array.

INTRODUCTION

Chemotherapy is an important method for the treatment of leukemia, but seldom eradicates the disease. Alternative approaches including molecular targeting therapy, stem cell transplantation and antisense technology are also currently used. However, these therapies are usually complicated, and their effectiveness is even controversial for some cases. In addition, there therapies are very costly.

Multidrug resistance (MDR) is a major cause of failure to chemotherapy in leukemia patients. MDR means leukemia cells resistant to one anti-leukemia drug become resistant to other drugs structurally and are mechanically different from the primary resistant drug. MDR can be primary (primary MDR), or secondary to the administration of an anti-leukemia drug (acquired MDR). The presence of MDR results in transient efficacies of anti-leukemia drugs, and rigorously limits the clinical use of chemotherapy. Many efforts have been focused on the research of MDR. For example, verapamil and cyclosporine A are useful for the reversion of MDR, but their accompanied side-effects and negative interactions with other anti-leukemia drugs limit their clinical use.

Flavonoids are a variety of natural compounds with

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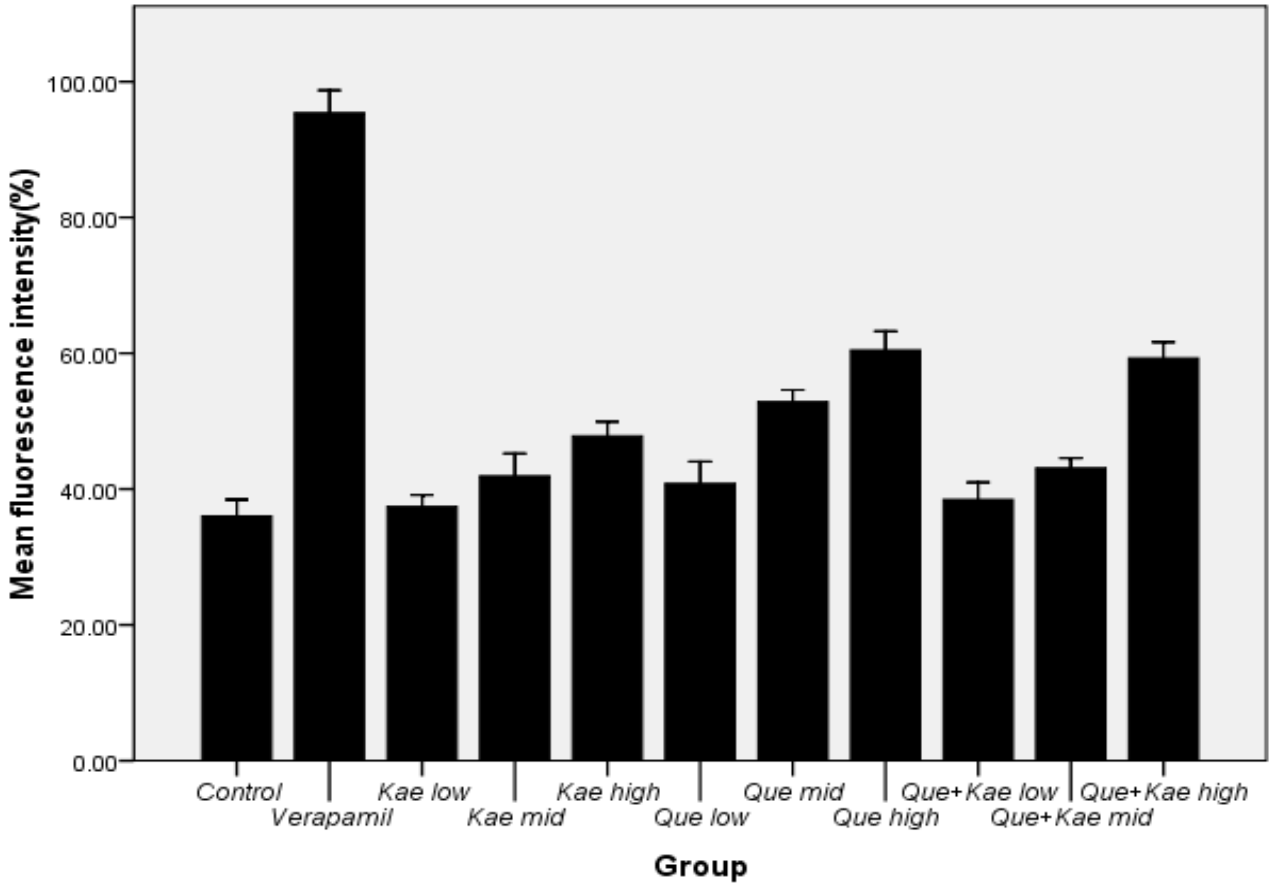

Figure 1. Molecular structures of quercetin and kaempferol.

similar molecular structures, which are present in berries and many other plants. About 6500 kinds of flavonoids have been described to date (Morris and Zhang, 2006). Based on their chemical structures, they can be classified as 15 subgroups including flavone, flavonol, flavonones, flavanonol, anthocyanidins, flavan-3,4-diols, xanthones, chalcones and biflavonoids, etc (Zhang et al., 2008). Natural flavonoids have significant pharmacological activities on tumors, cardiovascular system and endocrine system. *In vitro* studies have found that flavonoids have direct or indirect antitumor actions through interference of cell growth, inhibition of protein kinase activity, induction of apoptosis, suppression of matrix metalloproteinases secretion, and changes of tumor cell invasive behavior (Di Carlo et al., 1999). The antitumor activities of some flavonoids have also been confirmed in animal models (Mertens-Talcott, 2005)

Many studies have revealed that flavonoids, especially flavones and flavonols, exhibit cytotoxicity specifically towards cancer cells through inducing cell cycle arrest and apoptosis (Limtrakul et al., 2007). Some flavonoids could reverse MDR through increase of intracellular antitumor drug concentration by inhibiting the important drug transporter Pgp on cell membrane (Marilyn and Zhang, 2006; Marks et al., 1992). Quercetin (Que) and kaempferol (Kae) are flavonoids extracted and separated from Choerospondiatis, a kind of Chinese herbal medicine grown in North China and widely used in cardiovascular disease. Flavonoids are considered to be its effective components. In this study, we applied Que and Kae (Figure 1) to the adriamycin-resistant variant human K562/A cells, in order to find out the mechanism of MDR reversion and the targets useful for the management of MDR, we observed the changes of cell growth rate, increase of their sensitivity to adriamycin (ADR), apoptosis, and the expression changes of drug transporter and apoptosis related genes.

MATERIALS AND METHODS

Cell cultures

Human myelogenous leukemia K562 cells and the adriamycin-resistant variant K562/A cells (provided by Hematology Laboratory, Peking University First Hospital) were cultured in Iscove's Modified Dulbecco's Medium (IMDM) medium (Hyclone, USA) supplemented

with 10% fetal calf serum (Hyclone, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. K562/A cells were cultured in a medium containing 1 µg/ml ADR (Main Luck Pharmaceutical, Shenzhen, China), and were grown in drug-free medium for two weeks before the experiments.

Growth rate and ADR sensitivity evaluated by MTT assay

K562 or K562/A cells in an amount of 2×10^4/100 µl/well were cultured in 96-well plates (Corning, USA), and then cultured in a media containing various concentrations of drugs. The inhibition of cell growth rate was evaluated in cells treated with Que, Kae, or Que+Kae (National institute for the Control of Pharmaceutical and Biological Products, China) at the concentrations of 5 to 160 µmol/L. The changes of sensitivity to ADR were tested in cells treated with various concentrations of ADR, in combination with various concentrations of Que, Kae or Que+Kae. The experiments were also performed in cells treated with various concentrations of ADR, verapamil, and ADR+verapamil as test controls. After treatment for 48 h, 10 µl of 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5 -diphenyltetrazolium bromide (MTT) (Sigma, USA) was added to each well, and the cells were incubated for another 4 h. MTT product was solubilized by addition of 150 µl/well DMSO, and measured at 570 nm using an ELISA reader (Thermo, USA). The experiment was performed in triplicate. IC_{50} (concentration required for 50% inhibition) was calculated by the Logit method (Michieli et al., 1999).

Apoptosis determined by annexin V/PI analysis

For apoptosis measurement, K562 and K562/A cells were cultured in media containing various concentrations of Que, Kae, and Que+Kae for 72 h. Apoptosis of treated cells was measured by flow cytometry, using FITC-conjugated annexin V antibody and propidium iodide (KeyGen, China) following the manufacturer's instructions. The experiment was repeated in triplicate.

Gene expression determined by PCR array

K562/A cells were treated with 20 µmol/L Que, Kae, and Que+Kae for 72 h. Total RNA was extracted from the treated cells using TRIZOL Reagent (Invitrogen, USA). Extracted total RNA was further purified by RNeasy MinElute Cleanup Kit (Qiagen, Germany). Its concentration and purity was determined by the NanoDrop ND-1000. Its integrity was checked by electrophoresis in a 1.2% agarose gel, and then it was reverse-transcribed by SuperScript III reverse transcriptase (Invitrogen, USA). The first strand cDNA was diluted and mixed with 2 X SuperArray PCR master mix and water to a total volume of 1100 µl, and the mixture was added to the Human Drug Transporters PCR Array or Apoptosis PCR Array (Superarray, USA). The array was placed in a quantitative real-time PCR instrument (ABI PRISM 7900 system, Applied Biosystems, USA), using a thermal cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. The expression difference of a gene was calculated from the difference of the related Ct values.

Statistical analysis

Data was expressed as mean ± SD, and analyzed with student *t* test by SPSS 17.0 software. P-value below 0.05 was considered as statistically significant. All data points were reported as the mean of triplicates.

RESULTS

Que and Kae inhibited the growth of K562 and K562/A cells

K562 and K562/A cells were cultured in various concentrations of ADR. The calculated ADR IC_{50} was 1.48±0.34 µmol/L for K562 cells, and 76.82±4.14 µmol/L for K562/A cells. It can be seen that the ADR IC_{50} was 51.9 times higher for K562/A cells than for K562 cells. Que and Kae inhibited the growth of K562 and K562/A cells in a dose-dependent manner, and the two flavonoids were synergistic in inhibition of the two cell lines (Figure 2). Verapamil in the concentration range of 5 to 20 µmol/L showed no significant inhibition effect on the growth of the two cell lines.

Que and Kae increased the sensitivity of ADR on K562 and K562/A cells

When K562 and K562/A cells were incubated in media containing lower concentration ADR (cell growth inhibition rate <15%), the addition of Que and/or Kae to the media resulted in the increase of their sensitivity to ADR, especially in K562/A cells. Que and/or Kae induced the increase of sensitivity to ADR in K562/A cells at a concentration as low as 10 µmol/L and in a concentration dependent manner. The two compounds had similar effect on the sensitivity to ADR when they were used at lower and moderate concentrations ($P > 0.05$), but Que was more effective than Kae when they were used at higher concentrations ($P < 0.05$). The two compounds had a synergetic effect when both of them were applied to the cells. When the effect of Que and Kae on the sensitivity to ADR was compared with that of verapamil, they were less effective when they were used separately at 10 µmol/L, but were more effective when they were used together even at lower concentrations (5 µmol/L). Therefore, Que and Kae enhanced the antitumor effect of chemo-therapeutic agents, especially when they were used together (Table 1).

Que and Kae induced apoptosis

Both Que and Kae induced apoptosis in K562 and K562/A cells. Apoptosis induced by Que, Kae, and Que+Kae was mainly found to be at early stage, and was in a dose-dependent manner. Que and Kae had similar effect on apoptosis of K562 and K562/A cells, and Que+Kae treatment seemed to be more active in inducing apoptosis than Que treatment and Kae treatment alone (Figure 3). In addition, verapamil had no apoptosis of the two cell lines, suggesting the different mechanism of MDR reversion between verapamil and the two flavonoids.

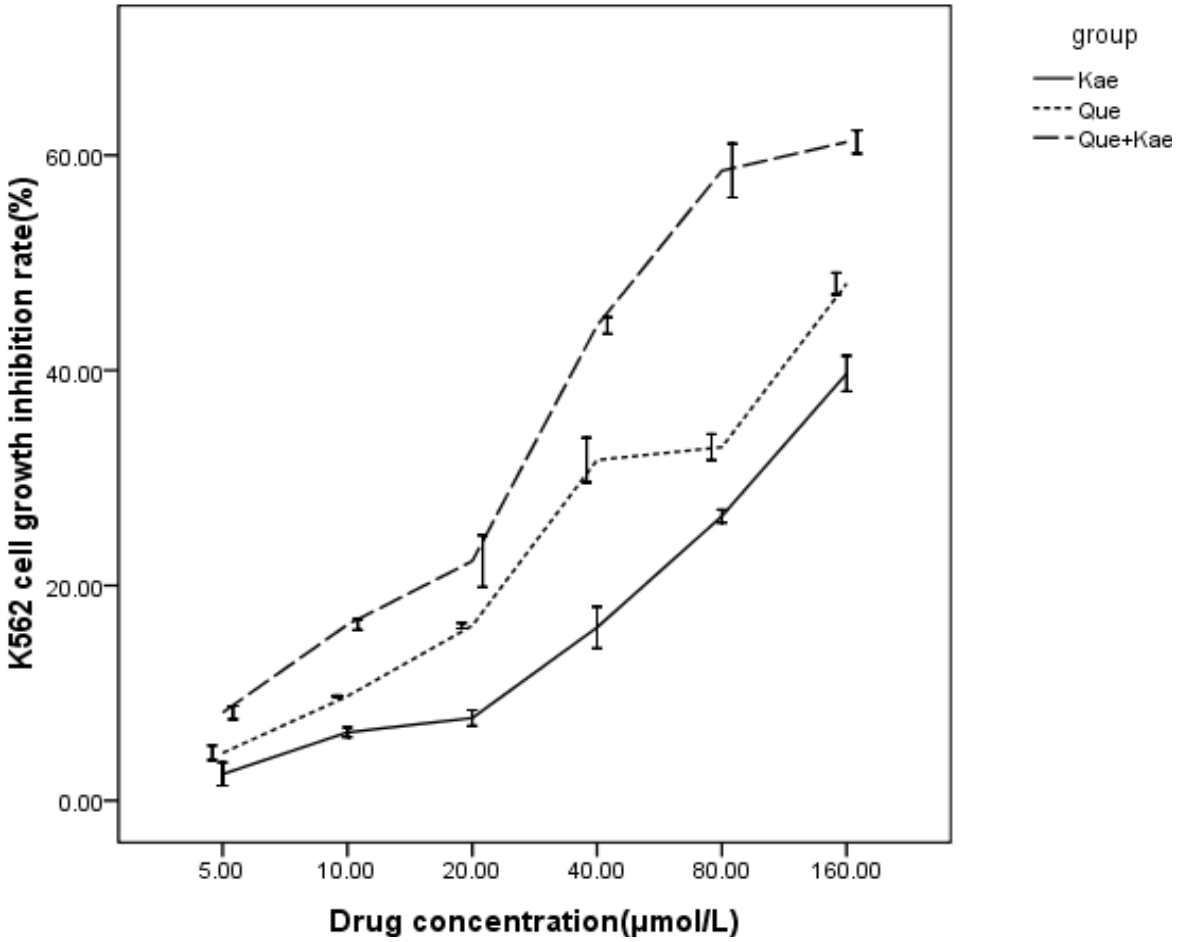

Figure 2. Que and Kae inhibited the growth of K562 and K562/A cells in dose dependent manners. K562 (panel A) and K562/A (panel B) cells were cultured in media containing various concentrations (5 to 160 μmol/L) of Que, Kae, or Que+Kae, and in medium without any drugs as a negative control for 48 h. Living cells were measured by MTT method. Cell growth inhibition rate on ordinate was determined from MTT assay data using the formula: (1-absorbance of experiment group/absorbance of control group) × 100%. Data were presented as mean ± SD of three independent experiments.

Table 1. Increase of intracellular ADR in K562 and K562/A cells treated with various concentrations of Que and/or Kae.

Group	ADR IC_{50} (μmol/L)		Increase of intracellular ADR (fold)	
	K562	K562/A	K562	K562/A
Control	1.48±0.34	76.82±4.14		
Verapamil(10 μmol/L)	0.62±0.08[a]	21.93±5.14[b]	2.39	3.50
Que low(10 μmol/L)	1.06±1.13[▲▲]	50.11±1.34[b▲]	1.40	1.53
Que mid(20 μmol/L)	0.98±0.08[▲▲]	32.44±4.04[b]	1.51	2.37
Que high(40 μmol/L)	0.85±0.07[a▲]	20.59±2.66[b]	1.74	3.73
Kae low(10 μmol/L)	1.47±0.40[▲]	52.59±4.82[b▲▲]	1.01	1.46
Kae mid(20 μmol/L)	1.31±0.50	46.66±4.51[b▲▲]	1.13	1.65
Kae high(40 μmol/L)	1.18±0.39	30.03±3.90[b]	1.25	2.56
Que+Kae low (5 μmol/L each)	1.35±0.28[▲]	19.23±3.47[b]	1.10	3.99
Que+Kae mid (10 μmol/L each)	0.54 ±0.22[a]	14.03±2.22[b]	2.74	5.48
Que+Kae high (20 μmol/L each)	0.48±0.09[b]	12.86±2.27[a▲]	3.08	5.97

[a] P<0.05 VS control; [b] P<0.01 VS control; [▲] P<0.05 VS verapamil; [▲▲] P<0.01 VS verapamil.

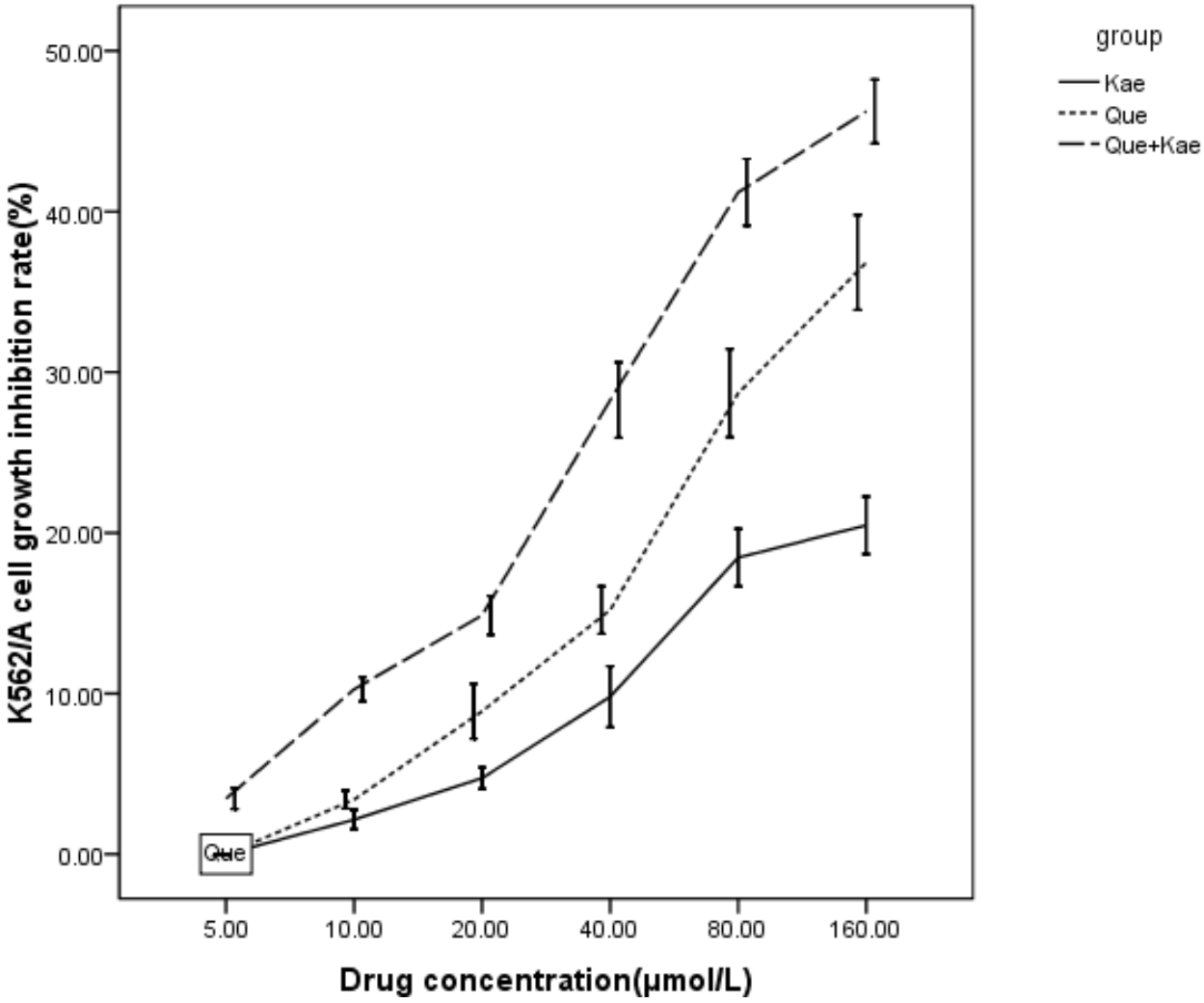

Figure 3. Apoptosis of K562 and K562/A cells induced by Que and/or Kae. Cultured K562 (panel A) or K562/A (panel B) cells (2×10^5/ml/well) were treated with 10 μmol/L Kae (Kae low), 20 μmol/L Kae (Kae mid), 40 μmol/L Kae (Kae high), 10 μmol/L Que (Que low), 20 μmol/L Que (Que mid), 40 μmol/L Que (Que high), 5 μmol/L/each Que+Kae (Que+Kae low), 10 μmol/L/each Que+Kae (Que+Kae mid) or 20 μmol/L/each Que+Kae (Que+Kae high) for 72 h; cells were cultured in medium without any drugs as a negative control and then cell apoptosis was examined by flow cytometry using Annexin V-FITC/PI staining method. Data was presented as mean ± SD of three independent experiments.

The Apoptosis PCR array profiles the expression of key genes involved in apoptosis. The array includes TNF ligands and their receptors; members of the Bcl-2 family, caspase, nhibitor of apoptosis (IAP), TNF associated-factor (TRAF), Caring for Relatives with Dementia (CARD), death domain, death effector domain, and cell death-inducing DNA fragmentation factor-alpha-like effector (CIDE) families; as well as genes involved in p53 and ataxia telangiectasia mutated (ATM) pathways. Que and Kae had different effect on genes related to apoptosis in K562/A cells. For example, Kae regulated the expression of 19 genes in all of the 84 genes on PCR array, but Que regulated only nine genes. Que and Kae down-regulated the expression of genes inhibited apoptosis such as Bcl-2, Bcl-w and up-regulated the expression of genes induced apoptosis such as BAD, and BIM in Bcl-2 family. Both of the flavonoids induced apoptosis though interfering with the balance of TNF and TNFR (Table 2).

Que and Kae influenced the expression of drug transporter genes

The Human Drug Transporters PCR array contains 84 transporter genes. Transporters play key roles in pharmacological behaviors of drugs, affecting entry and extrusion of drugs in and out of cells. This array includes

Table 2. Expression changes of apoptosis related genes in K562/A cells treated with Que and Kae for 72**h**

gene symbol	up or down regulation(fold)		gene description
	Que	Kae	
Bcl-2 family			
BAD	--	2.65	Bcl-2-antagonist of cell death
Bcl-2	-3.16	-4.03	B-cell CLL/lymphoma 2,inhibition apoptosis
BIM	3.23	7.13	Bcl-2-like 11,apoptosis facilitator
BCL-W	--	-2.34	Bcl-2-like 2
BIK	2.10	2.39	Bcl-2-interacting killer, apoptosis facilitator
BNIP1	--	-2.50	Bcl-2/adenovirus E1B interacting protein 1
IAP family/card			
BIRC3	-3.60	-2.91	Baculoviral IAP repeat-containing 3
XIAP	--	-3.37	X-linked inhibitor of apoptosis
TNF receptor			
CD40	2.53	2.62	TNF receptor super-family, member 5
TNF RSF10A	-6.03	-3.78	TNF receptor super-family, member 10a
TNF RSF10B	--	-3.50	TNFreceptorsuper-family, member 10b
TNF RSF1A	-2.54	-14.30	TNF receptor super-family, member 1A
TNF RSF9	-2.31	-2.08	TNF receptor super-family, member 9
TNF Ligand Family			
LTA	--	3.53	TNF super-family, member 1
TNF	--	20.59	TNF super-family, member 2
Death Domain Family			
DAPK1	--	3.26	Death associated protein kinase 1
p53 and DNA Damage	**Response**		
GADD45A	2.02	7.08	Growth arrest and DNA damage inducible, alpha
TP53 BP2	--	2.39	Tumor protein p53 binding protein, 2
TP73	--	2.28	Tumor protein p73

transporters important to absorption, distribution, metabolism and excretion of many drugs. In addition, transporters such as ABC and SLC gene families which contribute to sensitivity and resistance of tumor cells are also included in this array. Que and Kae regulated some of the genes in ABC transporter family, such as down-regulating the famous drug resistance gene ABCB1 (multidrug resistance1). The flavonoids also down-regulated the expression of genes relating to chemo-resistance in SLC family such as SLC28, SLC29, SLC7 and SLC3 sub-family (Table 3).

DISCUSSION

Our experiments revealed that the growth of K562 and K562/A cells was inhibited by Que and Kae, especially by Que, in a dose dependent manner, which are similar to the previous results reported by Chen et al. (2005) and Rusak et al. (2005). An earlier study has also suggested the anti-proliferative activity of Que on normal bone marrow and leukemic progenitors through the interaction with type II estrogen binding sites (Larocca et al., 1991). We also found that Que and Kae were synergetic with ADR to enhance the antitumor efficiency of ADR, which indicate that Que and Kae have the ability to increase the sensitivity to chemotherapeutic agents such as ADR in addition to the inhibition of tumor cell growth. However, Kae may have specific ability in anti-proliferation. Bestwick et al. (2007) observed that Kae had cytotoxic potentials to HL-60 cells as low concentration as 1 µmol/L, which was probably related to induce cell membrane injury and apoptosis. They also found that Kae inhibited the growth of HL-60 cells at more than 10 µmol/L through changing cell cycles.

MDR is a complicated phenomenon related to many processes. Membrane transporters play important roles in mediating chemo-sensitivity and drug resistance of tumor

Table 3. Expression changes of drug transporter genes in K562/A cells treated with Que and Kae for 72 h.

Gene symbol	Substrate	Up or down regulation (fold)	
		Que	Kae
ABC Transporters			
ABCA13	Unknown	-	-4.75
ABCA4	n-Retinylidiene-PE [10]	-13.53	-11.20
ABCB1	Anthracyclines, vinca alkaloids, imatinib [11]	-10.03	-38.11
ABCB11	Peptide[10]	-3.94	-4.59
ABCB4	Phosphatidylcholine [10]	2.00	3.10
ABCB6	Iron [10]	-3.46	-3.34
SLC Transporters			
SLC10A1	Bile acid [12]	-86.33	-12.51
SLC22A1	Verapamil, acyclovir, imatinib, oxaliplatin [13]	-6.22	-3.21
SLC28A2	Nucleoside[14]	-5.01	-2.80
SLC28A3	Pyrimidine and purine nucleoside [11]	-2.96	-
SLC29A1	Purine and pyrimidine nucleosides [11]	-	-2.33
SLC2A1	Glucose [15]	-2.01	-2.55
SLC2A2	Glucose [15]	-2.31	-
SLC2A3	Glucose [15]	-	-2.95
SLC31A1	Copper [11]	-2.37	-2.67
SLC38A2	Amino acid [16]	-3.46	-3.13
SLC38A5	Amino acid [16]	-2.37	-3.50
SLC3A1	Amino acid [16]	-	-3.01
SLC3A2	Amino acid [11]	-3.79	-6.70
SLC25A13	Citrin [17]	-	-2.43
SLC7A11	Cystine, L-glutamate [11]	-	-4.13
SLC7A5	Large neutral L-amino acids, BCH [11]	-2.50	-4.63
SLC7A8	Neutral and acidic amino acid [18]	-2.06	-2.09
SLCO1A2	Bile acids [19]	-	-3.02
SLCO2B1	Steroid hormone conjugates [20]	-2.08	-
Other Transporters			
AQP1	Water [16]	-	3.23
AQP7	Water, glycerol, urea [11]	-2.19	-
ATP6V0C	Unknown	-	-2.09
ATP7A	p-Chloromercuribenzoate [11]	-	-4.37
ATP7B	Copper [21]	-	-2.79

--: No obvious change.

cells. In addition to traditional ABC transporters which exclude cytotoxic drugs from cancer cells to lower their intracellular concentrations, SLC transporters increase chemo-sensitivity by uptaking hydrophilic drugs. Structurally, these drugs often resemble the natural substrates of the respective transporters. In particular, members of the SLC19 family (reduced folate carrier, RFC family), SLC28 and 29 families (CNT and ENT nucleoside transporters), SLC7A and 3A families (amino acid transporters) and SLC31A (CTR copper transporter family) are important determinants of chemosensitivity to anticancer drugs (Huang and Sadée, 2006).

In our real-time PCR array results, most ABC transporters including the famous drug resistance gene ABCB1 were down-regulated, but ABCB4 was up-regulated in K562/A cells after Que and Kae treatment. It may relate to the phenomenon that different transporters mediate different substrates' transportation across cell membrane. For example, some nutrient transporters (glucose, amino acids, organic anions, and peptides) in SLC family may be

up-regulated in chemo-resistant tumor cells because of increased energy and nutrition needs. Therefore, down-regulation of many gene members in SLC family by Que and Kae treatment may simply reflect the decreased nutrient uptake due to the growth inhibition of the tumor cells.

The therapeutic activity of many antitumor drugs has been found to rely on the activation of apoptosis pathways in tumor cells, and abnormalities in apoptosis is another important factor leading to multidrug resistance. Our results suggest that Que and Kae induce apoptosis through the expression change of some genes in bcL-2, TNF, and IAP families. However, Que and Kae are different in inducing apoptosis, and they block cell cycles at different phases (Gordana et al., 2005; Bestwick et al., 2007; Kandaswami et al., 2005). In acute myelocytic leukemia patients, for example, Que induced apoptosis by change of TRAIL signal pathway and caspase-3 expression (Mertens-Talcott, 2005; Kim et al., 2008; Liesveld et al., 2003). In contrast, Kae was less active than Que in cell growth inhibition and apoptosis, but was a potent reactive oxygen species scavenger in menadione-stressed cells (Gordana et al., 2005). Therefore, Kae may be useful for regulation of immunological function and prevention of diseases.

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