Macelignan: A New Modulator of P-Glycoprotein in Multidrug-Resistant Cancer Cells

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The effect of macelignan, a phytoestrogen, on P-gp function was investigated using multidrug resistant cancer cells overexpressing P-gp (NCI/ADR-RES) and the fluorescent P-gp substrates, daunorubicin and rhodamine 123. Macelignan (40 \( \mu \)M) increased the cellular accumulation of daunorubicin by approximately three-fold in NCI/ADR-RES cells, whereas it did not alter the cellular accumulation of daunorubicin in MCF-7/sensitive cells. Similarly, the presence of macelignan also enhanced significantly \((P < 0.05)\) the cellular accumulation of rhodamine 123 in a concentration-dependent manner in NCI/ADR-RES cells. Furthermore, cancer cells were more susceptible to the cytotoxicity of vinblastine, a P-gp substrate, in the presence of macelignan. Those results suggest that macelignan has inhibitory effects on P-gp mediated cellular efflux. However, P-gp activity did not affect the cellular accumulation of macelignan itself. Taken all together, macelignan was identified as a novel inhibitor of P-gp activity and may be a promising lead compound for the rational design of more efficacious drugs to reverse multidrug resistance in cancer.

INTRODUCTION

Lignans are a class of phytoestrogens having numerous potential pharmacological activities including anticancer, anti-inflammatory, antimicrobial, antioxidative, and immunosuppressive activities (1,2). Among the bioactive plant lignans ubiquitous in human diets, macelignan has been isolated from *Myristica fragrans*, widely used for the spice and flavor of foods, and structurally identified as a \((2R,3S)-1-(3,4-methylenedioxyphenyl)-2,3-dimethyl-4-(4-hydroxy-3-methoxyphenyl)-butane\) (Fig. 1) (3). Macelignan has been reported to possess the various biological activities that might be linked to certain therapeutic benefits in the treatment of human diseases (4–7). For example, macelignan showed strong inhibitory activity against *Streptococcus mutans*, cariogenic oral bacteria, implying the utility of macelignan as a natural antibacterial agent in oral care products (7). Macelignan also induced an apoptotic effect in human promyelocytic leukemia (HL-60) cells in a caspase-3 activation manner, suggesting the therapeutic potential as a cancer chemopreventive agent (5).

Cancer chemotherapy involves the use of anticancer drugs, which may be obtained from natural or synthetic sources. Recently, the interest in natural products has increased dramatically as an attractive approach for the discovery of new anticancer drugs (8–10). Particularly, the discovery of natural products that can modulate the activity of multidrug resistance proteins should be beneficial to improve the effectiveness of chemopreventive agents given that multidrug resistance is one of the big challenges in cancer chemotherapy. Although multidrug resistance can be mediated by multiple mechanisms, it has most often been linked to the overexpression of P-glycoprotein (P-gp), a drug efflux pump (11,12). So far, numerous studies have revealed the ability of dietary phytochemicals in vegetables and fruits to modulate P-gp function (13–17). For instance, some dietary phytochemicals including bioflavonoid quercetin, morin,
and EGCG have inhibitory effects on P-gp (14–17). However, although many studies have been performed with flavonoids, lignans have not been extensively studied yet, and only a few lignans have been identified as P-gp modulators (18,19). Therefore, in order to provide a new chemical lead for the discovery of effective P-gp modulators, the present study investigated the interaction characteristics of macelignan with P-gp in multidrug resistant cancer cells.

MATERIALS AND METHODS

Materials

Daunorubicin, rhodamine 123, vinblastine, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and BCA protein assay kit were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS), cell culture media, antibiotics, and all other reagents used in cell culture studies were purchased from Seolin Science Co. (Seoul, Korea). Macelignan was kindly provided by Amicogen Co. (Jinju, Korea). Human breast cancer cells (MCF-7/sensitive) and NCI/ADR-RES cells (previously designated as MCF-7/ADR-RES) were obtained from the National Cancer Institute (Frederick, MD). All other chemicals were of reagent grade, and all solvents were of HPLC grade.

Cell Cultures

MCF-7/sensitive and NCI/ADR-RES cells were routinely maintained in RPMI 1640 culture medium containing 10% FBS and penicillin (50 IU/ml)/streptomycin (50 µg/ml). All cells were maintained in an atmosphere of 5% CO₂ and 90% relative humidity at 37°C.

Effect of Macelignan on the Cellular Accumulation of Daunorubicin and Rhodamine 123

Cells, plated at a density of 10⁵ cells/cm², were incubated with 50 µM daunorubicin or 20 µM rhodamine 123 in the absence and the presence of macelignan (20 µM or 40 µM). After 2-h incubation, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with 1% sodium dodecyl sulfate (SDS) in PBS. Fluorescence intensity of each sample was measured by a spectrofluorometer. The excitation and emission wavelengths were 502 and 588 nm for daunorubicin and 485 and 527 nm for rhodamine 123, respectively. The protein amount of each sample was determined with BCA protein assay kit following the manufacturer’s instruction (Sigma Chemical Co., St. Louis, MO).

Effect of P-gp Overexpression on the Cellular Accumulation of Macelignan

Cells, plated at a density of 10⁵ cells/cm², were incubated with drug solution (50 µM daunorubicin or 10 µM macelignan). At the end of 1-h incubation, the drug solution was removed, and the cells were washed 3 times with ice-cold phosphate-buffered saline. After the cell lysis, cells were harvested and sonicated for 1 to 2 min. Acetonitrile was added to the cell lysate, vortexed rigorously, and centrifuged for 5 min at 3,000 rpm. After the filtration of the supernatant through a membrane filter (0.45 µm), the macelignan concentration of each sample was determined by HPLC. The concentration of daunorubicin was measured by a spectrofluorometer as described above.

Determination of Resistance to Vinblastine Cytotoxicity

The resistance of NCI/ADR-RES cells to vinblastine cytotoxicity was determined in the absence and the presence of macelignan. Cells were seeded into 96-well plates at a density of 5 × 10³ cells/well. After 24-h incubation in a CO₂ incubator at 37°C, various concentrations of vinblastine with and without macelignan (20 µM or 40 µM) were added to the cells, and the plates were incubated for 3 days. At the end of incubation, cell viability was determined by a modified colorimetric assay using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (20). Briefly, medium with the drug was removed and replaced by fresh medium (200 ul/well) containing 0.1 mg/ml MTT. After a 90-min incubation at 37°C, the medium was aspirated, and the cells were extracted with 150 ul/well of DMSO. The concentration of the extracted formazan metabolite was determined by the measurement of absorbance at 560 nm in a 96-well plate reader. The 50% cytotoxic concentration (CC₅₀) was determined from the nonlinear regression of a dose-response curve by using SigmaPlot® 9.0 (Systat Software Inc., Point Richmond, CA).

HPLC Analysis

Cellular concentrations of macelignan were determined by an HPLC assay. Felodipine was used as the internal standard for the assay. The chromatographic system consisted of a pump (LC-10AD), an automatic injector (SIL-10A), and a UV detector (SPD-10A) (Shimadzu Scientific Instruments, Tokyo, Japan). An octadecylsilane column (Gemini C18, 4.6 × 250 mm, 5 µm;
Phenomenex, Torrance, CA) was eluted with a mobile phase consisting of water:acetonitrile:methanol (30:35:35 vol/vol%). The flow rate was 1.0 ml/min, with the detection wavelength set at 240 nm. The calibration curve from the standard samples was linear over the concentration range of 0.01 µg/ml to 5 µg/ml. The limit of detection was 0.01 µg/ml.

Statistical Analysis

All the means are presented with their standard deviation. The statistical significance of the difference in the parameters was determined using ANOVA followed by a Dunnett’s post hoc test or by a Student’s t-test. A P value < 0.05 was considered statistically significant.

RESULTS

Effects of Macelignan on the Cellular Accumulation of Daunorubicin and Rhodamine 123

Previous studies have indicated that MCF-7/sensitive cells had no detectable level of P-gp, but NCI/ADR-RES cells overexpressed P-gp (14,24). Therefore, MCF-7/sensitive and NCI/ADR-RES cells were employed as the negative and the positive cells for P-gp-mediated efflux, respectively, and the inhibitory effects of macelignan on the accumulation of daunorubicin or rhodamine 123 were determined in both MCF-7/sensitive and NCI/ADR-RES cells. As illustrated in Fig. 2, the cellular accumulation of daunorubicin was approximately twofold lower in NCI/ADR-RES cells overexpressing P-gp than that in MCF-7/sensitive cells, indicating the P-gp-mediated efflux of daunorubicin. Macelignan (40 µM) increased the cellular accumulation of daunorubicin by approximately threefold in NCI/ADR-RES cells, whereas it did not alter the cellular accumulation of daunorubicin in MCF-7/sensitive cells. Similarly, the presence of macelignan also enhanced significantly (P < 0.05) the cellular accumulation of rhodamine 123 in a concentration-dependent manner in NCI/ADR-RES cells (Fig. 3).

Effect of P-gp Overexpression on the Cellular Accumulation of Macelignan

To examine whether P-gp activity affects the cellular accumulation of macelignan, the cellular uptake of macelignan was evaluated in MCF-7/sensitive and NCI/ADR-RES cells. As illustrated in Fig. 4, the cellular accumulation of macelignan was similar in both NCI/ADR-RES cells and MCF-7/sensitive cells, whereas the cellular accumulation of daunorubicin, a positive control, was reduced by approximately twofold with the overexpression of P-gp in NCI/ADR-RES cells.

Effects of Macelignan on Vinblastine Cytotoxicity

Cell growth inhibition curves of vinblastine were determined in the absence and the presence of macelignan in NCI/ADR-RES cells. Macelignan alone was not cytotoxic over the tested concentrations. As shown in Fig. 5, cells were more susceptible to the cytotoxicity of vinblastine, a P-gp substrate, in the presence of macelignan as compared to the control given vinblastine alone, implying the enhanced intracellular accumulation of vinblastine in the presence of macelignan.

DISCUSSION

Advances in molecular biology have accelerated the identification, cloning, and functional characterization of various drug transporters, resulting in the great awareness of the importance of drug transporters in the delivery of structurally diverse therapeutic compounds to the desired target organs. Particularly, efflux transporters pumping therapeutics out of cells actively involved in the development of multidrug resistance in cancer.
treatment; therefore, the modulation of those efflux transporters becomes more critical to improve the effectiveness of cancer chemotherapy. Among efflux transporters, the first mediator of multidrug resistance characterized at the molecular level was P-gp, one of the ATP-binding cassette (ABC) transporters (11,21). P-gp plays an important role in the absorption, distribution, and elimination of many commonly used drugs as well as multidrug resistance in cancer and consequently has great impact on the efficacy and toxicity of drugs. Therefore, over the past decades, there have been tremendous efforts to discover and develop the effective modulators of P-gp function. Whereas first- and second-generation inhibitors were limited by unacceptable toxicity or unpredictable pharmacokinetic interactions, third-generation inhibitors (e.g., tariquidar XR9576, zosuquidar LY335979, laniquidar R101933) possess high specificity for P-gp and have shown promising results in clinical trials (12,22). In addition to the synthetic compounds, there have been considerable amounts of work performed with dietary herbs. For instances, some dietary phytochemicals such as the bioflavonoid quercetin, morin, and EGCG have shown the inhibitory effects of P-gp activity (14–17). However, there should be continuous evaluations of those compounds to establish the true therapeutic potential of P-gp-mediated MDR reversal.

Lignans are one of three main groups of plant compounds classified as phytoestrogens, the other two being isoflavonoids and coumestans (23). All three groups possess a diphenolic
The cellular accumulation of daunorubicin increased significantly (23). Among phytoestrogens, isoflavones have been extensively studied with regard to P-gp inhibition effects; however, lignans have not been studied much yet. Therefore, the present study evaluated the interaction characteristics of macelignan with P-gp in multidrug resistant cancer cells, which may provide a new chemical backbone for the synthesis of derivatives against P-gp activity.

In the present study, the cellular accumulation of daunorubicin, a P-gp substrate, was approximately twofold lower in NCI/ADR-RES cells than that in MCF-7/sensitive cells as parallel to the observation that NCI/ADR-RES cells have significant expression of P-gp, whereas MCF-7/sensitive has no detectable expression of P-gp (14,24). The cellular accumulation of daunorubicin increased significantly ($P < 0.05$) in the presence of macelignan (40 $\mu$M) in NCI/ADR-RES cells but not in MCF-7/sensitive cells. Those results can be explained by the inhibitory effect of macelignan on P-gp mediated cellular efflux of daunorubicin. Although daunorubicin is a substrate of multidrug resistance protein 1 (MRP1) as well as P-gp, the involvement of MRP1 in daunorubicin accumulation in NCI/ADR-RES cells should be negligible since MRP1 is scarcely found in NCI/ADR-RES cells (14,24). Similarly, the cellular accumulation of rhodamine 123 was significantly enhanced in a concentration-dependent manner by the coincubation of macelignan in NCI/ADR-RES cells, supporting the role of macelignan as a P-gp inhibitor. However, P-gp activity did not appear to have a great impact on the cellular accumulation of macelignan itself, as the cellular accumulation of macelignan was not altered by the overexpression of P-gp.

Cell growth inhibition of vinblastine was also evaluated in the absence and the presence of macelignan. As generally expected with P-gp inhibitors, cells became more susceptible to the cytotoxicity of vinblastine in the presence of macelignan as compared to the control given vinblastine alone, indicating the reduced P-gp-mediated efflux of vinblastine in the presence of macelignan. In our cell culture systems, which include 10% serum, macelignan enhanced the cytotoxicity of vinblastine at the concentration as low as 20 $\mu$M. However, we observed that the effective concentration of macelignan could be shifted to 10 $\mu$M under the culture conditions containing 2% serum instead of 10% serum, implying that the effective concentration of macelignan might be varied in vivo depending on its protein-binding characteristics. Although the clinical implications are unknown and worthy of further study, the present study identified macelignan as a new P-gp inhibitor, which may also serve as a chemical backbone for designing the optimal P-gp inhibitors to attenuate the multidrug resistance in cancer. Therefore, a fruitful area of future studies may be in modifying natural macelignans or screening more lignans with structural diversity for more potent inhibition effects on multidrug resistance proteins.

**CONCLUSION**

Macelignan appears to be a novel inhibitor of P-gp transport activity and may be a promising lead compound for the rational design of more efficacious drugs to reverse multidrug resistance in cancer.

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**REFERENCES**


