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Original Paper

Inhibition of P-glycoprotein Activity and Reversal of Multidrug Resistance *In Vitro* by Rosemary Extract

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The transmembrane transport pump P-glycoprotein (Pgp) causes the efflux of chemotherapeutic agents from cells and is believed to be an important mechanism in multidrug resistance (MDR) in mammary tumours. In the present study we demonstrate that an extract of the common dietary herb rosemary (*Rosemarinus officinalis* Labiatae), increases the intracellular accumulation of commonly used chemotherapeutic agents, including doxorubicin (DOX) and vinblastine (VIN), in drug-resistant MCF-7 human breast cancer cells which express Pgp. Rosemary extract (RE) inhibits the efflux of DOX and VIN, which are known to be substrates of Pgp, but does not affect accumulation or efflux of DOX in wild type MCF-7 cells, which lack Pgp. Treatment of drug-resistant cells with RE increases their sensitivity to DOX, which is consistent with an increased intracellular accumulation of the drug. RE blocks the binding of the VIN analogue azidopine to Pgp. Thus, it appears that RE directly inhibits Pgp activity by inhibiting the binding of drugs to Pgp. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: rosemary, P-glycoprotein, multidrug resistance, breast cancer, doxorubicin
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INTRODUCTION

MULTIDRUG RESISTANCE (MDR), the cross-resistance of tumour cells to a variety of structurally and functionally unrelated anticancer drugs, is a major obstacle in cancer treatment [1]. Although there are several mechanisms which confer MDR, one that is often overexpressed in mammary tumours following drug treatment is the 170 kD plasma membrane-associated glycoprotein (Pgp). Pgp acts as an energy-dependent drug efflux pump that decreases intracellular drug accumulation, thereby decreasing the effectiveness of many chemotherapeutic agents [2]. Since Pgp can confer MDR on tumour cells, the development of agents which inhibit the Pgp-mediated efflux of drugs, and thus reverse MDR, has been intensively pursued [3]. A broad range of compounds have been identified that are able to reverse MDR by blocking Pgp activity *in vitro*. However, they have not been widely used in the treatment of cancer patients because the doses required to reverse MDR resistance are either toxic or not clinically achievable [4]. A 'second generation' of MDR reversal agents, such as the cyclosporin derivative PSC 833 [5] has recently been identified and are currently undergoing clinical trial.

Our laboratory has previously investigated the effect of several natural, plant-derived chemicals on the activity of Pgp. Several members of the flavonoids, a diverse group of structurally related polyphenolic compounds widely distributed in plants, were shown to increase the activity of Pgp towards carcinogens such as dimethylbenz[a]anthracene [6]. Since Pgp is expressed in normal tissues, we hypothesised that these flavonoids may have chemopreventive activity against environmental carcinogens by increasing the cellular efflux of these compounds from cells via Pgp. However, in a chemotherapeutic setting, an increase in Pgp activity would be counterproductive, and, indeed, we found that these flavonoids also increase the Pgp-mediated efflux of the chemotherapeutic drug doxorubicin (DOX) [7]. We, therefore, investigated other phytochemicals which might inhibit Pgp activity and reverse MDR. Extracts of rosemary (*Rosemarinus officinalis* Labiatae), a commonly used herb, has been previously shown to have potent chemopreventive activity *in vivo*

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[8, 9]. As a result, rosemary extract (RE) has been well studied in animal models and has been shown to be antimutagenic [10, 11] and nontoxic [12]. With RE already established as an effective chemopreventive agent and nontoxic in animal models, we investigated its effect on Pgp activity and MDR. In this report we demonstrate, for the first time, that RE inhibits Pgp-mediated drug efflux, resulting in an increase in the intracellular accumulation and cytotoxicity of chemotherapeutic drugs in drug-resistant human breast cancer cells *in vitro*.

MATERIALS AND METHODS

Materials

DOX, verapamil, vinblastine (VIN), kaempferol and dimethylsulphoxide (DMSO) were purchased from Sigma Chemical Company (St Louis, Missouri, U.S.A.). The RE compounds carnosic acid, carnosol and rosemarinic acid were gifts of Kelsec, Inc. (Kalamazoo, Michigan, U.S.A.). All radio-labelled compounds were purchased from Amersham (Arlington Heights, Illinois, U.S.A.).

Preparation of RE

RE was prepared according to the method of Wu and colleagues [13]. Briefly, 1 kg of powdered rosemary leaves were extracted with 6 l of methanol at 60°C for 2 h. The mixture was filtered and re-extracted with 4 l of methanol. The combined filtrate was bleached with 200 g of active charcoal, filtered and concentrated by rotary evaporation to 900 ml before filtering to remove precipitates. The filtrate was rotary evaporated to dryness to produce the RE. Purification of RE by this method gave a yield of 77 g from 1 kg of ground RE. RE was dissolved in DMSO with the final DMSO concentration used for experimentation adjusted to 0.1% (v/v) in media.

Cell culture

Three different MCF-7 human breast cancer cell lines were used in this study: the wild-type (WT), which does not express Pgp; R65, an MCF-7-derived line which expresses Pgp and has acquired resistance to DOX and VIN [14]; and Clone 10.9, a stable *MDR1*-transfected MCF-7 line with resistance to DOX and colchicine [15]. Cells were maintained in RPMI 1640 (BioFluids, Rockville, Maryland, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Rockville, Maryland, U.S.A.) and 2 mM L-glutamine (BioFluids) at 37°C in a humid atmosphere containing 5% CO_2. Cells were passed weekly using 0.05% trypsin/0.01% ethylenediaminetetra-acetic acid (EDTA).

Intracellular drug accumulation

WT and R65 cells were plated out at 200 000 cells per well and Clone 10.9 cells were plated out at 320 000 cells per well in 6-well plates and allowed to grow to confluence. Cells were pretreated with DMSO (control) or 82 μg of RE per ml of culture medium for 30 min, then exposed to 0.01 μCi [^{14}C]DOX/ml, 0.05 μCi [^{3}H]paclitaxel/ml, or 0.05 μCi [^{3}H]VIN/ml for 60 min. The medium was removed and the plates were washed extensively with phosphate-buffered saline (PBS). The cells were then trypsinised (0.25% trypsin/3 mM EDTA), transferred to scintillation vials to which 20 ml of Aquasol scintillation fluid (Beckman, Palo Alto, California, U.S.A.) was added, and the amount of intracellular radioactivity was determined.

Measurement of Pgp-mediated drug efflux

WT, R65, and Clone 10.9 cells were plated out as described for drug accumulation experiments and allowed to grow to confluence. Cells were pretreated with DMSO (control), 16.5 μg/ml or 85 μg/ml of RE (Figure 1), or 50 μM (16.5 to 18.0 μg/ml) of purified RE components for 30 min, then exposed to 0.01 μCi [^{14}C]DOX/ml for 60 min at 37°C. The medium was removed and the plates washed extensively with PBS. Fresh medium was added and the cells were incubated for 30 min at 37°C. The medium was removed, and the amount of [^{14}C]DOX in the medium that had been effluxed into the medium was measured by scintillation counting. The cells were trypsinised and also counted. Data are expressed as the per cent of total [^{14}C]DOX present in the medium.

Measurement of DOX cytotoxicity

WT and R65 cells were plated out at 20 000 cells per well and Clone 10.9 cells were plated out at 26 000 cells per well in 24-well plates. After 24 h, medium containing DMSO (control) or 16.5 μg of RE per ml of culture medium in the presence of various concentrations of DOX was added. The cells were incubated for 4 days at 37°C, and cell growth was assessed by sulphorhodamine assay [16].

Plasma membrane preparation and [^{3}H]azidopine labelling

Plasma membranes for azidopine labelling studies were prepared from confluent R65 cell cultures. Cells were scraped from the tissue culture flasks and pelleted by centrifugation. The cell pellet was resuspended in buffer consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and Complete protease inhibitor cocktail (Beckman) and sonicated. The sonicate was centrifuged at 120 000*g*, 4°C, for 60 min in an Optima XL-90 Ultracentrifuge (Beckman). The membrane pellet was resuspended in 50 mM Tris-HCl, pH 7.4; 16% (w/v) sucrose with Complete, and layered on to 31% (w/v) sucrose for centrifugation at 70 000*g*, 4°C, for 18 h. The partially purified Pgp membranes were dialysed overnight at 4°C against 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and Complete prior to use. Photoaffinity labelling of the partially purified membranes with [^{3}H]azidopine was carried out as previously described [14]. DOX, verapamil, kaempferol, and

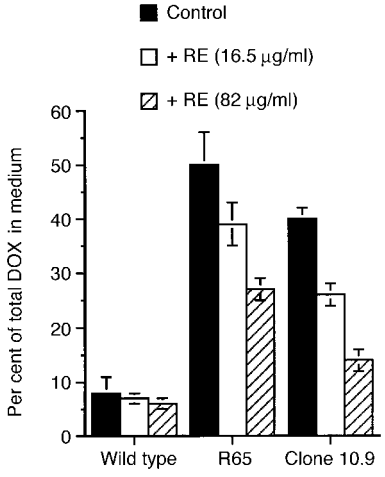

Figure 1. Effect of RE on the efflux of [^{14}C]DOX. $n = 3 \pm$ standard deviation (S.D.). There was a significant decrease in [^{14}C]DOX efflux in R65 and Clone 10.9 cells treated with RE, but no difference in WT cells ($P<0.05$).

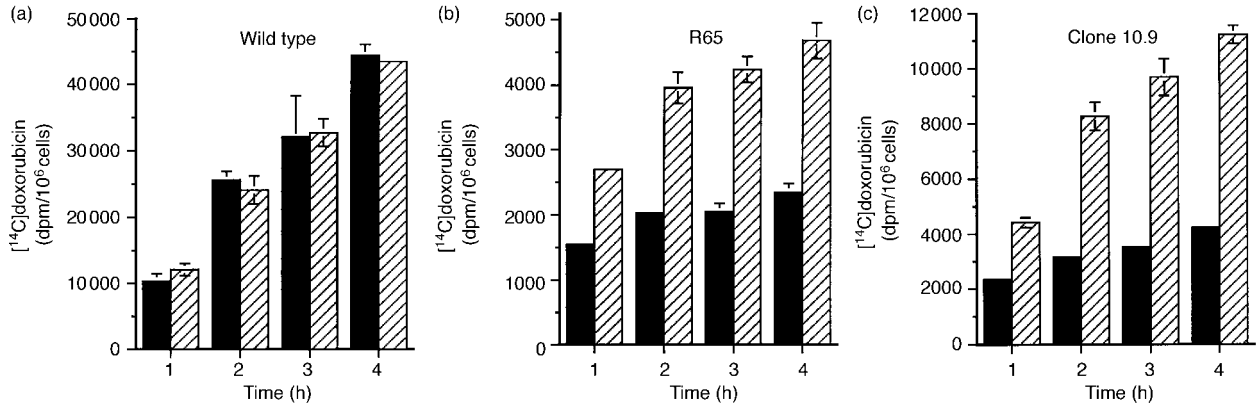

Figure 2. Effect of RE on the accumulation of [^{14}C]DOX in MCF-7 cells. $n = 3 \pm$ standard deviation (S.D.). There was a significant increase in [^{14}C]DOX accumulation in R65 and Clone 10.9 cells treated with RE compared with controls at every time point examined ($P<0.05$), but no difference in WT cells. ■ control (DMSO), ▨ RE treated.

VIN used in the [^{3}H]azidopine labelling were all at a concentration of 0.2 μM, which represents a 330-fold excess over the amount of [^{3}H]azidopine. RE was used at 0.066 μg/ml.

Statistical analysis

Statistical analyses were performed using StatView Statistical Analysis software (SAS Institute, San Francisco, California, U.S.A.). Differences between group mean values were determined by a one-factor analysis of variance (ANOVA), followed by Fisher PSLD *post-hoc* analysis for pairwise comparison of means.

RESULTS

Modulation of intracellular drug accumulation by RE

We have previously developed and characterised MDR cell lines derived from human breast cancer MCF-7 cells by continuous exposure to DOX [6, 14]. These cells overexpress Pgp, resulting in a 65-fold increase in DOX resistance compared to WT cells and are, therefore, designated as R65. WT and R65 cell lines, as well as Clone 10.9, a MCF-7 cell line which expresses Pgp as a result of a stable transfection with the *MDR1* gene, were used in the present study to examine the effect of RE on drug accumulation, Pgp activity, and MDR.

We examined the effect of RE on the intracellular accumulation of several commonly used chemotherapeutic agents.

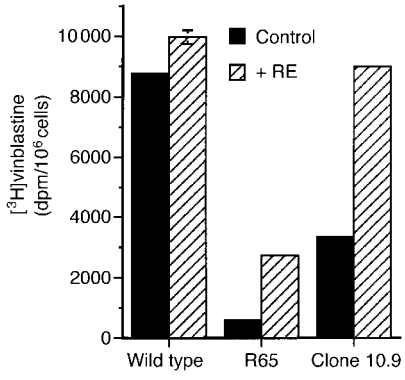

Figure 3. Effect of RE on the accumulation of [^{3}H]VIN. The accumulation of [^{3}H]VIN in WT, R65, and Clone 10.9 cells in the presence or absence of RE (82 μg/ml) was determined. $n = 3 \pm$ standard deviation (S.D.). There was a significant increase in [^{3}H]vinblastine accumulation in R65 and Clone 10.9 cells in the presence of RE ($P<0.05$).

As seen in Figure 2, incubation of WT cells with [^{14}C]DOX resulted in a time-dependent accumulation of DOX. This was not affected by exposure to RE. In R65 and Clone 10.9 cells there was no significant accumulation of [^{14}C]DOX in DMSO controls over the time period studied, but exposure to RE resulted in a significant increase ($P<0.05$) in DOX accumulation in R65 and Clone 10.9 cells compared with controls.

RE treatment also caused an increase in the cellular accumulation of [^{3}H]VIN in R65 cells and Clone 10.9 cells, but did not affect [^{3}H]VIN accumulation in WT cells (Figure 3). RE also caused a significant increase in the amount of [^{3}H]paclitaxel accumulation in R65 cells (data not shown).

Effect of RE on DOX efflux

The effect of RE on the Pgp-mediated efflux of DOX was examined. As shown in Figure 1, RE caused a decrease in the amount of DOX effluxed from R65 and Clone 10.9 cells, whilst having no effect on efflux in WT cells. Similarly, in normal human breast cells (Clonetics, San Diego, California, U.S.A.), which have little or no Pgp expression, there was no change in the accumulation or efflux of DOX in the presence of RE (data not shown).

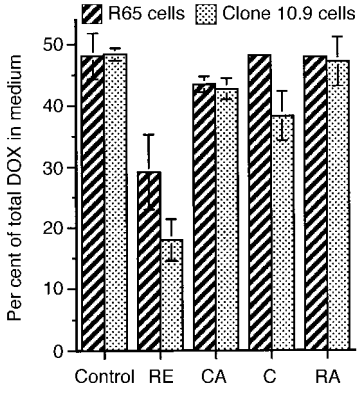

Figure 4. Effect of purified RE components on [^{14}C]DOX efflux. The efflux of [^{14}C]DOX in the presence of 50 μM (approximately 16.5 to 18 μg/ml) of the RE components carnosic acid (CA), carnosol (C), rosemarinic acid (RA), or 82 μg/ml RE was determined in R65 (striped bars) and Clone 10.9 cells (dotted bars). $n = 3 \pm$ standard deviation (S.D.). There was no significant difference in efflux in R65 or Clone 10.9 cells with any treatment except RE ($P<0.05$).

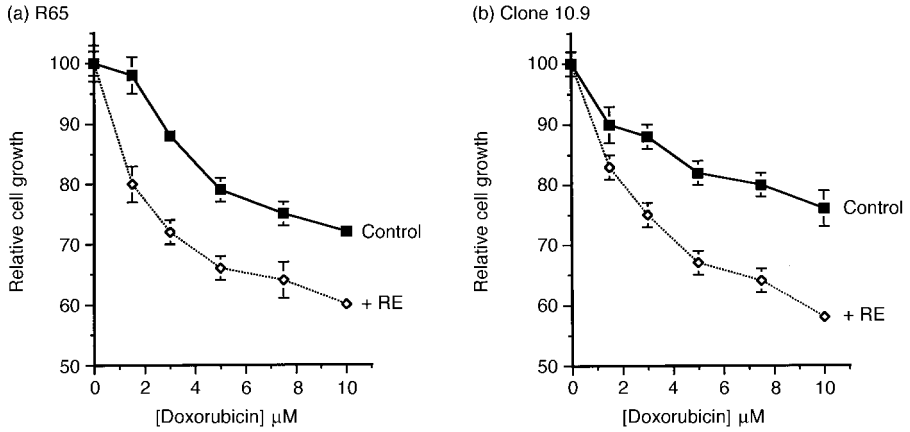

Figure 5. Effect of RE on the cytotoxicity of DOX. (a) R65 and (b) Clone 10.9 cells were grown in the presence of DMSO (control) or RE (16.5 μg/ml) in the presence of different concentrations of DOX. The amount of cell growth was determined after 3 days by sulphorhodamine. RE at this concentration had no significant inhibitory activity on cell growth by itself. $n = 3 \pm$ standard deviation (S.D.). There was a significant difference in cell growth in RE-treated cultures compared with controls in both cell lines ($P < 0.05$).

The RE prepared for these experiments is a complex mixture of compounds. Previous experiments on the antioxidant properties of RE led to the purification of the active antioxidant components carnosol, carnosic acid, and rosemarinic acid [13, 17]. To determine if any of these substances were the active component responsible for modulation of Pgp activity, we examined their effect on DOX efflux in R65 and Clone 10.9 cells. There was no significant inhibition of DOX efflux in R65 or Clone 10.9 cells treated with carnosic acid, carnosol, or rosemarinic acid (Figure 4).

Effect of RE on DOX cytotoxicity

Because RE increased the intracellular accumulation of DOX, we examined its effect on DOX-induced cytotoxicity as measured by inhibition of cell growth. Incubation of WT cells with increasing amounts of DOX resulted in a decrease in cell growth that was not affected by incubation with RE (data not shown). In R65 and Clone 10.9 cells, co-incubation of DOX with RE resulted in a significant increase in the cytotoxicity of DOX (Figure 5a and b). RE also decreased the IC_{50} of VIN in R65 cells (data not shown). A lower RE concentration was used in cytotoxicity experiments than in accumulation and efflux experiments because RE itself inhibited cells growth ($IC_{50} = 30$ μg/ml for all cell lines; data not shown). Higher concentrations of RE which lead to even greater accumulation of DOX could not, therefore, be tested with regard to cytotoxicity. The higher concentrations used in Figures 1–3 did not affect cell viability because the incubation time was short and the cells were confluent.

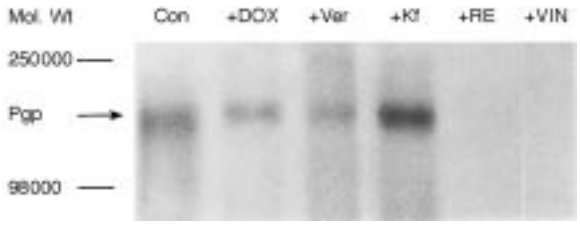

Figure 6. Photoaffinity labelling of Pgp with [^{3}H]azidopine in the presence of RE or other effectors of Pgp. Partially purified plasma membranes from R65 cells containing Pgp were subjected to photoaffinity labelling with the VIN analogue [^{3}H]azidopine in the presence of DMSO (Con), or a 330-fold excess of DOX, verapamil (Ver), kaempferol (kf), RE , or VIN.

Effect of RE on photoaffinity labelling of Pgp

Substrates and reversal agents of Pgp are known to inhibit photoaffinity labelling of Pgp by the VIN analogue [^{3}H]azidopine by competing with azidopine for the substrate binding site(s) of Pgp. In order to determine whether RE interacts directly with Pgp binding site(s), we examined photoaffinity labelling of Pgp in the presence of RE. The presence of a 330-fold excess of RE (compared with [^{3}H]azidopine) completely abolished binding of [^{3}H]azidopine to Pgp (Figure 6). RE was as effective as VIN at this concentration at inhibiting labelling, and more effective than DOX or the Pgp reversal agent verapamil. Kaempferol, a flavonoid which we have previously shown to increase Pgp activity [6, 14], increased [^{3}H]azidopine labelling.

DISCUSSION

The intracellular level of some chemotherapeutic drugs is decreased by the activity of Pgp, the ATP-dependent efflux pump encoded by the *MDR1* gene, which is up-regulated in many drug-resistant tumour cells [1, 18]. Much attention has, therefore, been devoted to the development of agents which inhibit Pgp activity and reverse MDR. Unfortunately, the most effective Pgp inhibitors *in vitro*, such as verapamil, have proven to be too toxic to be used clinically. A readily available, nontoxic Pgp inhibitor may prove to be efficacious when administered in combination with commonly used chemotherapeutic pleiotropic drugs whose effectiveness is compromised by Pgp, such as DOX, VIN, and paclitaxel. Therefore, the effects of many plant-derived compounds on Pgp were investigated. These phytochemicals have the advantage of being natural dietary compounds that are nontoxic in animals. In the present study we investigated the effects of RE on drug accumulation and Pgp activity *in vitro*. RE, a complex mixture of chemicals extracted from rosemary, has shown much promise in the prevention of chemically-induced carcinogenesis in animal models [8, 9, 17]. We carried out these experiments in a DOX-resistant MCF-7 cell line which expresses high levels of Pgp compared with WT cells. These cells were developed by continuous exposure to DOX over a period of months. To confirm these results, we also examined the effect of RE in Clone 10.9 cells, an MCF-7 cell line which is stably transfected with *MDR1*.

RE caused a substantial increase in the accumulation of DOX or VIN in cells expressing Pgp, and inhibited the efflux of DOX, but had no effect on WT cells which lack Pgp. RE also caused an increase in paclitaxel accumulation in these cells (data not shown). Since these drugs are known to be substrates for Pgp, we concluded that RE modulates intracellular drug levels by inhibiting Pgp. In agreement with these data, RE also increased the cytotoxicity of DOX in Pgp-expressing cell lines, but not WT cells. Exposure to RE also increased the cytotoxicity of VIN (data not shown). This demonstrates that RE can partially reverse MDR in cells which express Pgp.

Since the time of exposure of cells to RE in these experiments was short (1 to 4 h), it is unlikely that RE acts by down-regulating *MDR1* transcription and, therefore, reducing the amount of cellular Pgp. Nevertheless, we examined the effect of RE on the expression of Pgp at the protein (Western) and mRNA (Northern) levels. There was no difference in Pgp expression in any of the three cell lines used in this study when treated with RE for a period of 4 days (data not shown). Taken together, these data indicate that RE increases intracellular drug levels by modulating Pgp activity, not expression.

Several laboratories have isolated constituents present in RE that are primarily responsible for the antioxidant activity of RE. To attempt to identify the constituent of RE responsible for Pgp inhibition, we examined the effect of several of these phytochemicals on DOX efflux. The concentration of carnosic acid and carnosol in RE prepared by the method of Wu and colleagues [13] is approximately 7.5% each. The concentration of carnosic acid or carnosol in our assays of drug accumulation or efflux is, therefore, approximately 6 μg/ml. Neither carnosic acid, carnosol, nor rosemarinic acid significantly affected DOX efflux in R65 or Clone 10.9 cells, even at a concentration of 16.5 μg/ml (Figure 4). Thus, carnosic acid, carnosol, and rosemarinic acid do not contribute to the inhibitory effect of RE. Some other of the thousands of phytochemicals present in RE, or a combination of phytochemicals, must be responsible for this activity, but the 'active ingredient(s)' remains to be determined.

In order to determine the mechanism of RE's activity, we employed [^{3}H]azidopine photoaffinity labelling. [^{3}H]azidopine, a VIN analogue, binds to the substrate binding site(s) of Pgp. Inhibition of labelling by a compound indicates that the compound directly competes for the substrate binding site(s), thus blocking the binding of azidopine. In this manner, the Pgp substrates DOX and VIN, or the Pgp inhibitor verapamil, inhibit azidopine labelling of Pgp in partially purified plasma membranes (Figure 6). RE completely abolished photoaffinity labelling, indicating that RE directly binds to Pgp at the binding site(s). Thus, this suggests that the mechanism of RE's inhibitory activity is through a competitive inhibition of substrate binding. Whether the active component of RE itself undergoes transport by Pgp is unknown.

RE, unlike other Pgp inhibitors, is not only plentiful and inexpensive to prepare, but relatively nontoxic. Although the present experiments demonstrate that RE is an effective inhibitor of Pgp activity *in vitro*, animal experimentation is required to determine if RE has potential as an effective and safe 'chemosensitiser' for treating cancers expressing Pgp-mediated MDR. Furthermore, despite intense interest in rosemary extract as an inhibitor of experimental carcinogenesis in animal studies, the physiologically relevant concentrations attainable in humans have not been reported. It is important to consider that the levels of MDR and Pgp expression in the cell lines used in this study are much greater than that developed in human tumour cells. Thus, RE may be an even more effective MDR reversal agent in actual tumours. The effect of RE on other drug efflux mechanisms such as the multidrug resistance protein [19] is currently under investigation.

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