

# Overcoming of P-glycoprotein-mediated multidrug resistance in K562/A02 cells using riccardin F and pakyonol, bisbibenzyl derivatives from liverworts

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## Summary

Riccardin F and pakyonol, macrocyclic bisbibenzyls from *Plagiochasm intermedium*, have been confirmed to possess antifungic activities against *Candida albicans*. Herein, we evaluated their anti-tumor activity *in vitro* by employing K562 and K562/A02 cells, the well-known adriamycin (ADR)-induced multidrug resistance (MDR) tumor cell lines over-expressing P-glycoprotein (P-gp). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays showed that riccardin F and pakyonol ranging from 0 to 6 µg/mL exhibited no inhibitory effects on the growth of the two cell lines. However, in the presence of 3 µg/mL riccardin F or pakyonol (non-cytotoxic concentration), the IC<sub>50</sub> of ADR against K562/A02 cells decreased by 2.51- and 4.78-fold, respectively. Flow cytometry showed that riccardin F and pakyonol significantly enhanced the accumulation of ADR in K562/A02 cells. Furthermore, fluorescence intensity detection revealed that the two natural products remarkably increased the retention of rhodamine-123 in K562/A02 cells rather than in K562 cells, indicating that the major cause for riccardin F and pakyonol to reverse P-gp-mediated MDR in K562/A02 cells is probably due to the constrained transport activity of P-gp. This study explores the potential application of bisbibenzyl type compounds as modulators of P-gp-mediated MDR in tumor cells.

**Keywords:** P-glycoprotein (P-gp), bisbibenzyl, riccardin F, pakyonol, K562/A02 cells, multidrug resistance (MDR)

## 1. Introduction

Multidrug resistance (MDR), a major obstacle to successful tumor chemotherapy in the clinic, refers to the resistance of cancer cells to multiple structurally and mechanically unrelated hydrophobic anticancer drugs (1). Over-expression of P-glycoprotein (P-gp), a 170 kDa transmembrane glycoprotein, is widely considered to be the cause of MDR under most circumstances. P-gp, an ATP-dependent drug transporter, unilaterally expels intracellular drugs out of cells, resulting in drug

resistance. A large number of well known anti-cancer drugs such as paclitaxel and vincristine, substrates of P-gp, were limited by P-gp-mediated MDR in clinical application (2). To circumvent efficaciously drug resistance in tumor chemotherapy, it is essential to develop either novel anticancer agents to overcome P-gp-mediated MDR and/or inhibitors that specifically block the function of P-gp as drug transporter. A number of synthetic and natural substances, such as verapamil, dihydropyridine analogs, quinidine and cyclosporin A, have been confirmed for their abilities to overcome P-gp-mediated MDR by inhibiting the transport activity of P-gp (3). However, these compounds have failed in clinic applications due to their serious side effects and poor pharmacokinetics (4). Therefore, exploration of alternatives to current P-gp inhibitors with higher selectivity and stronger potency remains a major goal in this field.

Natural products with bioactivities and various structural properties are becoming the major source

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of novel agents with pharmacological interests (5). A considerable number of natural polyphenolic compounds, including curcuminoids, curcumin and eigallocatechin gallate (EGCG) have been revealed to modulate P-gp efflux activity (6-8). Bisbibenzyls, a class of plant metabolites produced exclusively in liverworts, is a family of phenolic compounds belonging to stilbenoids (9). They have attracted more attention for their wide-range of bioactivity and medicinal potency, including antibacterial, antifungal, antioxidative, muscle-relaxing and cytotoxic activities as well as inhibitory effects on 5-lipoxygenase, cyclooxygenase and calmodulin (10).

In the previous study, we demonstrated that plagiochin E, a macrocyclic bisbibenzyl compound isolated from *Marchantia polymorpha*, not only enhanced the sensitivity of resistant *Candida albicans* toward fluconazole but also reversed P-gp-mediated MDR in resistant tumor cells (11,12). Recently, we isolated riccardin F and pakyonol from *Plagiochasm intermedium*, and substantiated their antifungal activity against *C. albicans* (13,14). In the present study, we further evaluate their reversal efficacy on P-gp-mediated MDR by employing the K562/A02 cell line, which is known for over-expressing P-gp (15).

## 2. Materials and Methods

### 2.1. Chemicals

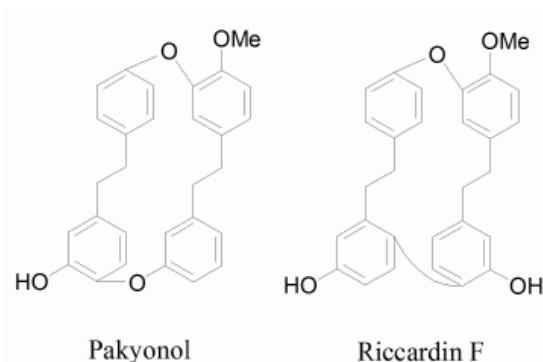
The tested macrocyclic bisbibenzyl compounds riccardin F and pakyonol (Figure 1) were isolated from *Plagiochasm intermedium*, collected in Shandong Province. Their structures were identified by interpretation of their mass spectrum (MS) and 1D- and 2D-nuclear magnetic resonance (NMR) data as well as by comparison with reported values. They were dissolved in dimethylsulfoxide (DMSO) and then diluted in RPMI-1640 medium for *in vitro* assays.

### 2.2. Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Rhodamine-123, adriamycin (ADR) and DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium and fetal bovine serum (FBS) were purchased from HyClone (Logan, UT, USA).

### 2.3. Cell lines and cell culture

The human myeloid leukemia cell line K562, and its MDR counterpart K562/A02, were obtained from the Department of Pharmacology, the Institute of Hematology of Chinese Academy of Medical Sciences (Tianjin, China). K562 and K562/A02 cells were



**Figure 1. The chemical structures of riccardin F and pakyonol.**

maintained in complete RPMI-1640 medium in the absence and presence of 1  $\mu\text{g}/\text{mL}$  ADR at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$ , respectively. Resistant cells were cultured for two weeks in drug-free medium prior to use in experiments (16).

### 2.4. MTT assay

Cytotoxicity of riccardin F and pakyonol toward K562 and K562/A02 cells was first measured by the MTT method. Briefly, K562/A02 cells ( $1\sim 2 \times 10^4$  cells per well) were seeded in 96-well plates. After 24 h incubation, cells were treated with various concentrations of riccardin F and pakyonol for 48 h, respectively. Light absorbance of the solution was measured at 570 nm on a plate reader (TECAN, Grodig, Salzburg, Austria). Cell viability was assessed by MTT assay as reported (17).

The reversal effect of riccardin F and pakyonol was further investigated with a similar procedure. K562/A02 and K562 cells were treated for 48 h with varying concentrations of ADR in absence or presence of riccardin F or pakyonol at doses of minimum inhibitory effect on cell growth, respectively. The inhibitory rate of cell growth was obtained through previously described procedures (18), and  $\text{IC}_{50}$  values for ADR were calculated from plotted results using untreated cells as reference. The reversal fold (RF) values, as potency parameter of reversal, were calculated from dividing  $\text{IC}_{50}$  of ADR alone by  $\text{IC}_{50}$  of ADR in combination with riccardin F or pakyonol. Triplicate experiments with triplicate samples were performed. Control medium included an equivalent amount of DMSO (as solvent control), but the applied dosage of riccardin F or pakyonol did not exhibit modulation effects on cell growth or drug sensitivity. In all experiments, verapamil was used as a positive control.

### 2.5. Intracellular ADR accumulation assay

On the basis of ADR autofluorescence, effects of riccardin F and pakyonol on accumulation of ADR

inside K562 and K562/A02 cells were assessed by measuring the mean fluorescence intensity (MFI) associated with ADR (19). Briefly, the cells at  $5 \times 10^5$  per well pretreated with 3  $\mu\text{g}/\text{mL}$  of each sample for 1 h were incubated in medium containing 3  $\mu\text{g}/\text{mL}$  ADR at 37°C for 90 min, respectively. MFI associated with ADR was measured using FACScan Caliber (Beckton Dickinson, USA) at 488 nm for emitted fluorescence and 615 nm for collected fluorescence. Data analysis was performed using CellQuest software.

### 2.6. Intracellular rhodamine-123 accumulation assay

Rhodamine-123 is used widely as the specific fluorescence indicator of P-gp. Therefore, regulation of the effect of riccardin F and pakyonol on drug-transport activity of P-gp was determined by measuring intracellular accumulation of rhodamine-123 in resistant cancer cells. K562 cells and K562/A02 cells in exponential growth at  $5 \times 10^5$  per well were seeded in 6-well plates and pretreated with 3  $\mu\text{g}/\text{mL}$  of each sample for 1 h, followed by incubating with 2.5  $\mu\text{g}/\text{mL}$  of rhodamine-123 in culture medium at 37°C with 5%  $\text{CO}_2$  for 90 min. The MFI associated with rhodamine-123 was measured with FACScan flow cytometry at 488 nm emitted fluorescence and 530 nm collected fluorescence (20). Data analysis was performed using CellQuest software.

### 2.7. Data analysis

Student's *t*-test was used to compare data and a *p*-value of 0.05 was taken as the minimal level of significance. All data are expressed as mean  $\pm$  S.D.

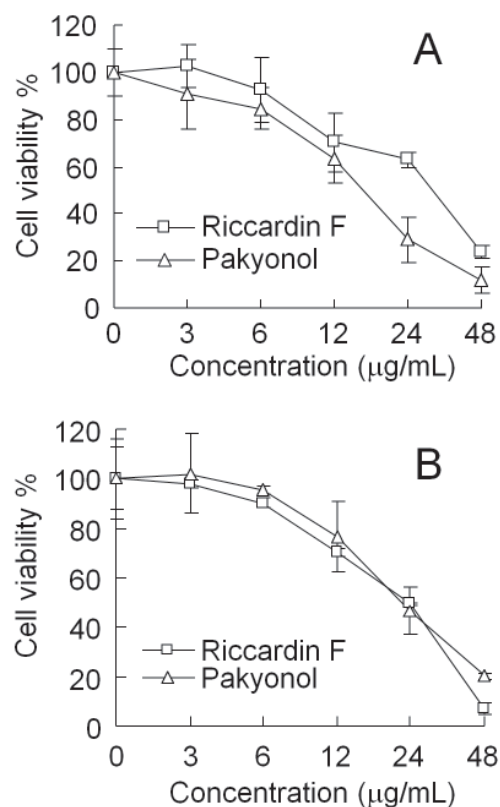
## 3. Results

### 3.1. Determination of non-cytotoxic concentration of the compounds to K562 and K562/A02 cells

In the assay, we assessed inhibitory effects of riccardin F and pakyonol on growth of K562 and K562/A02 cells using the MTT colorimetric method. As shown in Figure 2, concentrations of riccardin F and pakyonol ranging from 6 to 48  $\mu\text{g}/\text{mL}$  remarkably decreased the viability of the multidrug resistant cell line K562/A02 as well as the sensitive cell line K562, while treatments with 0 to 6  $\mu\text{g}/\text{mL}$  exhibited no significant effects on cell growth. Therefore, riccardin F and pakyonol concentrations of 0-6  $\mu\text{g}/\text{mL}$  were taken as the optimal doses for the reversal investigation of drug resistance of K562/A02.

### 3.2. Effects on potency of ADR

As shown in Table 1, treatments with riccardin F or pakyonol at 3  $\mu\text{g}/\text{mL}$  (non-toxic dosage) significantly



**Figure 2.** The effect of riccardin F and pakyonol on the proliferation of K562 and K562/A02 cells. Cells were treated with various doses (0, 3, 6, 12, 24, 48  $\mu\text{g}/\text{mL}$ ) of riccardin F and pakyonol for 48 h, respectively. Data are expressed as mean  $\pm$  S.D. of three independent experiments. (A) K562 cells; (B) K562/A02 cells.

**Table 1.** The effect of combinations of the test compounds with ADR on growth of K562/A02 cells

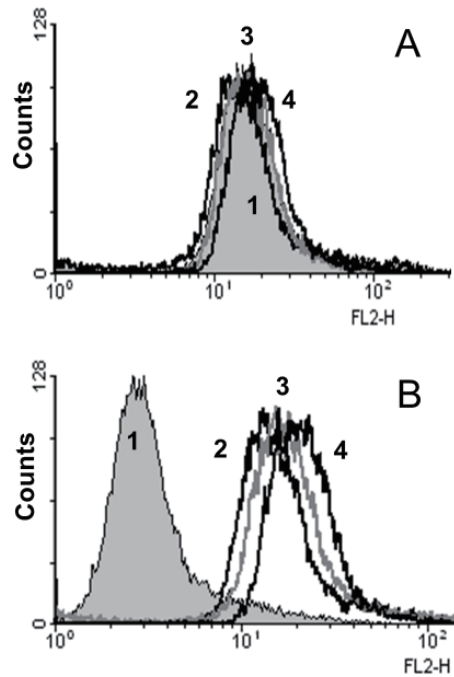
| Treatment         | K562/A02 cells                               |      | K562 cells                                   |      |
|-------------------|--|------|--|------|
|                   | IC <sub>50</sub> ( $\mu\text{g}/\text{mL}$ ) | RF   | IC <sub>50</sub> ( $\mu\text{g}/\text{mL}$ ) | RF   |
| ADR               | 5.78 $\pm$ 0.76                              | –    | 0.17 $\pm$ 0.056                             | –    |
| ADR + riccardin F | 2.34 $\pm$ 0.54*                             | 2.51 | 0.16 $\pm$ 0.086                             | 1.06 |
| ADR + pakyonol    | 1.21 $\pm$ 0.41*                             | 4.78 | 0.15 $\pm$ 0.077                             | 1.13 |
| ADR + verapamil   | 2.39 $\pm$ 0.37*                             | 2.42 | 0.16 $\pm$ 0.061                             | 1.06 |

Data are presented as mean  $\pm$  S.D. from three independent experiments. \* *p* < 0.01 vs. ADR alone. The RF of drug resistance, the IC<sub>50</sub> of ADR alone divided by the IC<sub>50</sub> of combination ADR with each compound tested, respectively, and cells.

decreased the IC<sub>50</sub> value of ADR against K562/A02 cells in comparison to the treatment of ADR alone, while this fact was not observed in K562 cells. It suggested that the two natural products at non-toxic concentrations enhanced the sensitivity of the resistant K562/A02 cells to ADR. Moreover, the RF of pakyonol against K562/A02 cells was about two times higher than that of riccardin F.

### 3.3. Modulation on intracellular ADR accumulation

Reversal activities were evaluated by flow cytometry red fluorescence histograms (Figure 3). Figure 3A

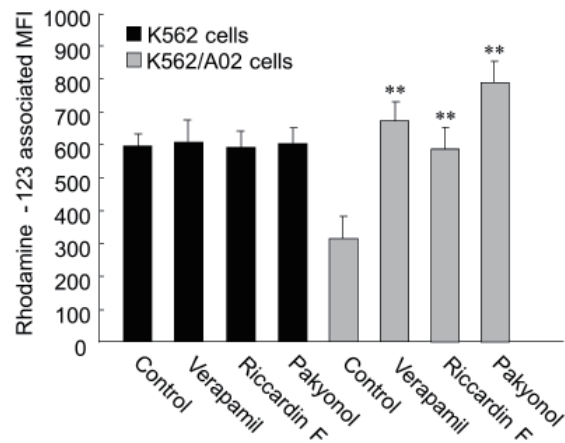


**Figure 3. Effects of riccardin F and pakyonol on intracellular accumulation of ADR in K562 and K562/A02 cells, respectively.** Cells were incubated with 3  $\mu\text{g}/\text{mL}$  of ADR for 90 min following pretreatment with 3  $\mu\text{g}/\text{mL}$  of riccardin F and pakyonol respectively for 60 min. The MFI (mean fluorescence intensity) was measured by flow cytometry. Data were obtained from three independent experiments. (A) K562 cells; (B) K562/A02 cells. (1) Control, (2-4) Treatment combinations of ADR with verapamil, riccardin F and pakyonol, respectively.

indicates that ADR accumulated in parent K562 cells efficiently, with a maximum drug accumulation plateau at 90 min incubation. Addition of riccardin F and pakyonol didn't influence ADR accumulation in K562 cells. In contrast, the accumulation level of ADR in K562/A02 cells was much lower, but it was increased by 3.8, 4.3 and 5.7-fold in the presence of verapamil, riccardin F and pakyonol, respectively. These results indicate that the synergistic effects of riccardin F or pakyonol with ADR against K562/A02 cells were related to the increased accumulation of ADR in resistant tumor cells.

### 3.4. Regulation of rhodamine-123 accumulation

Rhodamine-123, a fluorescence substrate for P-gp, is widely used to estimate the drug transportation capability of P-gp. To further investigate the mechanism of riccardin F and pakyonol to modulate the ADR accumulation level inside K562/A02 cells, the intracellular accumulation of rhodamine-123 was assessed by monitoring its fluorescence intensity. As shown in Figure 4, verapamil, riccardin F and pakyonol exhibited no influence on the rhodamine-123 accumulation ability of ADR-sensitive K562 cells. However, in K562/A02 cells, all the tested compounds



**Figure 4. Effects of riccardin F and pakyonol on intracellular accumulation of rhodamine-123 in K562 and K562/A02 cells.** Cells were incubated with 2.5  $\mu\text{g}/\text{mL}$  of rhodamine-123 for 90 min following pretreatment with 3  $\mu\text{g}/\text{mL}$  of riccardin F and pakyonol, respectively, for 60 min. The MFI was measured by flow cytometry. Data were obtained from three independent experiments and expressed as mean  $\pm$  S.D., and \*\*  $p < 0.01$  vs. K562/A02 cells control.

increased the rhodamine-123 accumulation efficiency compared with the reference, indicating the desired reversal of MDR. Among the three compounds, pakyonol exhibited the highest reversal activity, followed by verapamil and riccardin F. Since rhodamine-123 is a substrate of P-gp, the successful reversal of MDR using novel pakyonol and riccardin F reveals that the role of these two compounds in recovery of ADR accumulation is correlated with inhibition of P-gp activity.

## 4. Discussion

Inhibiting activity of P-gp is an ideal way to overcome tumor MDR. Many natural phenolic compounds, such as curcuminoid and EGCG, have been reported to restrain P-gp activity through blocking its drug-transport function or down-regulating its expression (6-8). Bisbibenzyl derivatives including riccardin F and pakyonol are dimeric bibenzyls which are chemically composed of two lunularin moieties with diarylether and/or biphenyl linkages (21). This type of natural product has been confirmed to confer a selective feature on plants against competition from the other plants and microbial attacks. On the other hand, they boast a wealth of medicinally important activities. Such a class of natural products was validated to have antioxidative potential and radical scavenging activity (21,22). In this paper, we report reversal activities of riccardin F and pakyonol against P-gp-mediated MDR by employing K562/A02 cells for the first time. Riccardin F and pakyonol as potent reversal agents of MDR were supported by the following evidence: (a) riccardin F or pakyonol at 3  $\mu\text{g}/\text{mL}$  (non-toxic dosage toward K562 and K562/A02 cells) significantly

enhanced cytotoxicity of ADR toward K562/A02 cells, while such a result was not observed in K562 cells; (b) increased ADR accumulation occurred in P-gp positive cells, but not in P-gp negative cells; and (c) increased intracellular rhodamine-123 stagnation in K562/A02 cells suggested that riccardin F and pakyonol inhibited the transport activity of P-gp. Such findings were not observed in ADR-sensitive K562 cells, which indicated that enhanced accumulation of ADR is correlated with the blocking of P-gp efflux activity.

As reported previously, the uptake of rhodamine-123, is the result of passive inward diffusion, while its efflux is known to be P-gp-dependent. Consequently, rhodamine-123 has been used extensively as an indicator for examining the efflux activity of P-gp in drug-resistant cell lines over-expressing P-gp (23,24). In P-gp activity assays, K562/A02 cells were pretreated with riccardin F and pakyonol, and intracellular rhodamine-123-associated MFI was increased by 90.3% and 151.7%, respectively. The increased accumulation of rhodamine-123 demonstrated that riccardin F and pakyonol could retrieve P-gp activity in P-gp-mediated resistant cancer cells. It was reported that a number of phenolic compounds such as flavones (hesperetin, limettin and 7-OH-coumarin) decreased P-gp efflux activity in human colon carcinoma Caco-2 cells through directly binding to unspecified sites of P-gp or down-regulating P-gp expression (25). In addition, methoxylated flavones, including tangeretin, hepatomethoxyflavone and nobiletin, have been demonstrated to show an inhibitory effect on P-gp-mediated delivery in the above-mentioned cancer cell line as well (26,27). Riccardin F and pakyonol structurally have a methoxyl at lunularin moieties. However, pakyonol possesses much stronger reversal activities than riccardin F since it might be easier to enter tumor cells due to fewer hydroxyl groups caused by lipophilic features. In the P-gp activity assay of this study, the time of exposure of cells to riccardin F and pakyonol was short (1 h), and enhanced accumulation of ADR or rhodamine-123 by riccardin F and pakyonol in K562/A02 cells is likely carried out by suppressing directly P-gp efflux activity.

In conclusion, the present results confirmed that riccardin F and pakyonol exhibited potentially *in vitro* inhibitory effects on P-gp function and the reversal of activity of P-gp-mediated MDR. This study exploited a class of bisbibenzyl compounds with inhibitory activities against P-gp-mediated MDR. The reversal effects and mechanisms of riccardin F and pakyonol in different resistant cancer cell lines and their *in vivo* pharmacokinetics are in progress.

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