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Reversal of cancer multidrug resistance by green tea polyphenols

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Abstract

The aim of this study was to examine the effect and mechanism of green tea polyphenols (TP) on reversal of multidrug resistance (MDR) in a carcinoma cell line. Using the MTT assay, TP was examined for its modulating effects on the drug-resistant KB-A-1 cells and drug-sensitive KB-3-1 cells. When 10 μg mL^{-1} (–)-epigallocatechin gallate (EGCG) or 40 μg mL^{-1} TP were present simultaneously with doxorubicin (DOX), the IC50 of DOX on KB-A-1 cells decreased from 10.3 ± 0.9 μg mL^{-1} to 4.2 ± 0.2 and 2.0 ± 0.1 μg mL^{-1}, respectively. TP and EGCG enhanced the DOX cytotoxicity on KB-A-1 cells by 5.2- and 2.5-times, respectively, but did not show a modulating effect on KB-3-1 cells. This indicated that both TP and EGCG had reversal effects on the MDR phenotype in-vitro. A KB-A-1 cell xenograft model was established, and the effect of TP on reversing MDR in-vivo was determined. Mechanistic experiments were conducted to examine the uptake, efflux and accumulation of DOX. Cloning and expression of the nucleotide binding domain of the human MDR1 gene in *Escherichia coli* was established, and by using colorimetry to examine the activity of ATPase to hydrolyse ATP, the ATPase activity of target nucleotide binding domain protein was determined. TP exerted its reversal effects through the inhibition of ATPase activity, influencing the function of P-glycoprotein, and causing a decreased extrusion of anticancer drug and an increased accumulation of anticancer drug in drug resistant cells. Using reverse transcription-polymerase chain reaction, the inhibitory effect of TP on MDR1 gene expression was investigated. Down-regulation of MDR1 gene expression was the main effect, which resulted in the reversal effect of TP on the MDR phenotype. TP is a potent MDR modulator with potential in the treatment of P-glycoprotein mediated MDR cancers.

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Introduction

Multidrug resistance (MDR) is characterized by the ability of cells exposed to a single drug to produce resistance to a broad range of structurally and functionally distinct drugs. Since clinical resistance to chemotherapeutic drugs is still a major problem in cancer treatment (Simon & Schindler 1994), a large number of studies has been devoted to elucidating the mechanisms leading to MDR in tumour cells. One of the best understood is the classical MDR mediated by P-glycoprotein (P-gp), a 170-kDa transmembranous ATP-dependent efflux pump, which decreases cellular drug accumulation in resistant cells (Juliano & Ling 1976; Kartner et al 1983). P-gp is encoded by the MDR1 gene, which when activated in resistant malignant cells, confers on them the MDR phenotype. The efflux action of P-gp is able to exclude a number of naturally occurring, though structurally unrelated cytotoxics, including anthracyclines, epipodophyllotoxins and vinca alkaloids (Baines et al 1992; Nooter & Sonneveld 1994; Germann 1996).

Reversal of MDR by non-toxic agents that block the transport activity of P-gp has been a challenging area for pharmaceutical development. When co-administered with a non-toxic agent, known as a MDR modulator, the net accumulation of relevant cytotoxic drugs within tumour cells can be enhanced. Determination of the level of P-gp expression in tumour cells and reversal of its MDR effects by prior use of a MDR modulator could be a useful way to improve the chemotherapeutic effects of drugs. The use of modulators to increase the effectiveness of antitumour agents increases the number of medications and thus adds to the patient's burden. Modulators based on natural food or beverage components could be useful in enhancing the activity of antitumour drugs without side-effects, and thus improving the quality of life of patients.

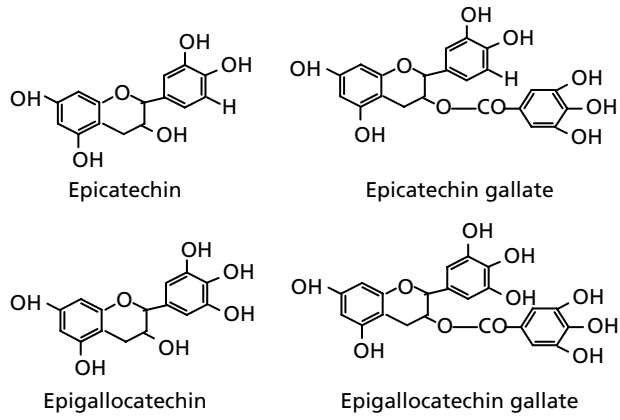

Figure 1 Chemical structure of four components of green tea polyphenol.

Consumption of green tea has been reported to afford protection against carcinogenesis of human oesophagus, fore stomach, duodenum, colon, liver and lung (Kato et al 1990; Gao et al 1994; Dreosti et al 1997). The chemopreventive effect against cancer has also been demonstrated in mouse and rat models (Huang et al 1992; Narasiwa & Fukaura 1993; Katiyar & Mukhtar 1996). The main responsible component of green tea is tea polyphenol (TP), especially flavonols of the catechin-type (Figure 1), (–)-epigallocatechin gallate (EGCG) in particular (Ahmad & Mukhtar 1999). It has been assumed that the chemopreventive effects of these compounds involve antioxidant and free radical scavenging activity, and stimulation of detoxification systems through selective induction or modification of phase 1 and phase 2 metabolic enzymes. In addition, green tea may inhibit biochemical markers of tumour initiation and promotion, including the rate of cell replication, and thus inhibition of the growth and development of neoplasms, and prevention of mutagenicity and genotoxicity (Zloch 1996; Weisburger 1999). Recent studies have indicated an inverse association between green tea consumption and cancer risk, and support a possible chemopreventive effect of green tea (Ohno et al 1995; Yu et al 1995; Buschman 1998). However, there are few reports about using green tea and its main components as anticancer agent modulators (Sugiyama & Ueda 1991; Gerd & Manfred 1997; Yasuyuki et al 2000). Given that green tea is inexpensive, non-toxic, and is a popular beverage consumed worldwide (Yang & Wang 1993), the aim of this study was to investigate if the main components of green tea (TP and EGCG) are suitable modulators of MDR.

Materials and Methods

Cells

A human oral epidermoid carcinoma drug-sensitive cell line (KB-3-1) and a human oral epidermoid carcinoma drug-resistant cell line (KB-A-1), which is a MDR subclone derived from KB-3-1 by inducing with doxorubicin (DOX) (Sigma, St Louis, MO, USA), were kindly provided by Ira Pastan and Michael M. Gottesman (National Institutes of Health, USA). KB-A-1 was cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc., MA, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco, Carlsbad, CA, USA) and 1 $\mu g\,mL^{-1}$ DOX. KB-3-1 was cultured in DMEM supplemented with 10% (v/v) fetal bovine serum. Both cell cultures were incubated at 37°C in a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO_2.

Measurement of cell growth and viability

Cell growth and viability were measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. Cells from exponentially growing cultures were harvested and dispensed within 96-well culture plates in 200 μL of medium at a concentration of 10^4 cells/well for KB-3-1 and KB-A-1 cell lines. The cells were exposed to drugs previously determined for each cell line. Cell viability was measured after 3 days of culture using the MTT assay (Mosmann 1983; Carmichael et al 1987). MTT stock solution (10 μL; 5 $mg\,mL^{-1}$ in phosphate-buffered saline) was added to each well and, after 4 h incubation at 37°C, the supernatant was removed and replaced with 150 μL dimethylsulfoxide. After formazan solubilization, the extinction of each well was measured using a Bio-Rad 550 Automated Microplate Reader (Bio-Rad, Hercules, CA, USA). The cell viability was calculated by the following equation: cell viability (%) = (mean extinction of one group/mean extinction of the control) × 100. The reversal index is equal to the IC50 without modulator versus the IC50 with modulator.

Measurement of DOX concentration in KB cells

Cells from exponentially growing cultures were harvested and dispensed in 96-well culture plates in 200 μL of medium at a concentration of 10^4 cells/well for KB-3-1 and KB-A-1 cell lines. After 3 days incubation, the cells were exposed to 10 $\mu g\,mL^{-1}$ DOX and 6, 8 and 10 $\mu g\,mL^{-1}$ EGCG; the control was treated with 10 $\mu g\,mL^{-1}$ DOX alone. After incubation of 1.5 h for KB-3-1 and 3.0 h for KB-A-1 cells, the cells were washed three times with cold phosphate-buffered saline and then resuspended with 0.5 M HCl in 50% (v/v) ethanol. The concentration of DOX in the organic phase was determined with a fluorescence plate reader (Thermo Labsystem, Helsinki, Finland) (excitation 485 nm; emission 538 nm). DOX concentrations were given by the DOX standard curve.

Measurement of DOX efflux and influx from KB-A-1 cells

To examine the effects of EGCG (Sigma) and TP (in which the composition of EGCG was greater than 65%; Lvbao Natural Additives Co., Ltd, Wuxi, China) on the DOX efflux from KB-A-1 cells, cells from exponentially growing cultures were harvested and dispensed in 96-well culture plates in 200 μL of medium at a concentration of 10^4 cells/well for KB-A-1 cells. After 3 days incubation, the cells were

pre-incubated in the medium at 37°C for 30 min. The cells were then exposed to 10 μg mL^{-1} DOX combined with 10 μg mL^{-1} EGCG and 40 μg mL^{-1} TP; the control was treated with 10 μg mL^{-1} DOX alone. After 0–180 min incubation, the cells were washed three times with cold phosphate-buffered saline and then resuspended with 0.5 M HCl in 50% (v/v) ethanol. The concentration of DOX in the organic phase was determined with a fluorescence plate reader (excitation 485 nm; emission 538 nm). DOX concentrations were given by the DOX standard curve. In addition, to examine the effects of TP and EGCG on the DOX influx from KB-A-1 cells, the cells were exposed to 10 μg mL^{-1} DOX combined with 10 μg mL^{-1} EGCG and 40 μg mL^{-1} TP; the control was treated with 10 μg mL^{-1} DOX alone. Subsequent procedures were the same as those describe above.

RNA extraction and cDNA synthesis

Total RNA was extracted by the TROZOL method, according to the manufacturer's instructions (TelTest Inc., Friendswood, TX, USA). RNA purity and quantity were assessed by UV absorbance (A260/A280 > 1.8). The reverse transcription of poly(A)$^+$ mRNA to cDNA was performed using a reverse transcription kit (Promega, Madison, WI, USA). A coding sequence of the MDR1 gene nucleotide binding domain (NBD) fragment was obtained by polymerase chain reaction (PCR) (forward primer: 5′ CGG GAT CCA TGC CGA ACA CAT TG 3′; reverse primer: 5′ CGG AAT TCT CAC TGG CGC TTT G 3′). The PCR conditions were as follows: (i) denaturation at 92°C for 4 min; (ii) 35 cycles of denaturation at 92°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 60 s plus 1 s increase per cycle; and (iii) a final elongation at 72°C for 10 min. The PCR product, showing the expected size of 775 bp on agarose gel electrophoresis, was digested with BamHI and EcoRI to generate ends compatible with in-frame insertion into the pGEX-4T-1 plasmid.

Construction of the expression plasmid encoding CNBD

The pGEX-4T-1 plasmid was linearized with BamHI and EcoRI and ligated with CNBD cDNA to generate a plasmid coding for an in-frame fusion protein composed of glutathione *S*-transferase and NBD2. The design of pGEX-4T-1 included a thrombin cleavage site and an upstream glycine kinker engineered between the glutathione *S*-transferase and NBD2 sequences. *Escherichia coli* strain BL21 (DE3) was transformed with the ligation product and transformants were selected on Luria-Bertani broth agar plates containing 100 μg mL^{-1} ampicillin. Correct recombinants were identified by sequencing.

Expression, refolding and purification of CNBD

E. coli cells containing CNBD were cultured in 200 mL of 2 YTA medium (16 g L^{-1} tryptone, 10 g L^{-1} yeast extract, 5 g L^{-1} NaCl and 100 μg mL^{-1} ampicillin) at 37°C with vigorous shaking until the optical density reached 1.5 units at 600 nm. The culture was then induced with 10 g L^{-1} lactose and continued for an additional 12-h period at 30°C. Afterwards, the culture was centrifuged at 9300 *g* in a Beckman JA-10 rotor (Beckman, Fullerton, CA, USA) for 10 min at 4°C. The drained pellet can be stored at −80°C for up to 2 weeks or subjected to subsequent cell disruption.

Pelleted bacteria were suspended in 60 mL Tris-HCl buffer (pH 8.0) with EDTA (10 mM) and lysozyme was added to 0.5 mg mL^{-1}. The mixture was stirred (30 min, 4°C), followed by sonication and separation of soluble and insoluble fractions by centrifugation (16 000 *g*, 30 min). Sedimented inclusion bodies were suspended in cold distilled water containing 0.1% (v/v) Triton X-100 and centrifuged three times, until all impurities were removed. This was followed by three more washes in water not containing Triton X-100. Inclusion body pellets were homogenized in solubilization buffer (6 M guanidine hydrochloride/0.1 M Tris, pH 8.0/2 mM EDTA) and stored at room temperature for at least 2 h. Solubilized inclusion body protein was harvested by centrifugation at 20 000 *g* for 30 min. Renaturation was initiated by a rapid 100-fold dilution of the solubilized protein into refolding buffer (0.1 M Tris, pH 8.0/0.5 mL-arginine/2 mM EDTA) and maintained at 10°C for 12 h. Renatured protein was dialysed against 20 mM Tris, pH 8.0/100 mM NaCl (50 L) overnight. Afterwards, the sample was diluted with 3.6 mL of 50 mM Mes buffer, pH 6.2, and applied to a CM-Sepharose column (0.7 × 2.5 cm), previously equilibrated with buffer 2 (50 mM NaCl, 50 mM Mes, pH 6.2). The column was washed through with the same medium and then eluted with 20 mL of a linear NaCl gradient (50–500 mM) at a flow rate of 60 mL h^{-1}; 0.5-mL fractions were collected. Absorbance of the column eluent was monitored continuously at 280 nm.

Measurement of ATPase activity using a colorimetric assay

P-gp associated ATPase activity of recombinant proteins was determined by the release of inorganic phosphate using a colorimetric assay. Protein solution (100 μg mL^{-1}) was incubated at 37°C for 5 min in a reaction mixture containing 50 mM Tris (pH 7.5), 5 mM sodium azide, 2 mM EGTA (pH 7.0), 2 mM ouabain, 50 mM NaCl and 6 mM $MgCl_2$. The reactions were initiated by adding 5 μL of ATP to give a final concentration of 2 mM in a 50-μL final volume and incubated at 37°C for 20 min. The reactions were terminated by adding 50 μL of 6% (w/v) sodium dodecyl sulfate, 3% (w/v) ascorbate and 0.5 M sodium molybdate in 0.5 M HCl. Products were stabilized by adding 50 μL of 2% (w/v) sodium citrate, 2% (w/v) sodium meta-arsenite and 2% (v/v) acetic acid. After incubation at 37°C for 10 min the absorbance was read at 850 nm.

Measurement of MDR1 gene expression

RNA from KB-A-1 cells was extracted by the TROZOL method, and reverse transcription-polymerase chain reaction (RT-PCR) was performed using a one-step kit (Promega). For MDR1, the forward primer sequence used was 5′ CCC ATC ATT GCA ATA GCA GG 3′ and the reverse primer

sequence was 5′ GTT CAA ACT TCT GCT CCT CA 3′, corresponding to residues 2596–2615 and residues 2733–2752, respectively, of the published cDNA sequence. Using these primes, PCR yielded a 156-bp product. Evaluation of the control sequence expression was carried out by using the forward primer sequence 5′ GCC ATT CTC ACC GGA TTC ACT CGT C 3′ and the reverse primer sequence 5′ AGC CGC CGT CCC GTC AAG TCA G 3′, which yielded a 323-bp product. Amplification was performed via 40 cycles of sequential denaturation (94°C, 1 min), annealing (65°C, 0.5 min), and extension (72°C, 1 min). A total of 10 μL of each PCR product was electrophoresed in 1 × Tris/acetate/EDTA electrophoresis buffer on a 1% (w/v) agarose gel. Gels were stained with 2 $\mu g\,mL^{-1}$ ethidium bromide.

Reversal of MDR in the KB-A-1 cell xenografts

Animal experiments were consistent with national and international ethical protocols for animal rights and animal experiments. KB-A-1 cells (10^6/animal) were transplanted under the shoulder of nude mice for the chemotherapeutic studies. After subcutaneous implantation of the cells, when the subcutaneous tumour was approximately 5 × 5 mm in size, mice were randomized into the control group and four treatment groups: DOX alone (2 $mg\,kg^{-1}$ i.p.) every 2 days for 4 days, TP alone (40 $mg\,kg^{-1}$ i.p.) every 2 days for 4 days, DOX (2 $mg\,kg^{-1}$) + TP (20 $mg\,kg^{-1}$ i.p.) (1 day DOX + 1 day TP) for 4 days, or DOX (2 $mg\,kg^{-1}$) + TP (40 $mg\,kg^{-1}$ i.p.) (1 day DOX + 1 day TP) for 4 days. The animal weight and tumour volume were measured every 3 days. The tumour volume was measured in two perpendicular diameters (A and B). Tumour volume (V) was estimated according to the formula: $V = p/6[(A + B)/2]^3$. The tumour growth curve was drawn according to tumour volume and time of implantation. The mice were killed on Day 21 and tumour weight was measured. The rate of inhibition was calculated according to the formula: inhibition rate (%) = (1 − tumour weight in the drug treatment group/tumour weight in the control group) × 100.

Statistical analysis

The effect of the various compounds on the IC50, the concentration of DOX entered and intracellular concentration of DOX, the change in bodyweight and tumour weight, the intracellular concentration of DOX, the control ATPase activity, and the volume of tumour were all analysed using the Kruskal–Wallis test. Individual differences between the concentrations were then evaluated using Dunn's test. A value of $P < 0.05$ was considered statistically significant.

Results

MDR reversal by TP and EGCG in KB-A-1 cells

Based on the TP or EGCG concentration that had no growth inhibition on KB-A-1 and KB-3-1 cells, combined with EGCG or TP, the IC50 values of DOX on KB-A-1 cells decreased from $10.3 \pm 0.9\,\mu g\,mL^{-1}$ to 4.2 ± 0.2 and $2.0 \pm 0.1\,\mu g\,mL^{-1}$. The reversal index values were 2.5 and 5.2, respectively (Table 1). These results indicate that TP and EGCG could reverse the MDR phenotype in-vitro (Mei et al 2003).

Table 1 Reversal effects of tea polyphenol (TP) and (–)-epigallocatechin gallate (EGCG) on multidrug resistance in the KB-A-1 cell line

Group	IC50 of DOX ($\mu g\,mL^{-1}$)	Reversal index
Control	10.3 ± 0.9	–
EGCG (10 $\mu g\,mL^{-1}$)	4.2 ± 0.2*	2.5
TP (40 $\mu g\,mL^{-1)}$	2.0 ± 0.1*	5.2

Cells were treated for 72 h in the presence of doxorubicin (DOX) combined with EGCG or TP in DMEM/10% fetal bovine serum. Cell viability was determined by the MTT assay and the IC50 value was expressed as the mean (± s.e.m.) of four separate experiments. Statistical significance was taken at $*P < 0.05$. The reversal index is equal to the IC50 without modulator versus the IC50 with modulator.

Effect of EGCG on the intracellular DOX accumulation in KB-A-1 and KB-3-1 cells

When treated with DOX, the intracellular accumulation of DOX in drug sensitive KB-3-1 cells was 1.6-times greater than that in drug resistant KB-A-1 cells (Figure 2), indicating that KB-A-1 cells have the typical MDR phenotype: decreased steady-state drug accumulation. When KB-A-1 and KB-3-1 cells were exposed to 10 $\mu g\,mL^{-1}$ DOX combined with 6, 8 and 10 $\mu g\,mL^{-1}$ EGCG for 3 and 1.5 h, respectively, the intracellular concentrations of DOX in KB-A-1 cells were increased 1.6-, 1.7- and 1.8-times, respectively,

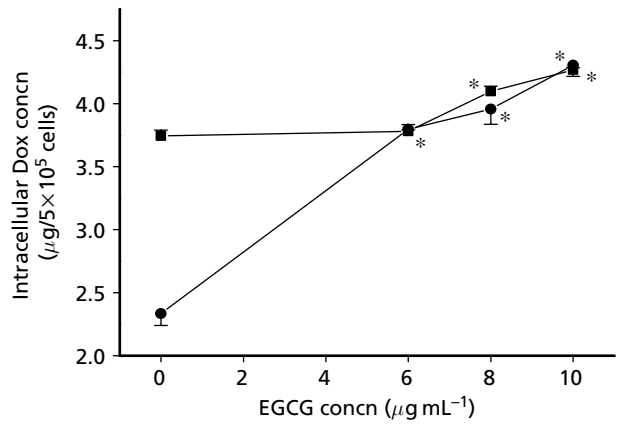

Figure 2 Effect of (–)-epigallocatechin gallate (EGCG) on the intracellular doxorubicin (DOX) accumulation in KB-3-1 (■) and KB-A-1 (●) cells. KB-A-1 and KB-3-1 cells were treated for 3 or 1.5 h, respectively, in the absence or presence of different concentrations of EGCG. The intracellular DOX accumulation was then determined by fluorescence assay and was expressed as the mean (± s.e.m.) of four separate experiments. Significant differences are indicated by $*P < 0.05$.

Table 2 Effect of (–)-epigallocatechin gallate (EGCG) on the intracellular doxorubicin (DOX) accumulation in KB-A-1 and KB-3-1 cells

Group	Concentration of DOX entered ($\mu g\,mL^{-1}$)	Intracellular concentration of DOX ($\mu g\,mL^{-1}$)	Reversal multiple
DOX	10	2.335 ± 0.094	
DOX + 6 $\mu g\,mL^{-1}$ EGCG	10	3.798 ± 0.006*	1.6
DOX + 8 $\mu g\,mL^{-1}$ EGCG	10	3.958 ± 0.122*	1.7
DOX + 10 $\mu g\,mL^{-1}$ EGCG	10	4.305 ± 0.088*	1.8

Each value represents the mean (± s.e.m.) of four independent experiments. Statistical significance was taken at $*P < 0.05$. The reversal multiple of each concentration of EGCG was calculated by: concentration of DOX in the combined group/concentration in the DOX alone group.

compared with DOX alone treatment. The intracellular concentrations of DOX in KB-3-1 cells were not significantly increased (Table 2), and after combination treatment, the intracellular accumulation of DOX in drug-resistant cells was increased to the level of drug-sensitive cells. The results showed that EGCG could increase the intracellular concentration of DOX in MDR cells and therefore reverse the MDR phenotype.

Effects of TP and EGCG on DOX influx and efflux in KB-A-1 cells

The effects of TP and EGCG on DOX influx in KB-A-1 cells are shown in Figure 3. The TP and EGCG treatment increased the rate of DOX influx into KB-A-1 cells. After

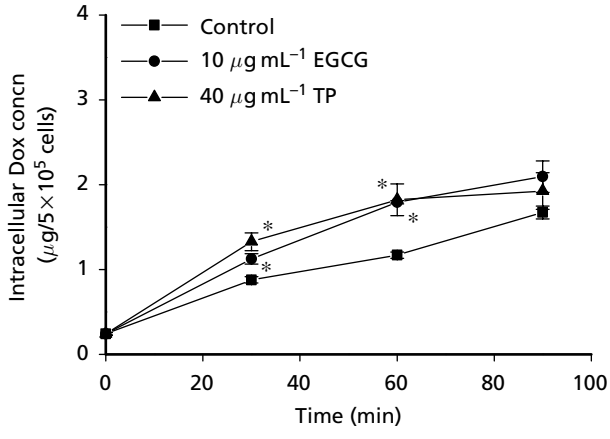

Figure 3 Effect of tea polyphenol (TP) or (–)-epigallocatechin gallate (EGCG) on the time course of doxorubicin (DOX) accumulation in KB-A-1 cells as to the DOX influx system. Each point represents the mean (± s.e.m.) of four independent experiments. Significant differences are indicated by $*P < 0.05$.

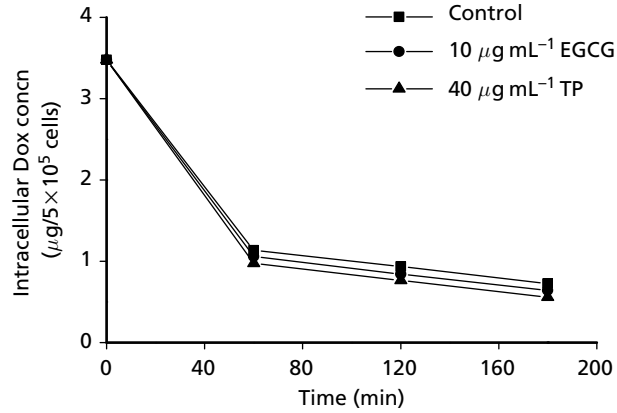

Figure 4 Effect of tea polyphenol (TP) or (–)-epigallocatechin gallate (EGCG) on the time course of doxorubicin (DOX) accumulation in KB-A-1 cells as to the DOX efflux system. Each point represents the mean of four independent experiments.

45-min combination treatments, the intracellular concentration of DOX in KB-A-1 cells had already increased to the accumulation level of the 90-min treatment of DOX alone. After 90 min, EGCG and TP increased the rate of DOX influx by 15.1% and 25.3%, respectively, compared with the DOX alone group. The effects of TP and EGCG on DOX efflux from KB-A-1 cells are shown in Figure 4. The TP and EGCG treatment decreased the rate of DOX efflux in KB-A-1 cells. After 180 min, EGCG and TP decreased the rate of DOX influx by 15.2% and 30.3%, respectively, compared with the DOX alone group. The results suggest that, through an increase of the influx rate of DOX into drug-resistant cells and a decrease of the efflux rate of DOX from drug-resistant cells, TP and EGCG could increase the intracellular accumulation of DOX in drug-resistant cells and therefore reverse MDR.

Inhibitory effects of TP and EGCG on the ATPase activity of P-gp

Cloning and expression of the NBD of the human MDR1 gene in *E. coli* was established and, by using colorimetry to examine the ability of ATPase to hydrolyse ATP, the ATPase activity of the target NBD protein was examined. Using this method, we found that TP and EGCG inhibited the ATPase activity of P-gp in dose-dependent manner (Figure 5) compared with the classic modulator, verapamil (data not shown; verapamil cannot influence the ATPase activity of P-gp). Therefore it can be concluded that TP and EGCG exert their reversal effect on MDR through modulation of the ATPase activity of P-gp.

Effects of TP and EGCG on MDR1 expression

To verify if TP and EGCG could modulate the spontaneous P-gp expression (mRNA) occurring in-vitro, 10 $\mu g\,mL^{-1}$ EGCG and 40 $\mu g\,mL^{-1}$ TP were added to the culture medium and the cultures were examined at 72 h. Using RT-PCR, a decrease in MDR1 expression was found (Figure 6). Thus,

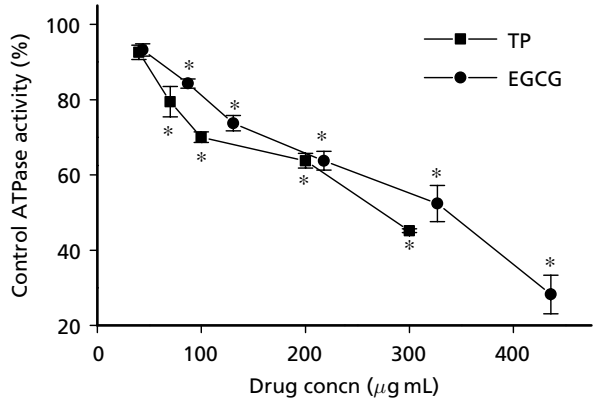

Figure 5 Inhibitory effects of tea polyphenol (TP) and (–)-epigallocatechin gallate (EGCG) on the ATPase activity of P-glycoprotein. Each value represents the mean (± s.e.m.) of three separate experiments. Significant differences are indicated by $*P < 0.05$.

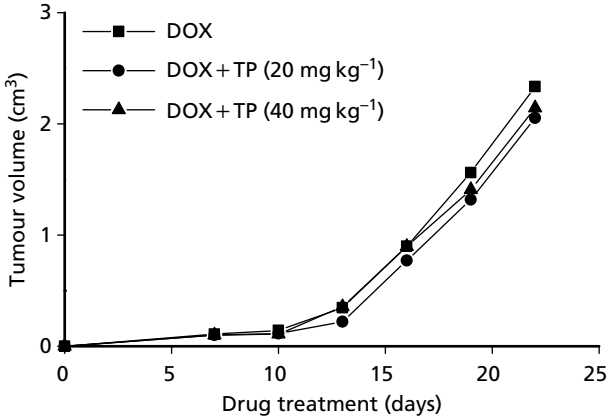

Figure 7 Modulation by tea polyphenol (TP) of the inhibitory effect of doxorubicin (DOX) on tumour growth in-vivo.

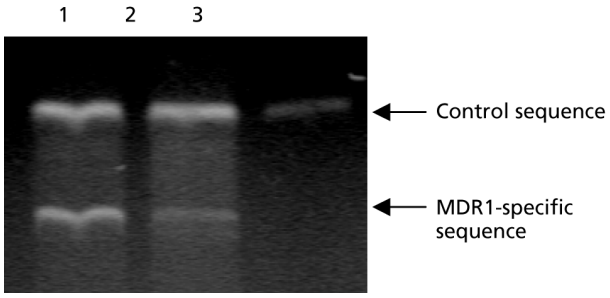

Figure 6 Effect of (–)-epigallocatechin gallate (EGCG) and tea polyphenol (TP) on the MDR1 expression of KB-A-1 cells. Lane 1: control from untreated KB-A-1 cells; lane 2: treatment of 10 $\mu g\,mL^{-1}$ EGCG; lane 3: treatment of 40 $\mu g\,mL^{-1}$ TP.

it can be concluded that TP and EGCG down-regulated MDR1 gene expression, which resulted in the reversal effect of TP on the MDR phenotype.

MDR reversal by TP in xenografts

When MDR tumours were treated with TP, tumour growth suppression was observed in the in-vivo model established by the nude mice xenografts. Figure 7 shows that tumour growth curves in the xenografts of KB-A-1 cells. Tumour growth was much slower for the group given DOX and TP compared with the DOX alone group. Tumour weights were 1.513 g, 1.544 g, 1.284 g, 0.906 g and 0.938 g in the control group, TP alone, DOX alone and co-administration groups at Day 21 after implantation, respectively (Table 3). The results showed that TP alone had no anticancer effect in the nude mice xenografts of KB-A-1 cells, and the DOX treatment only had 14.1% inhibition rate (Table 3). However, the combination of DOX and TP significantly inhibited the growth of the xenograft and the inhibition rate (versus the control weight of tumour) was 41.1% and 38.0% (Table 3). The results demonstrated that TP could reverse MDR in-vivo.

Importantly, the combination of DOX and TP did not cause death in any of our experiments. The bodyweights of the mice after the experiments were greater than before the experiments in the combination group, and the rate of weight increase was greater than in the DOX alone treatment group, suggesting that the treatment regimen did not result in increased toxic side-effects.

Discussion

Nearly 50% of human cancers are either completely resistant to chemotherapy or respond only transiently, after

Table 3 Effect of tea polyphenol (TP) on the reversal of multidrug resistance in the xenograft model of KB-A-1 cells in nude mice

Group	Animal weight before experiment (g)	Animal weight after experiment (g)	Change (%)	Tumour weight (g)	Inhibition rate (%)
Control	21.7 ± 1.98	27.1 ± 2.09	24.9	1.513 ± 0.19	
TP (40 mg kg^{-1})	22.3 ± 2.02	27.9 ± 2.05	25.1	1.544 ± 0.21	–
DOX (2 mg kg^{-1})	23.1 ± 2.02	23.7 ± 1.99	2.6	1.284 ± 0.18	15.1
DOX + TP (20 mg kg^{-1})	22.9 ± 2.11	25.0 ± 2.04	9.2	0.906 ± 0.20	41.1
DOX + TP (40 mg kg^{-1})	22.4 ± 2.08	24.2 ± 2.07	8.0	0.938 ± 0.18	38.0

DOX, doxorubicin.

which they are no longer affected by commonly used anticancer drugs. This phenomenon is referred to as MDR. In the present study, MDR was modelled by KB-A-1 cells. The KB-A-1 phenotype was selected for by subjecting KB-3-1 cells in a step-wise fashion to increasing concentrations of DOX. KB-A-1 cells overexpress the MDR1 gene, which results in the cross-resistance to anticancer agents compared with KB-3-1 cells (data not shown). Since KB-A-1 and KB-3-1 cell lines have been characterized extensively with respect to MDR as well as MDR1 gene expression, they were used in the present study to assess the reversal effect of TP on MDR.

The effect of TP and EGCG on the antineoplastic activity of DOX from a biochemical modulation approach was determined in-vitro. Combined with TP or EGCG, the antitumour activity of DOX on KB-A-1 cells was enhanced 5.2- and 2.5-times, respectively, but no similar reversal effect was found in KB-3-1 cells. In-vivo, the combination of TP and DOX had a potent inhibitory action. The inhibitory rate was 41.1% at most for the growth of the KB-A-1 xenografts, compared with the groups treated with the DOX alone or TP alone. Furthermore, the combination of TP and DOX did not cause death among the animals and no decrease in bodyweight was seen. Thus, it can be concluded that TP reversed MDR in-vivo and did not increase toxic side-effects of antitumour drugs. Therefore, TP may be an efficient and suitable modulator of MDR in-vitro and in-vivo.

The classic MDR phenotype is characterized by overexpression of P-gp, a 170 kDa plasma membrane glycoprotein that mediates efflux of several different cytotoxic compounds and decreases intracellular drug levels. Thus, it is paramount that the accumulation of anticancer drugs in MDR cells is improved if MDR is to be reversed. In the present study, the accumulation of DOX was greatly increased in drug-resistant KB-A-1 cells in the presence of TP. The increase in the antitumour agent concentration caused by a combination of drugs has been considered to occur through promotion of the influx or the inhibition of the efflux of the antitumour agent. This demonstrates that the circumvention of MDR mediated by TP is associated with an increased accumulation of the anticancer drug through modulation of the drug influx and efflux from MDR cells.

The effect of TP on P-gp ATPase activity was investigated. Cloning and expression of the NBD of the human MDR1 gene in *E. coli* was established and the ATPase activity of the target NBD protein was examined. Optimal ATPase activity is most important in the function of P-gp. The inhibition of ATPase activity may have potentially important implications. The inhibitory effect of TP on ATPase activity could result in interference with the drug transport capability of P-gp. This could lead to an increased intracellular level of cytotoxic agents, including DOX, thereby leading to a potentiation of the anticancer drug cytotoxicity. Our data suggest that one of the pathways of MDR modulation by TP resulted from the inhibition of ATPase activity, which then influenced the function of P-gp, caused a decrease in the extrusion of anticancer drugs and an increased accumulation of anticancer drugs in drug resistant cells. Using RT-PCR, the effect of TP on MDR1 gene expression was investigated. Down-regulation of MDR1 gene expression was the main effect, which resulted in the reversal effect of TP on MDR phenotype.

In conclusion, it is suggested that TP is a useful biochemical modulator and is effective for resistant tumours in-vitro and in-vivo. TP may modulate MDR by decreasing extrusion of anticancer drugs and increasing the intracellular anticancer drug accumulation through inhibition of ATPase activity of P-gp and down-regulation of MDR1 gene expression.

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