Modulation of Anticancer Drug-Induced P-Glycoprotein Expression by Naringin

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Multidrug resistance (MDR) is a phenomenon that is often associated with decreased intracellular drug accumulation in the tumour cells of a patient, resulting from enhanced drug efflux. It is often related to the overexpression of P-glycoprotein (P-gp) on the surface of tumour cells, thereby reducing drug cytotoxicity. In the present study, naringin (the predominant flavonone found in grapefruit and other related citrus species) was tested for its potential ability to modulate the expression of P-gp in a short-term animal bioassay, in comparison with verapamil (a calcium channel blocker and positive MDR reversal agent). Western blot analysis showed that pre-treatment by i.p. administration of 5 mg naringin/kg body weight for 3 consecutive days prior to doxorubicin (the most common used anticancer drug which induces MDR) administration was able to significantly lower the P-gp expression reaching nearly the level of animals treated with verapamil. Moreover, pre-treatment with naringin prior to doxorubicin increased the sensitivity to the drug. Naringin inhibited the doxorubicin-stimulated ATPase activity demonstrating that naringin may interact directly with the transporter. In addition, the results demonstrated that induction of both glutathione (GSH) and glutathione-S-transferase (GST) by doxorubicin is consistent with an increased ATP-dependent doxorubicin transport. Thus, naringin seems to modulate the *in vivo* expression of P-gp. In summary, the present study describes the dual modulation of P-gp expression and function by the flavonoid naringin, which may be an attractive new agent for the chemosensitization of cancer cells.

Key words: P-Glycoprotein, Multidrug Resistance, Naringin

Introduction

When patients suffering from cancer are treated with a cytotoxic agent, the pharmacological goal is to deliver as much active drug as possible to the molecular target in the cancer cells, so that sufficient molecular damage to lead to cell death. On the other hand, the occurrence of drug resistance renders cells to be resistant not only to the drug used in the chemotherapy, but also to a broad spectrum of unrelated cytotoxic drugs as well. Cancer cells may develop a multidrug-resistant phenotype. When human tumour cells express this phenotype, they often overexpress the drug export protein called plasma membrane P-glycoprotein (P-gp) with a molecular mass of approx. 150–170 kDa. This plasma membrane phosphoglycoprotein, which belongs to the superfamily of ATP-binding cassette (ABC) transporters, consists of two homologous halves that share a high degree of sequence similarity (Ambudkar *et al.*, 1999).

Several classes of compounds that inhibit the P-gp-mediated efflux and enhance the accumulation and efficacy of anticancer compounds have been identified. Multidrug resistance (MDR)-reversing agents include calcium channel blockers (verapamil), calmodulin inhibitors (phenothiazines), indole alkaloids (reserpines), and nonpolar cyclic oligopeptides with immunosuppressant activity (cyclosporin A) (Tan *et al.*, 2000). Although P-gp mediates the transport of many structurally and functionally diverse compounds, many potent MDR-inhibiting compounds share common physical characteristics such as cyclicity, lipophilicity, and a positive or neutral charge at physiological pH. Many synthetic MDR modulators, including reversins 121 and 205 (Sharom *et al.*, 1999)

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and the cyclosporin D analogue valspodar (PSC 833) (Sikic, 1999), successfully reverse the MDR phenotype *in vitro*. However, the efficacy of these compounds in animal studies and clinical trials has been disappointing due to dose-limiting toxicity. Accordingly, much effort is currently being expanded toward identifying natural compounds from plant origins that inhibit P-gp, reverse the MDR phenotype, and sensitize cancer cells to conventional chemotherapy without undesired toxicological effects. The other approach for MDR modulation is the modulation of the MDR1 gene. Studies on the MDR1 gene promoter sequence suggest that modulation of P-gp expression at the genetic level may be possible (Hu *et al.*, 1996). These types of MDR modulators may either block the induction of MDR1 gene expression or inhibit its promoter and down-regulate the P-gp expression.

Naringin (glycoside) is the predominant flavonone found in grapefruit and other related citrus species (Swiader and Zarawska, 1996). Like most flavonoids, naringin has a wide range of biological and pharmacological activities, including metal-chelating, antioxidant and free radical-scavenging properties (Chen *et al.*, 1990) and has been reported to offer some protection against lipid peroxidation (Maridonneau-Parini *et al.*, 1986). In addition, naringin is a potent cholesterol-lowering agent (Jeon *et al.*, 2001), anti-atherogenic agent (Lee *et al.*, 2001), and antimutagenic agent (Calomme *et al.*, 1996). Although, the previous beneficial effects of naringin have been established, no attention has been given to the role of naringin in the inhibition of P-gp overexpression and ist possible utility as a dietary adjuvant in the treatment of cancer.

Due to its wide range of biological and pharmacological effects, lack of toxicity in animal models, cyclicity, and lipophilicity, naringin was examined in the present study to determine possible interactions with P-gp expression and function. We demonstrated that naringin down-regulates the P-gp expression and reduces the P-gp-mediated efflux in animals treated with doxorubicin. In summary, these results suggest that naringin may have chemosensitizing properties on the MDR phenotype as a result of its ability to modulate both the expression and function of MDR1.

Material and Methods

Chemicals

Naringin (7-[[2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,3-dihydro-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one) (Berhow and Vandercook, 1989) and verapamil (5-[*N*-(3,4-dimethoxyphenethyl)methylamino]-2-(3,4-dimethoxy-phenyl)-2-isopropylvaleronitrile) were procured from Sigma Chemical Co. (St. Louis, MO, USA). Doxorubicin, vinblastine, and ouabain were obtained from ICN Biochemicals (Eschwege, Germany). Anti P-gp monoclonal antibody clone JSB1 was procured from Boehringer Mannheim GmbH (Germany). RNA-Clean kit and Taq DNA polymerase kit were purchased from Hybaid/AGS (Heidelberg, Germany). Oligonucleotides were obtained from Genset (Paris, France). Restriction endonucleases were purchased from MBI Fermentas (St. Leon-Roth, Germany). AMV-reverse transcriptase kit was from Promega (California, USA). All other chemicals were of the highest purity and purchased from either Merck (Darmstadt, Germany) or Sigma-Aldrich Chemie (Deisenhofen, Germany).

Animals and treatment

The animal care and handling was done according to the guidelines set by the World Health Organization, Geneva, Switzerland and according to the approval of the committee for animals care at the National Research Centre, Egypt. Adult male Sprague Dawley rats (150–180 g body wt) were selected for the study. The animals were kept for a quarantine period of 1 week in an air-conditioned room at a temperature of (25 ± 2) °C, relative humidity of (57 ± 2)% and with a photocycle of 12 h light and dark. Water and food pellets were provided *ad libitum*. For the study the animals were divided into different experimental and control groups, each comprising eight animals as shown in Table I. The animals were sacrificed 24 h after the last treatment. Liver specimens were excised, rinsed with buffered physiological saline, immediately frozen in liquid nitrogen, and stored at –20 °C until use. The protein concentration was determined as described by Lowry *et al.* (1951) using bovine serum albumin as standard.

Table I. Details of the experimental and control groups used.

Group	Treatment
I (control)	No treatment.
II (naringin)	Naringin at a dose of 5 mg/kg body wt by i.p. administration for 3 consecutive days.
III (verapamil)	Verapamil at a dose of 10 mg/kg body wt by i.p. administration for 3 consecutive days in 0.9% saline.
IV (doxorubicin)	Doxorubicin at a dose of 10 mg/kg body wt by i.p. administration for 3 consecutive days in 0.9% saline.
V (verapamil + doxorubicin)	Verapamil at a dose of 10 mg/kg body wt by i.p. administration for 3 consecutive days. Doxorubicin at a dose of 10 mg/kg body wt by i.p. administration 1 h after the last dose of verapamil.
VI (naringin + doxorubicin)	Naringin at a dose of 5 mg/kg body wt by i.p. administration for 3 consecutive days. Doxorubicin at a dose of 10 mg/kg body wt by i.p. administration 1 h after the last dose of naringin.

Tissue preparation

A portion of liver was precooled to 4 °C in TMEP buffer (50 mM Tris, pH 7.0, with HCl, containing 50 mM mannitol, 2 mM EGTA, 2 mM β-mercaptoethanol, 10 mM leupeptin, 8 mg/ml aprotinin and 0.5 mM phenylmethylsulfonyl fluoride) and homogenized. Undisrupted tissue and other debris were removed by centrifugation (5000 × *g* for 10 min) and the supernatant was centrifuged at 45000 × *g* for 45 min. The pellet, consisting of mitochondria, plasma-, ER- and nuclear membrane fragments, was suspended in reaction buffer: 50 mM Tris, pH 6.8, with 2-(*N*-morpholino) ethanesulfonic acid (MES), containing 50 mM KCl, 2 mM dithiothreitol, 2 mM EGTA, 5 mM sodium azide, and 1 mM ouabain. ATPases other than P-gp were blocked by this cocktail of inhibitors (ouabain is an inhibitor of the Na^+,K^+-channel, sodium azide blocks the F_0-F_1-ATPase, and EGTA inhibits calcium-dependent ATPases) which did not interfere with the mammalian P-gp (Ambudkar *et al.*, 1992; Sarkadi *et al.*, 1992). The sample was further diluted with reaction buffer to a protein concentration of 0.5 mg/ml and subsequently used for the ATPase assay. The membrane suspensions were divided into aliquots and stored at –20 °C until use.

P-gp ATPase activity assay

ATPase activity was measured basically as described by Sarkadi *et al.* (1992) by determining the liberation of inorganic phosphate from ATP with a colorimetric reaction. Briefly, membranes (25 μg) were incubated at 37 °C for 5 min in 30 μl Tris-MES buffer, pH 6.8 (50 mM Tris-MES, pH 6.8, 50 mM KCl, 5 mM sodium azide, 2 mM EGTA) and 20 μM of each test compound in the presence or absence of 200 μM sodium orthovanadate in duplicate wells of a 96-well plate. The reaction was started by addition of 30 μl 10 mM ATP and was stopped 20 min later by addition of 30 μl of 10% SDS-containing antifoam A. Detection reagent [180 μl, 35 mM ammonium molybdate/15 mM zinc acetate (1:4), pH 5.0, and 10% ascorbic acid, pH 5.0] was added to all wells and incubated at 37 °C for 20 min. The absorbance at 800 nm was measured via a microplate spectrophotometer. The ATPase activity (nmol min^{-1} mg $protein^{-1}$) was determined as the difference between the amounts of inorganic phosphate released from ATP in the absence and presence of vanadate. Phosphate standards were prepared in each plate (Litman *et al.*, 1997).

Determination of P-gp by Western blotting

Western blots were carried out as described by Towbin *et al.* (1979) in plasma membrane-enriched fractions of rat hepatocytes. Membrane proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and transfected on nitrocellulose membranes. Blots were blocked with 3% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20, for 6 h, and incubated with anti P-gp monoclonal antibody clone JSB1 at a concentration of 0.25 mg/ml. Immunoblots were detected by horse radish peroxidase conjugated anti mouse IgG (1:600 dilution) according to the manufacturer's instructions. The colour was developed by adding 3,3'-diaminobenzidine tetrahydrochloride

and H_2O_2 in Tris-HCl (0.05 M, pH 7.3) buffer. Visualized bands were photographed and the intensity was quantified by densitometry.

RNA extraction and quantitative RT-PCR

RNA from liver cells was isolated with the TRIzol reagent®, and RT-PCR was performed using one-step RT-PCR (Gibco-BRL, California, USA). The MDR1 forward primer sequence GCCTGGCAGCTGGAAGACAAATACACAAAATT and the reverse primer sequence CAGACAGCAGCTGACAGTCCAAGAACAGGACT were used corresponding to residues 406–437 and 657–688, respectively, of the published cDNA sequence (Murphy *et al.*, 1990). Using these primers, PCR gave a 283-bp product. Amplification was performed for 30 cycles of sequential denaturation (94 °C, 1 min), annealing (55 °C, 1 min), and extension (72 °C, 1 min). A total of 10 μl of each PCR product was electrophoresed in 1× Tris/acetate/EDTA (TAE) electrophoresis buffer on a 1% agarose gel. Gels were stained with 2 μg/ml of ethidium bromide and photographed with a Polaroid positive-negative film. The negative films were analyzed by scanning densitometry.

Glutathione-S-transferase (GST) activity

The total GST enzyme activity was assayed by spectrophotometric determination of 1-chloro-2,4-dinitrobenzene (CDNB) conjugation with glutathione (GSH) (Habig and Jakoby, 1981). The GST enzyme activity was expressed in nmol min^{-1} mg $protein^{-1}$.

Reduced glutathione (GSH) level

The GSH content was determined as previously described (Beutler *et al.*, 1963); the method is based on determination of a yellow hue that develops when 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) is added to sulfhydryl compounds. Briefly, 0.2 ml of sample was added to 1.80 ml

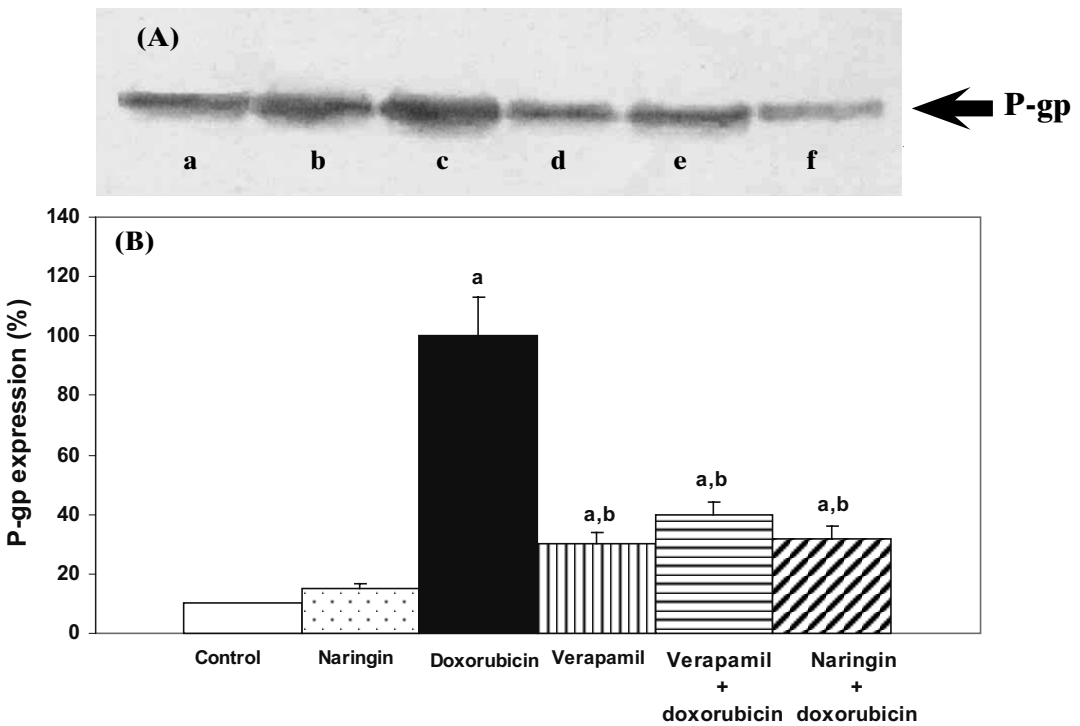

Fig. 1. (A) The P-gp protein level was determined by Western blotting using anti P-gp monoclonal antibody clone JSB1 and quantified by densitometry. Lane a, naringin; lane b, verapamil; lane c, doxorubicin; lane d, verapamil + doxorubicin; lane e, naringin + doxorubicin; lane f, control (untreated). (B) Data are expressed as means ± SE (n = 8). a and b indicate a significant difference from control and doxorubicin-treated groups, respectively, at $P < 0.05$ using Student's t-test.

of distilled water. The sample was precipitated with 3 ml of precipitating solution (1.67 g glacial metaphosphoric acid + 0.2 g EDTA + 30 g NaCl/100 ml distilled water). The mixture was allowed to stand for 5 min and was then filtered. A quantity of 2 ml of the filtrate was added to 8 ml of 0.3 mol/l Na_2HPO_4 solution + 1 ml of DTNB reagent (40 mg/100 ml distilled water of 1% sodium citrate). The developed colour was measured spectrophotometrically at 412 nm. Results were expressed as nmol mg protein^{-1}.

Statistical analysis

Data were expressed as means ± SE and analyzed statistically using Student's *t*-test. Results were considered to be statistically significant when $P < 0.05$.

Results

Effect of naringin on P-gp/MDR1 expression

Western blot analysis of the level of P-gp in the studied groups revealed measurable amounts of P-gp in normal rat liver (Fig. 1A, lane f), which were found to be significantly increased with exposure to verapamil and doxorubicin (Fig. 1A, lanes b and c, respectively). This increased level of P-gp with exposure to verapamil and doxorubicin was found to be reversed to the normal levels by the treatment with naringin (Fig. 1A, lane e). Administration of naringin alone was similar to P-gp expression in the control group (Fig. 1A, lane f). The P-gp levels were decreased by 70% in the group treated with verapamil alone and by 85% in the control group, while they were decreased by 60% and 68%, respectively, in groups pre-treated with verapamil and naringin prior to doxorubicin compared with the doxorubicin-treated group (Fig. 1B).

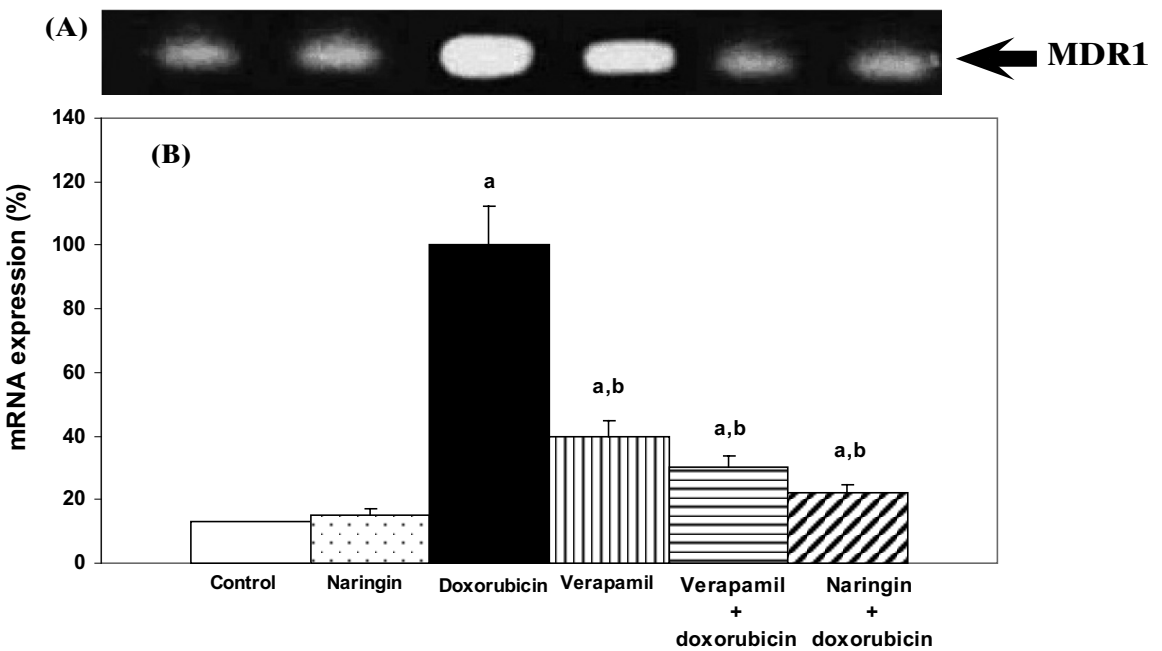

Fig. 2. (A) Analysis of MDR1 mRNA levels in the studied groups. From left to right: control, naringin, doxorubicin, verapamil, verapamil + doxorubicin, naringin + doxorubicin as determined by RT-PCR. The PCR products (283 bp MDR1) were run on 1% agarose gel. (B) The bands from the autoradiographs were quantified by densitometry. Data are expressed as means ± SE ($n = 8$). a and b indicate a significant difference from control and doxorubicin-treated groups, respectively, at $P < 0.05$ using Student's *t*-test.

Table II. Hepatic ATPase and GST activities as well as GSH level in the studied groups.

Group	ATPase [nmol min^{-1} mg $protein^{-1}$]	GST [nmol min^{-1} mg $protein^{-1}$]	GSH [nmol mg $protein^{-1}$]
Control	20.40 ± 2.75	280.50 ± 22.60	30.60 ± 2.80
Naringin	19.70 ± 2.16	170.70 ± 18.30[a]	21.50 ± 2.30[a]
Verapamil	26.80 ± 2.85	300.40 ± 28.70[a]	39.20 ± 3.75[a]
Doxorubicin	38.60 ± 4.30[a]	360.30 ± 35.10[a]	50.30 ± 6.20[a]
Verapamil + doxorubicin	26.50 ± 3.10[a,b]	310.30 ± 34.80[a,b]	35.90 ± 4.30[a,b]
Naringin + doxorubicin	28.90 ± 3.30[a,b]	330.30 ± 32.60[a,b]	40.70 ± 5.80[a,b]

Values are means ± SE; $n = 8$ rats.
[a] $P < 0.05$ significant with respect to control; [b] $P < 0.05$ significant with respect to doxorubicin.

To verify the expression of MDR1 mRNA in the studied groups, PCR products were measured after amplification. The levels of MDR1 mRNA, as shown in Fig. 2, revealed a decrease in the level of MDR1 mRNA by 60% in the group treated with verapamil alone and by 87% in the control group; while pre-treatment with verapamil or naringin prior to doxorubicin treatment resulted in levels reduced by 70% and 78%, respectively, as compared to the doxorubicin-treated group.

ATPase activity

As shown in Table II, in contrast to the verapamil-treated group, doxorubicin treatment produced a significant increase ($P < 0.05$) in the ATPase activity as compared to the control group, while in the naringin-treated group there was no change in the ATPase activity. It has to be noted that the relative ATPase activity levels were lower in the case of verapamil treatment than for doxorubicin treatment, which is most probably reflecting a lower turnover rate of the former transporter protein. Pre-treatment with verapamil and naringin prior to doxorubicin treatment significantly ($P < 0.05$) inhibited the ATPase activity as compared to the doxorubicin-treated group. The results revealed that the relative ATPase activity levels in the naringin-pre-treated group were comparable with the verapamil-pre-treated group.

GST activity and GSH level

The results of GST activity and GSH level tests revealed that treatment with verapamil or doxorubicin produced a significant increase ($P < 0.05$) in the GST activity and GSH level as compared to the control group, while in the group treated with naringin both GST and GSH significantly decreased ($P < 0.05$) (Table II). On the other hand, in the verapamil or naringin pre-treated groups the activity of GST and the level of GSH showed a significant decrease ($P < 0.05$) as compared to the doxorubicin group pre-treated with verapamil or naringin.

Discussion

P-gp-mediated MDR is one of the major obstacles in cancer chemotherapy (Roninson, 1992). Therefore, much of the current research efforts are focused on the identification of the compounds capable of reversing MDR without much toxicological consequences (Sikic *et al.*, 1997).

Results of the present investigation revealed that doxorubicin-induced P-gp levels were significantly inhibited by naringin, a predominant flavanone found in grapefruit. Naringin resulted in about 78% reversal of the doxorubicin-induced MDR1 mRNA expression in comparison to 70% reversal by verapamil, a well-known MDR-reversing agent (Muller *et al.*, 1994; Bennis *et al.*, 1995). Also the levels of P-gp were decreased by 68% and 60% in the doxorubicin group pre-treated with naringin and verapamil, respectively. Tsuruo *et al.* (1981) showed that verapamil reversed the approximately 30-fold resistance to vincristine and 7-fold resistance to vinblastine displayed by the MDR murine leukemia cell line P388/VCR. But its clinical use has been hampered by the severe cardiovascular effects, which occur at doses required for effectiveness (Salmon *et al.*, 1991).

Most of the MDR-reversing compounds share some common physical characteristics such as cyclicity, lipophilicity, and a positive or neutral charge at physiological pH value (Zamora *et al.*, 1988). Based on its hydrophobic nature and aromatic components, naringin was considered as a

potential chemosensitizing agent resulting in a much higher inhibition in P-gp expression in comparison to verapamil.

The anticarcinogenic properties of naringin have been well documented (Wattenberg and Coccia, 1991; Fuhr *et al.*, 1993; Jagetia and Reddy, 2002), still the mechanism of its anticarcinogenic action is not fully understood. The present study, for the first time, showed the inhibitory effect of naringin on the doxorubicin-induced P-gp overexpression in *in vivo* models and thus its potential as an MDR-reversing agent. However, further studies are required to provide more evidences for the MDR-reversing efficacies of naringin based upon the mechanism and the kinetics of inhibition. Thus, a remarkably low level of toxicity of naringin coupled with its MDR-reversing activity may contribute to the known anticarcinogenic properties of this compound.

It is clear from our results that GSH is required for P-gp function (drug efflux). Depletion of the cellular GSH levels by treatment with naringin (Table II) increases the accumulation of doxorubicin inside the cells accompanied with depletion in the expression of P-gp protein (Fig. 1A, lane e) compared to the treatment with doxorubicin alone (Fig. 1A, lane c) which causes an increase in the level of GSH according to the capture the free radicals formed by doxorubicin. The mechanism by which GSH enables the ATP-dependent doxorubicin transport by P-gp is not clear. The results also revealed that doxorubicin increased the activity of GST (catalyzes the interaction between GSH and drug), while treatment with naringin reduced the activity of GST. These results agree with those of Tew and Klapper (1988) who mentioned that GST catalyzes interactions between glutathione and alkylating drugs, increasing the rate of drug detoxification. So activation of these enzymes can cause cellular drug resistance. Resistance of tumour cells to drugs of the P-gp-MDR group (anthracyclines, vincristine) can also be connected with alterations of the GSH system (Stavrovskaya, 2000). In cells with P-gp-MDR, increased levels of a GST isoenzyme, GSTp, are frequently found (Tew and Klapper, 1988). In the present study, we also investigated the effects of naringin on the ATPase activity and the results revealed that naringin also effectively inhibited the doxorubicin-stimulated ATPase activity of P-gp (Table II).

In conclusion, the present investigation suggests that naringin, one of the predominant flavonones found in grapefruit and other related citrus species, is a highly effective and potent modulation agent for multiple drug resistance *in vivo*, and could be considered as a promising compound for the design of more efficacious multiple drug resistance chemosensitizers.

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