Resveratrol and P-glycoprotein Inhibitors Enhance the Anti-Skin Cancer Effects of Ursolic Acid

Jacob J. Junco1, Anna Mancha1, Gunjan Malik1, Sung-Jen Wei1,2, Dae Joon Kim1,2, Huiyun Liang1, and Thomas J. Slaga1

Abstract

Ursolic acid, present in apples, rosemary, and other sources, is known to inhibit tumor formation and tumor cell viability in multiple systems, including skin. However, various cancers are resistant to ursolic acid treatment. Herein, skin carcinoma cells (Ca3/7) as compared with skin papilloma cells (MT1/2) displayed more resistance to ursolic acid-induced cytotoxicity. Interestingly, Ca3/7 cells had elevated levels of P-glycoprotein (P-gp), an ATP-dependent efflux pump that mediates resistance to chemotherapy in preclinical and clinical settings, and not only accumulated less but also more rapidly expelled the P-gp substrate rhodamine 123 (Rh123) indicating ursolic acid is transported by P-gp. To determine whether P-gp inhibition can enhance ursolic acid-mediated cytotoxicity, cells were challenged with P-gp inhibitors verapamil or cyclosporin A. Alternatively, cells were pretreated with the natural compound resveratrol, a known chemotherapy sensitizer. Verapamil and resveratrol enhanced the effects of ursolic acid in both cell lines, whereas cyclosporin A only did so in Ca3/7 cells. Similarly, verapamil inhibited Rh123 efflux in both lines, whereas cyclosporin A only inhibited Rh123 efflux in Ca3/7 cells. Resveratrol did not inhibit Rh123 efflux in either line, indicating the synergistic effects of resveratrol and ursolic acid are not manifest by inhibition of P-gp–mediated efflux of ursolic acid. These results indicate that the anti-skin cancer effects of ursolic acid are enhanced with P-gp inhibitors. In addition, resveratrol and ursolic acid interact synergistically, but not through inhibition of P-gp.

Implications: Resveratrol and/or p-glycoprotein inhibitors in combination with ursolic acid are an effective anti-skin cancer regimen. Mol Cancer Res; 11(12); 1521–9. ©2013 AACR.

Introduction

In many cancer therapies, tumors have devised ways to resist treatment. Multidrug resistance (MDR) in tumors can be mediated by a large number of factors, depending on tumor location or tumor type as well as the etiology of mutations. MDR can be mediated by factors including alterations in p53 or related pathways and decreased susceptibility to apoptosis via upregulation of antiapoptotic factors like bcl-2 (1, 2). Increased activity of NF-kB may also induce the MDR phenotype by decreasing p53 activity as well as transcribing various inhibitors of apoptosis (3). The tumor microenvironment, specifically the disorganized vascular system surrounding the tumor, can promote chemoresistance by creating high interstitial pressure in the tumor to limit drug influx, or by inducing hypoxia or low pH (4). MDR is also associated with increased levels of efflux pumps, which have normal physiologic functions but also mediate resistance to chemotherapy by actively removing anticancer compounds from cells. Efflux pumps which can mediate MDR in different tumor types include breast cancer resistance protein (BCRP), multidrug resistance protein (MRP1), and the highly-characterized P-glycoprotein (P-gp) (5). P-gp, encoded by the gene ABCB1 is composed of two subunits, each containing six transmembrane regions and an ATP-binding domain (6, 7). P-gp is transcribed by a number of factors implicated in MDR, including NF-kB (8). P-gp is upregulated in many cancer forms and can efflux a variety of drugs to mediate resistance to chemotherapy. P-gp is highly expressed in both normal and cancerous liver, kidney, and colon tissues, making these tumors intrinsically chemoresistant (9). In addition, other cancer types, including hematologic malignancies and breast cancers increase P-gp as a result of tumor progression and in response to chemotherapeutic challenge, leading to decreased therapeutic responses (10, 11). Studies of P-gp inhibitors, in vitro, in vivo, and in some clinical trials have shown they can enhance the effects of traditional chemotherapeutics (11–14). Chemotherapy of existing skin tumors may be a less scarring alternative to surgery and provide benefit for those with higher risk of metastasis (15). However, a number of studies have shown that metastatic squamous cell carcinoma...
(SCC) can resist certain chemotherapies, but become sensitized upon inhibition of specific pathways (15, 16). In addition, P-gp was found to be upregulated in human SCC tissue and SCC cell lines as compared to epidermis and normal keratinocytes (17). Therefore, P-gp inhibition may enhance the effects of SCC chemotherapies that are also P-gp substrates.

Natural phytochemical ursolic acid has been shown to have anticancer effects in different tumor models, including those of the breast (18), lung (19), and skin (20), and likely targets many different processes in tumorigenesis (21). However, a number of studies have revealed a variety of resistance mechanisms to ursolic acid. In one report, ursolic acid treatment of HT-29 human colorectal cancer cells induced increases in proinflammatory COX-2 and activation of upstream COX-2 regulator p38 MAPK in association with apoptosis. COX-2 siRNA or inhibition of p38 MAPK enhanced ursolic acid-mediated apoptosis (22). A similar study in HT-29 cells and DU145 human prostate carcinoma cells showed ursolic acid-mediated apoptosis is enhanced by inhibition of upstream inducers of p38 such as Src (23). Also, while one report showed a number of chemoresistant cancer cell types were sensitive to ursolic acid, ursolic acid-mediated cytotoxicity was still stronger in the parental cell types (24), which have lower levels of P-gp (25, 26). In another, another study showed that ursolic acid inhibits substrate efflux through P-gp at high concentrations. However, ursolic acid also enhanced the ATPase function of P-gp, indicating it is a substrate for P-gp and likely inhibits efflux of other substrates through a competitive interaction (7). These results show ursolic acid may be a substrate for P-gp. Therefore, in addition to sensitization by inhibition of other resistance mechanisms, the anti-cancer effects of ursolic acid may be enhanced by P-gp inhibition.

As ursolic acid has been shown to have antitumor effects in chemically mediated skin cancer (20), we conducted experiments to determine whether modulation of P-gp could enhance the effects of ursolic acid in skin cancer-relevant systems. This was done using verapamil and cyclosporin A, which both function as P-gp substrates and competitive P-gp inhibitors (7, 14, 27). We also tested ursolic acid in combination with the phytochemical resveratrol, which has been shown to sensitize cancer cells to chemotherapeutics by multiple mechanisms, potentially including enhancement of apoptotic pathways and inhibition of P-gp-mediated efflux (28, 29). Ursolic acid with or without P-gp inhibitors may provide another treatment avenue besides surgery, and may also provide therapeutic leads for related epithelial cancers, such as lung cancer.

Materials and Methods

Reagents

Ursolic acid, verapamil, cyclosporin A, resveratrol, doxorubicin hydrochloride, thiazolyl blue tetrazolium bromide (MTT reagent), rhodamine 123 (Rh123), and a kit for Annexin V/propidium iodide staining were obtained from Sigma. Stock solutions of ursolic acid (up to 40 mmol/L), resveratrol (up to 400 mmol/L), and Rh123 (100 mmol/L) were prepared in dimethyl sulfoxide (DMSO). Stock solutions of cyclosporin A (100 mmol/L in EtOH), verapamil (100 mmol/L in H2O), and doxorubicin hydrochloride (40 mmol/L in H2O) were also prepared. For Western blotting, primary antibodies used were for phosphorylated (Ser536) p65 and total p65 (Cell Signaling Technology), P-gp (Santa Cruz Biotechnology), and β-actin (Abcam).

Cell culture

MT1/2 mouse skin papilloma cells and Ca3/7 mouse skin carcinoma cells were maintained in Joklik MEM containing 8% FBS, 50 μg/mL penicillin, 50 ng/mL streptomycin, 10 μg/mL transferrin, 50 μg/mL gentamicin sulfate, 5 μg/mL insulin, 5 μg/mL EGF, 10 μmol/L o-phosphorylethanolamine, and 10 μmol/L 2-aminoethanol. B16F10 metastatic mouse melanoma cells were maintained in Dulbecco’s modified Eagle medium (DMEM) containing 10% FBS, 50 μg/mL penicillin, and 50 ng/mL streptomycin. A549 human lung adenocarcinoma cells were grown in DMEM/F12 containing 10% FBS, 50 μg/mL penicillin, and 50 ng/mL streptomycin. All cells were grown in an incubator at 5% CO2 and 37°C. MT1/2 are papilloma-producing cells derived from SENCAR mouse skin initiated with N-methyl-N’-nitro-N-nitrosoguanidine (MNNNG) and treated with 12-O-tetradecanoylphorbol-13-acetate (TPA; ref. 30). Ca3/7 were obtained from carcinomas grown using the 7,12-dimethylbenz(a)anthracene (DMBA)/TPA skin carcinogenesis protocol in SENCAR mice, and reform metastatic carcinomas when implanted into nude mice (31). B16F10 cells were provided by the lab of Dr. Tyler Curiel [The University of Texas Health Science Center at San Antonio (UTHSCSA), San Antonio, TX]. A549 cells were provided by the lab of Dr. Sung-Jen Wei (UTHSCSA).

MTT assay

Cells were treated with various doses of ursolic acid, doxorubicin hydrochloride, or 0.1% vehicle for 24 hours. In combination experiments, cells were pretreated with verapamil, cyclosporin A, resveratrol, or 0.1% vehicle for 1 hour followed by various doses of ursolic acid or 0.1% vehicle for 24 hours. In the study with A549 cells, cells were treated for 48 hours. MTT reagent (0.5 mg/mL) was added, and cells were incubated for an additional 2.5 hours. Media was removed and formazan crystals were dissolved with DMSO. Samples were measured at 570 nm with background subtraction at 650 nm, using a Biotek Synergy HT spectrophotometer (Biotek).

Annexin V/propidium iodide staining

Ca3/7 cells were treated with indicated doses of resveratrol, ursolic acid, or 0.1% DMSO vehicle for 12 hours. Media and trypsinized cells were collected and spun at 150 × g for 3 minutes, rinsed and spun again, and resuspended in 1 × binding buffer containing Annexin V and propidium iodide according to the manufacturer’s instructions. Cells were run on an LSRII flow cytometer (Becton Dickinson) with dead cells gated out.
Western blotting

MT1/2 and Ca3/7 cells were treated with indicated doses of verapamil, cyclosporin A, resveratrol, or 0.1% vehicle for 24 hours. Media was aspirated and cells were rinsed twice with cold PBS before lysis in buffer containing 1% Triton X-100, 0.5% IGEPAL, 0.05 mol/L TrisHCl and 0.1 mol/L NaCl as well as protease/phosphatase inhibitors and 5 mmol/L EDTA. Proteins were extracted by centrifugation and quantified by using the Bradford reagent method. Proteins were denatured in XT Sample Buffer containing XT reducing agent. Proteins were separated on 4% gradient gels, transferred onto polyvinylided difluoride membranes, blocked in 5% bovine serum albumin or 5% milk in TBST for 1 hour before incubation overnight in primary antibody solution. Blots were rinsed, incubated in secondary antibody for 1 hour, and developed using ECL2 Western blotting substrate (Pierce). Digital images of films were acquired using EPSON Scan (EPSON). Densitometry was measured using UN-SCAN-IT (Silk Scientific).

Rh123 assay

Cells suspended in Hank’s balanced salt solution (HBSS) were incubated with 10 μmol/L Rh123 for 1 hour. Cells were centrifuged and rinsed twice, followed by efflux for the desired time points at 37°C. Cells were rinsed and kept on ice in the dark in HBSS. Cells were rinsed and resuspended in PBS before analysis. For flow cytometry, live cells were gated and fluorescein isothiocyanate channel mean fluorescence was measured. At least 2,500 live cells per group per experiment were analyzed. Results were normalized to the Rh123 initial ("accumulation") level in the MT1/2 cells.

Alternatively, in experiments with P-gp inhibitors or resveratrol, Rh123 levels were measured using a plate reader. MT1/2 and Ca3/7 cells were treated with vehicle or 100 μmol/L verapamil, cyclosporin A, or resveratrol for 24 hours. Ten μmol/L Rh123 was added for 1 hour. Plates were rinsed twice with cold PBS and resupplied with the compounds in warm media for 30 minutes. Plates were rinsed twice with cold PBS and lysed with lysis buffer. Proteins were extracted by centrifugation and quantified by Bradford method. Fifty microliters extracted lysate per sample was transferred to a black plate and read at 485 nm/530 nm using a Biotek Synergy HT spectrophotometer. Rh123 levels for each sample were normalized to the protein concentration.

Statistical analyses

Differences between individual groups were determined by ANOVA. IC_{50} values were calculated with GraphPad Prism. SD is given except where otherwise noted.

Results

Effect of ursolic acid and doxorubicin hydrochloride on skin cancer cell viability

Preliminary cell viability experiments showed Ca3/7 cells are more resistant to the effects of ursolic acid than are MT1/2 cells (Fig. 1A). Ursolic acid had an IC_{50} of 8.17 ± 0.49 in MT1/2 cells versus an IC_{50} of 19.66 ± 3.69 in Ca3/7 cells (P < 0.001). In addition, the Ca3/7 cells are also chemoresistant to doxorubicin hydrochloride, a P-gp substrate chemotherapeutic, relative to MT1/2 cell lines (Fig. 1B). Doxorubicin hydrochloride reduced viability of MT1/2 cells with an IC_{50} of 0.95 ± 0.29 versus an IC_{50} of 45.2 ± 3.47 in Ca3/7 cells (P < 0.005). While ursolic acid is less potent than doxorubicin hydrochloride in MT1/2 cells, ursolic acid achieves greater cell viability decreases than does doxorubicin hydrochloride in Ca3/7 carcinoma cells (P < 0.005).

Ca3/7 cells have higher basal P-gp levels and activity

Factors typically involved in chemoresistance were measured by Western blotting. We found that Ca3/7 cells expressed higher levels of P-gp, relative to MT1/2 cells (Fig. 2A and C). We also found Ca3/7 cells have higher levels of activated NF-κB, indicated by phosphorylation status of the active subunit p65 (Fig. 2B and C). NF-κB contributes to transcription of P-gp, which may partially explain the chemoresistant effects of NF-κB activation (8). In addition, we analyzed cells for basal levels of cyclin D1.
Molecular Cancer Research

Minutes (data not shown). Similar correlations between

cells (average of 3 experiments, basal NF-

cells also had a higher rate of ef

accumulation level of Rh123 (data not shown) indicated that

minutes after rinsing (Fig. 2D). Data normalized to the

intracellular levels of Rh123 were also evident 15, 30, and 60

Rh123 with a plate reader (data not shown). The decreased

results were obtained in preliminary studies measuring

Flow cytometry experiments indicated Ca3/7 cells accumu-

the increase in P-gp levels led to an increase in P-gp activity.

We also used P-gp substrate Rh123 to determine whether

Ca3/7 cells have higher basal P-gp levels than MT1/2 cells

Figure 2. A, Ca3/7 cells have higher basal P-gp levels than MT1/2 cells (average of 3 experiments, n = 3/experiment). B, Ca3/7 cells have higher basal NF-κB activity (measured by phosphorylation of p65) than MT1/2 cells (average of 3 experiments, n = 3/experiment). C, representative Western blot analysis for the data in (A) and (B). D, Ca3/7 cells have higher basal P-gp activity than MT1/2 cells. This was indicated by decreased accumulation and increased efflux of Rh123, a P-gp substrate, measured by flow cytometry (average of 4 experiments, n = 2/exp). For these experiments, significance between cell lines is noted by **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

and bcl-2, but found no difference between cell lines (data not shown).

We also used P-gp substrate Rh123 to determine whether

the increase in P-gp levels led to an increase in P-gp activity. Flow cytometry experiments indicated Ca3/7 cells accumu-

lated less Rh123 than did MT1/2 cells (Fig. 2D). Similar results were obtained in preliminary studies measuring

Rh123 with a plate reader (data not shown). The decreased intracellular levels of Rh123 were also evident 15, 30, and 60

minutes after rinsing (Fig. 2D). Data normalized to the accumulation level of Rh123 (data not shown) indicated that

Ca3/7 cells also had a higher rate of efflux at the 15- and 30-

minute time points. Preliminary experiments using 30-, 60-, and 120-minute time points indicated overall Rh123 levels had equalized by 120 minutes, but Ca3/7 cells still had a significantly higher efflux rate than MT1/2 cells at 30

minutes (data not shown). Similar correlations between
efflux of Rh123 and relative protein or mRNA levels of

P-gp have been shown for different cancer cell lines (32).

P-gp inhibitors enhance the cytotoxic effects of ursolic acid

The differences in sensitivity to ursolic acid between cell lines, combined with differences in P-gp expression and activity, indicate that ursolic acid may be a P-gp substrate in these skin cancer cells. We next examined pharmacologic inhibitors of P-gp to see if they could enhance the cytotoxic effects of ursolic acid.

Verapamil and cyclosporin A have been shown to inhibit

P-gp in multiple systems, leading to enhanced chemother-

apeutic effects (12–14, 33). In addition, resveratrol has been

shown to sensitize cancer cells to traditional chemothera-

peutics, perhaps by inhibiting P-gp expression and/or activity (28, 29). We pretreated MT1/2 and Ca3/7 cells with a

range of doses of verapamil, cyclosporin A, or resveratrol for

1 hour before incubation with different doses of ursolic acid.

For these studies, the viable cells in each treatment group

were normalized to vehicle control, and IC50 values for ursolic acid in the presence of vehicle or 5 to 100 μmol/L

of verapamil, cyclosporin A, or resveratrol were calculated. In

our experiments, we found that verapamil significantly and dose-dependently enhanced ursolic acid-mediated decreases in viability of both MT1/2 and Ca3/7 tumor cells. This effect was significant with as low as 50 μmol/L verapamil in MT1/2 cells (Table 1) and as little as 5 μmol/L verapamil in Ca3/7 cells (Table 2). Similarly, cyclosporin A dose-depen-
dently and significantly enhanced ursolic acid-mediated decreases in viability of Ca3/7 cells. This effect was significant with as little as 5 μmol/L cyclosporin A (Table 2). However, the enhancing effects of cyclosporin A were not evident in MT1/2 cells, even up to 100 μmol/L cyclosporin A (Table 1). Finally, resveratrol starting at 25 μmol/L enhanced the antiproliferative effect of ursolic acid in the Ca3/7 cell line in a dose-dependent manner (Table 2). Resveratrol also enhanced the effects of ursolic acid in MT1/2 cells, starting at 25 μmol/L; however, this effect did not increase at higher doses (Table 1). Also, the syner-
gistic effects of resveratrol in Ca3/7 cells were significantly stronger (P < 0.05) than in MT1/2 cells at 100 μmol/L. Representative experiments for the data in Tables 1 and 2 are shown in Supplementary Fig. S1.

Stronger synergistic effects, indicated by an average fold IC50 decrease of 3.26 ± 0.45, were observed in the Ca3/7 cell line pretreated with 200 μmol/L resveratrol (Supplementary Fig. S2). The synergistic effects of resveratrol and ursolic acid in Ca3/7 cells were confirmed by flow cytometry, which showed these compounds induced a mixed apoptotic/necrotic cell death (Supplementary Fig. S3). A synergistic cytotoxic effect of resveratrol and ursolic acid was also manifest in the metastatic mouse melanoma cell line B16F10 (average fold IC50 decrease of 1.84 ± 0.13; Supplementary Fig. S4), as well as in the transformed mouse epidermal line JB6 P+ (John DiGiovanni lab, personal communication, data not shown) and in the human lung cancer cell line A549 (Supplementary Fig. S5).

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In preliminary experiments, 200,000 MT1/2 cells significantly increased NF-κB activity, while 100 μM resveratrol increased P-gp levels in Ca3/7 cells (Fig. 3). In preliminary experiments, 200 μM resveratrol also significantly enhanced P-gp levels in Ca3/7 cells (P < 0.05) by a factor of 2.11 ± 0.29 (average of 2 independent experiments).

Other studies have shown cell line-specific effects of verapamil, cyclosporin A, and resveratrol on P-gp protein and mRNA levels. Resveratrol has been shown to decrease ABCB1 mRNA expression in HeLa, HepG2, and KBv200 cells but not MCF-7 cells (28, 29). Interestingly, cytotoxic doses of verapamil increased ABCB1 mRNA levels in leukemia cells, while nontoxic doses decreased ABCB1 mRNA levels (38). Similarly, studies in human colon carcinoma showed verapamil and cyclosporin A, at slightly growth-suppressing doses, increased ABCB1 mRNA levels (39). These inhibitors as well as resveratrol may increase P-gp expression as part of a cell stress response to efflux harmful chemicals, as cytotoxic non-P-gp substrates have been shown to increase P-gp expression (40).

Verapamil and cyclosporin A, but not resveratrol, inhibit Rh123 efflux

To confirm that verapamil, cyclosporin A, and resveratrol are functioning via inhibition of P-gp, levels of Rh123 in MT1/2 and Ca3/7 cells after 30 minutes efflux were measured in the presence of these chemicals. The effect of each individual chemical was normalized to their

Table 1. Verapamil and resveratrol enhanced ursolic acid-mediates cytotoxicity in MT1/2 cells, indicated by a decrease in the IC50 for ursolic acid (average of 3–4 independent experiments, n = 3/exp)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose</th>
<th>Fold decrease of IC50</th>
<th>P</th>
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<tbody>
<tr>
<td>+Veh</td>
<td>1</td>
<td></td>
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<tr>
<td>+5 μM/L</td>
<td>1.14 ± 0.16</td>
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<tr>
<td>+10 μM/L</td>
<td>1.17 ± 0.19</td>
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<tr>
<td>+25 μM/L</td>
<td>1.53 ± 0.31</td>
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<tr>
<td>+50 μM/L</td>
<td>1.99 ± 0.41</td>
<td>&lt;0.01</td>
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<tr>
<td>+100 μM/L</td>
<td>2.52 ± 0.53</td>
<td>&lt;0.05</td>
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<tr>
<td>+Veh</td>
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<tr>
<td>+5 μM/L</td>
<td>1.00 ± 0.02</td>
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<td>Verapamil</td>
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<td>+Veh</td>
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<tr>
<td>+5 μM/L</td>
<td>1.03 ± 0.11</td>
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<td>+25 μM/L</td>
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<td>+50 μM/L</td>
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<td>+100 μM/L</td>
<td>1.24 ± 0.23</td>
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<td>+Veh</td>
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<tr>
<td>+5 μM/L</td>
<td>1.01 ± 0.06</td>
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<tr>
<td>Cyclosporin A</td>
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<td>1.01 ± 0.07</td>
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<tr>
<td>+25 μM/L</td>
<td>1.38 ± 0.2</td>
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<tr>
<td>+50 μM/L</td>
<td>1.35 ± 0.17</td>
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<tr>
<td>+100 μM/L</td>
<td>1.3 ± 0.11</td>
<td>&lt;0.005</td>
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<td>+Veh</td>
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<tr>
<td>+5 μM/L</td>
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<td>Resveratol</td>
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<td>+100 μM/L</td>
<td>1.3 ± 0.11</td>
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NOTE: The cytotoxicity of ursolic acid was enhanced by verapamil and resveratrol in MT1/2 cells.

The discrepancies for the sensitization effects of verapamil, cyclosporin A, and resveratrol in ursolic acid-treated cells may be explained by potential differences in substrate dependence of P-gp inhibitors. Studies have shown that different tea catechins can affect the transport of some P-gp substrates but not others, indicating the potential of P-gp modulation via allosteric sites (34). Others have also found P-gp has multiple distinct sites for interaction with compounds (6, 35, 36). In addition, these cell lines may express variants of P-gp, which could differentially affect the ability of inhibitors to interact with P-gp at these multiple sites (37).

Verapamil, cyclosporin A, and resveratrol do not decrease P-gp levels

Western blotting was performed to determine whether the effects of these compounds on potentiating ursolic acid-induced cytotoxicity were due to a decrease in P-gp expression. Levels of phosphorylated p65, indicative of NFκB activity, were also measured to explain potential changes in P-gp levels. However, none of the treatments significantly decreased P-gp levels or NF-κB activity. Some treatments, including cyclosporin A in both cell lines and resveratrol in MT1/2 cells significantly increased NFκB activity, while 100 μM resveratrol increased P-gp levels in Ca3/7 cells (Fig. 3). In preliminary experiments, 200 μM resveratrol

Table 2. Verapamil, cyclosporin A, and resveratrol enhanced ursolic acid-mediated cytotoxicity in Ca3/7 cells, indicated by a decrease in the IC50 for ursolic acid (average of 3–4 independent experiments, n = 3/exp)

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<tr>
<td>+Veh</td>
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<tr>
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<td>1.34 ± 0.06</td>
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<td>Verapamil</td>
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<tr>
<td>+5 μM/L</td>
<td>1.53 ± 0.2</td>
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<td>+25 μM/L</td>
<td>1.82 ± 0.27</td>
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<td>+50 μM/L</td>
<td>1.96 ± 0.43</td>
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<td>1.93 ± 0.39</td>
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<td>+Veh</td>
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<td>+5 μM/L</td>
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<td>Cyclosporin A</td>
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<td>1.96 ± 0.43</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>+100 μM/L</td>
<td>1.93 ± 0.39</td>
<td>&lt;0.05</td>
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<tr>
<td>+Veh</td>
<td>1</td>
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<tr>
<td>+5 μM/L</td>
<td>0.91 ± 0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resveratol</td>
<td>+10 μM/L</td>
<td>1.0 ± 0.29</td>
<td></td>
</tr>
<tr>
<td>+25 μM/L</td>
<td>1.35 ± 0.15</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>+50 μM/L</td>
<td>1.42 ± 0.17</td>
<td>&lt;0.005</td>
<td></td>
</tr>
<tr>
<td>+100 μM/L</td>
<td>2.31 ± 0.52</td>
<td>&lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: The cytotoxicity of ursolic acid was enhanced by verapamil, cyclosporin A, and resveratrol in Ca3/7 cells.
vehicle (H₂O, EtOH, or DMSO). Each individual vehicle did not affect the relative Rh123 efflux differences between cell lines (Supplementary Fig. S6). As expected, verapamil significantly decreased efflux of Rh123 in both cell lines. Cyclosporin A significantly decreased Rh123 efflux in Ca3/7 cells while showing a trend towards this effect in MT1/2 cells. Interestingly, resveratrol significantly increased Rh123 efflux in MT1/2 cells, but had no effect in Ca3/7 cells (Fig. 4).

These results mirror those found with sensitization of ursolic acid-mediated cytotoxicity, which revealed verapamil sensitized cells of both lines while cyclosporin A only significantly sensitized Ca3/7 cells to ursolic acid (Tables 1 and 2; Supplementary Fig. S1). However, these results show that resveratrol did not increase Rh123 levels. This, combined with the fact resveratrol does not decrease P-gp levels (Fig. 3) indicates resveratrol does not inhibit P-gp to enhance the effects of ursolic acid in these skin-relevant systems.

**Discussion**

In this study we attempted to determine whether the cytotoxic effects of the phytochemical ursolic acid in skin cancer cell lines could be enhanced with various chemosensitizing compounds. These included verapamil and cyclosporin A, inhibitors of the efflux pump P-gp, and resveratrol, a phytochemical which can sensitize cells to chemotherapeutics through various mechanisms. In addition, we used doxorubicin hydrochloride, a chemotherapeutic compound effective in a multitude of tumor types including aggressive skin carcinoma (41), as a positive control P-gp substrate. The cell lines used were MT1/2, a
cell line derived from chemically induced mouse skin papilloma (30), and Ca3/7, derived from chemically induced mouse skin carcinoma (31).

Our results indicated that the Ca3/7 carcinoma line was significantly more chemoresistant to both ursoic acid and doxorubicin hydrochloride, relative to the MT1/2 papilloma cell line. In addition, ursoic acid more strongly inhibited viability of the skin cancer cell line than doxorubicin hydrochloride. To determine the mechanism of relative resistance, pathways associated with chemoresistance were analyzed. We found Ca3/7 cells exhibit higher basal P-gp expression and NF-kB activity than MT1/2 cells. As described earlier, the increased P-gp levels may be driven by higher NF-kB activity (8), which contributes to transcription of a wide variety of genes involved in progression to carcinomas (42). In addition, Rh123 accumulation and efflux assays showed Ca3/7 cells have higher P-gp activity than MT1/2 cells.

Because P-gp appeared to be a contributing factor to resistance to ursoic acid, we combined ursoic acid with well-characterized P-gp inhibitors verapamil and cyclosporin A as well as the chemosensitizing phytochemical resveratrol. While verapamil, cyclosporin A, and resveratrol all enhanced the cytotoxic effects of ursoic acid in Ca3/7 cells, only verapamil and resveratrol synergized with ursoic acid in MT1/2 cells. One explanation for these discrepancies is the potential that verapamil and cyclosporin A bind and inhibit P-gp at different sites (43). In addition, studies of different ABCB1 variants have shown no differences in basal activity but differences in the ability of inhibitors to block P-gp activity (37). The differing sensitivities of MT1/2 and Ca3/7 to doxorubicin hydrochloride or ursoic acid may be mediated by P-gp amounts, whereas different interactions between P-gp and inhibitor across cell lines may be explained by ABCB1 polymorphisms as well as separate inhibitor binding sites.

To determine whether verapamil, cyclosporin A, and resveratrol are inhibiting P-gp function in these cells, Western blotting for total P-gp levels as well as Rh123 efflux assays for P-gp activity was performed. None of the chemosensitizers significantly decreased P-gp levels or NFκB activity. However, the Rh123 efflux assay indicated verapamil increased Rh123 levels in both cell lines, while cyclosporin A only increased Rh123 levels in Ca3/7 cells. These results parallel that seen with sensitization to ursoic acid-mediated cytotoxicity in these cell lines. Interestingly, the Rh123 efflux assay also showed resveratrol actually decreased Rh123 levels in MT1/2 cells while having no effect in Ca3/7 cells. Regardless, these results indicate that while verapamil and cyclosporin A can sensitize skin cancer cells to ursoic acid via P-gp inhibition, the synergistic effects of resveratrol and ursoic acid occur through a different mechanism. Because resveratrol synergizes with ursoic acid through a separate mechanism than P-gp inhibitors, we also propose future studies examining combinations of resveratrol, ursoic acid, and P-gp inhibitors in skin cancer-relevant systems.

Other studies have shown resveratrol may sensitize cells by blocking chemotherapy-induced NF-kB activity (44). However, in our cancer cell lines, resveratrol did not inhibit NF-kB activity (data not shown). Our other preliminary studies showed that resveratrol and ursoic acid actually synergistically increased NF-kB activity in Ca3/7 cells, measured by Western blotting for phosphorylated p65 (data not shown). Another potential explanation for the synergistic activities of resveratrol and ursoic acid is through resveratrol-mediated potentiation of apoptotic pathways or inhibition of metabolizing enzymes (44). While the metabolism of ursoic acid is not well characterized, related compounds such as glycyrrhetinic acid (45) and protepanaxatriol ginsenosides (46) are metabolized by the cytochrome p450 isoform CYP3A4. In addition, a number of studies have shown resveratrol inhibits the activities of this isoform (47). This indicates resveratrol may enhance the effects of ursoic acid by preventing its metabolism and subsequently maintaining its intracellular concentration.

Contrary to some positive results, a number of other clinical trials have shown that P-gp inhibitors do not enhance response to chemotherapy. However, many of these studies suffered from various design flaws, including failure to enrich the patient population for those whose tumors overexpress P-gp, show increased P-gp activity through the use of radioimaging, or have demonstrated resistance to previous chemotherapy. In addition, some clinical trials have also
combined P-gp inhibitors with a chemotherapy that was not a P-gp substrate (5). As a consequence, recent research and subsequent clinical trials have focused on specific pathway inhibitors and moved away from chemotherapy plus P-gp inhibitors as a treatment modality. However, many of these targeted therapies (gefitinib, gleevec, etc.) are substrates for P-gp themselves (48). We suggest clinical trials using P-gp inhibitors, perhaps in combination with tolerated natural compounds like ursolic acid, need to be explored in chemoresistant tumor types.

Ursolic acid has been shown to inhibit tumor formation in a variety of animal models, including cancers of the breast (18), lung (19), and skin (20) among others. In addition, ursolic acid is reasonably tolerated in humans, and can achieve concentrations as high as 7.57 μmol/L in the serum when supplied intravenously in nanoliposomes (49). We suggest use of ursolic acid in addition to approved P-gp inhibitors may be a valuable method to prevent or reverse tumor formation in P-gp-expressing human tumors. Also, topically applied ursolic acid plus P-gp inhibitors may provide an easily administered, non-scarring alternative to traditional surgery for resectable SCC. In addition to enhancing ursolic acid accumulation in P-gp-expressing SCC, P-gp inhibition will help limit topical applications of ursolic acid or other substrate chemotherapeutics to the epidermis. Studies have shown that knocking out P-gp in mouse skin kept P-gp substrates Rh123 and itraconazole localized to the epidermis. Topical application of P-gp inhibitors propranolol and verapamil had a similar effect on itraconazole distribution (50).

References

Enhancement of Ursolic Acid Tumor Cytotoxicity In Vitro


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Jacob J. Junco, Anna Mancha, Gunjan Malik, et al.


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