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Polyphyllin D is a potent apoptosis inducer in drug-resistant HepG2 cells

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Abstract

In a search for new anticancer agents, we identified a novel compound polyphyllin D (PD) (diosgenyl α-L-rhamnopyranosyl-(1→2)-(α-L-arabinofuranosyl)-(1→4)]-[β-D-glucopyranoside) that induced DNA fragmentation and phosphatidyl-serine (PS) externalization in a hepatocellular carcinoma cell line HepG2 derivative with drug resistance (R-HepG2). PD is a saponin originally found in a tradition Chinese medicinal herb *Paris polyphylla*. It has been used to treat liver cancer in China for many years. We evaluated the cell-killing mechanisms of this compound in R-HepG2 and its parental cells. The mitochondrial apoptotic pathway was found to be involved in the PD-induced apoptosis because PD elicited depolarization of mitochondrial transmembrane potential ($\Delta\Psi$m), generation of H_2O_2, as well as release of cytochrome *c* and apoptosis-inducing factor in a dose- and time-dependent manner. In conclusion, we show for the first time that PD is a potent anticancer agent that can overcome drug resistance in R-HepG2 cells and elicit programmed cell death via mitochondrial dysfunction.

Keywords: Polyphyllin D; Apoptosis; Drug resistance; HepG2

Abbreviations: AIF, apoptosis-inducing factor; BA, bongkrekic acid; CDDP, cisplatin; DCF, dichlorofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; Dox, doxorubicin; FCS, fetal calf serum; HCC, hepatocellular carcinoma; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; NAC, *N*-acetyl-cysteine; PD, polyphyllin D; PI, propidium iodide; PS, phosphatidyl-serine; PVDF, poly vinylidene fluoride; ROS, reactive oxygen species; $\Delta\Psi$m, mitochondrial transmembrane potential.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. The high incidence of the liver cancer has been attributed to factors such as persistent infection with hepatitis virus and contact with hepatocarcinogens like nitrosamines and aflatoxins [1]. Because of the mutlifocal nature of liver carcinoma, most cancer patients are considered non-resectable at the presentation of case. In these patients, chemotherapy is the only choice of treatment. Unfortunately, development of drug resistance in tumor after treatment is always a major obstacle to the successful management of liver cancer [2]. Thus, developing new therapeutic agents that can overcome drug resistance becomes an urgent need for cancer patient.

Saponin polyphyllin D (PD) (diosgenyl α-L-rhamnopyranosyl-(1→2)-(α-L-arabinofuranosyl)-(1→4)]-[β-D-glucopyranoside) (Fig. 1) is an active component of *Paris polyphylla* which has been used to treat liver cancer in China for a long time. However, the underlying mechanism of the anticancer effect of *Paris polyphylla* remains unknown. Recently, we have chemically synthesized PD [3,4] and examined its efficacy in the induction of apoptosis in a HCC cell line HepG2 and its derivative R-HepG2 with drug resistance. Our findings illustrate that PD is a potent cytotoxic agent. Our results, for the first time, indicate that PD was able to circumvent drug resistance and elicited apoptosis in HepG2 and R-HepG2 cells via mitochondrial damages.

2. Materials and methods

2.1. Materials

JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) and DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate) were purchased from Molecular Probes. Antibodies against cytochrome *c*, apoptosis-inducing factor (AIF) and secondary antibody conjugated to horseradish peroxidase were obtained from Santa Cruz. All other reagents were from Sigma. PD was synthesized as described previously [3,4].

2.2. Cell culture

HepG2 cells, obtained from American Type Culture Collection, were cultured in RPMI 1640 medium (Sigma) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco) at 37 °C, 5% CO_2. For the development of doxorubicin (Dox)-resistant cells, HepG2 cells were co-cultured with Dox and survival cells were treated with a higher concentration of Dox during cell passages. After several rounds of selection, a clone named R-HepG2 was obtained [5]. To maintain the Dox-resistance, R-HepG2 cells were cultured with 1.2 μM Dox during passages. From time to time, the sensitivity of cells to Dox was analyzed for their resistance to cell death.

2.3. MTT and alamar blue assay

Cell viability was determined by MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) assay. After treatment, 20 μl of MTT (5 mg/ml) in PBS was incubated with cells in a 96-well plate for 2 h at 37 °C. Subsequently, the medium containing MTT was removed, and 100 μl of acidified isopropanol (0.04 N HCl) added. Spectrophotometric absorbance of each sample was measured at 470 nm using a microplate reader (Bio-Rad, model 3550). For the alamar blue assay, cells after treatment were incubated with alamar blue (10%, w/v) for 3 h and fluorescence was measured with a fluorescence plate reader (CytoFluor 2350, Millipore) with excitation at 530 nm and emission 590 nm.

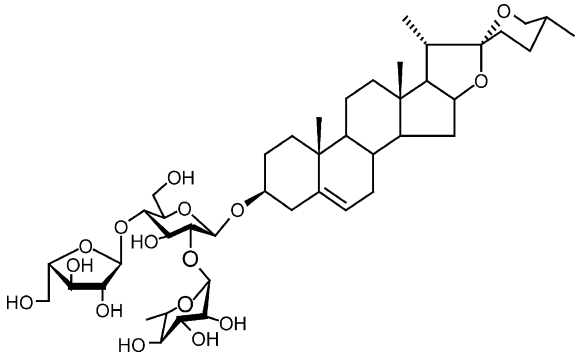

Fig. 1. Chemical structure of polyphyllin D.

2.4. Agarose gel electrophoresis for analysis of DNA fragmentation

Cells (1×10^6 ml^{-1}) after treatment were washed and suspended in 400 μl cell lysis buffer (5 mM Tris–HCl, 10 mM NaCl, 100 mM EDTA, 1% (w/v) SDS, 50 μg/ml proteinase K, pH 7.4). RNase (final concentration 100 μg/ml) was then added and incubation was continued at 45 °C for 45 min. After incubation overnight at 37 °C for complete digestion, DNA was extracted twice with equal volumes of phenol followed by two extractions with chloroform. The DNA was then precipitated by adding two volumes of absolute ethanol followed by centrifugation at 14,000 rpm for 20 min.

Extracted DNA was dissolved in 20 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.6). Loading buffer (10 mM EDTA, 0.25% (w/v) bromophenol blue, 30% (v/v) glycerol) was then added at a 1:5 ratio. Samples were then loaded onto a 1.5% agarose gel and electrophoresis was carried out at 60 V in TBE buffer (2 mM EDTA, 90 mM Tris–HCl, 90 mM boric acid, pH 8.0). One hundred base-pair ladder molecular weight markers (Gibco) were used. After electrophoresis, DNA was visualized by soaking the gel in TBE buffer containing 1.5 μg/ml ethidium bromide.

2.5. Western blot analysis of cytochrome c and AIF release

Cytochrome *c* or AIF released from mitochondria was isolated from cytosol as previously described [6]. Briefly, HepG2 or R-HepG2 cells (1×10^6 ml^{-1}) treated with agents were incubated in lysis buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose, 1 mM PMSF, 5 μg/ml leupeptin, 21 μg/ml aprotinin) containing digitonin (25 μg$\times10^6$ cells) at room temperature for 30 s to permeabilize the plasma membrane. Supernatants were obtained after centrifugation at 10,000 rpm for 1 min. Proteins in the supernatants were then resolved on 12% SDS polyacrylamide gels with the same amount of protein per lane. Proteins so separated were blotted onto PVDF (poly vinylidene fluoride) membranes and the amount of cytochrome *c* or AIF released into the cytoplasm was detected by enhanced chemiluminescence method (Amersham).

2.6. Assessment of apoptosis

Flow cytometry was used to assess loss of membrane asymmetry (externalization of (PS) and membrane integrity, respectively, by FITC-labeled annexin-V and propidium iodide (PI), as previously described [7]. Briefly, cells (5×10^5 ml^{-1}) after treatment were washed with PBS and then stained with 100 μl labeling solution containing FITC-labeled annexin-V and PI for 15 min at room temperature. Subsequently, signals from cells were determined by flow cytometry (FACSort, Becton Dickinson). Cell debris, characterized by a low forward scatter/side scatter, was excluded from analysis. Fluorescence was detected in fluorescence channels FL1 (488 nm excitation and 530 nm emission for FITC-labeled annexin-V) and FL3 (488 nm excitation and 600 nm emission for PI). For one single analysis, the fluorescence properties of 10,000 cells within gates were collected. Data acquisition and analysis were performed using program Cell-Quest (Becton Dickinson). Positioning of quadrants on annexin-V/PI plots was performed to distinguish living cells (annexin-V^-/PI^-), early apoptotic cells (annexin-V^+/PI^-), late apoptotic/secondary necrotic cells (annexin-V^+/PI^+). Digitonin (2 mg/ml) was used to permeabilize untreated cells to serve as a standard for annexin-V^+/PI^+ cells to position the quadrants for analysis.

2.7. Detection of the mitochondrial transmembrane potential ($\Delta\Psi m$)

The $\Delta\Psi$m was analyzed using JC-1. JC-1 is capable of selectively entering mitochondria, where it forms monomers and emits green fluorescence when $\Delta\Psi$m is relatively low. At a high $\Delta\Psi$m, JC-1 aggregates and gives red fluorescence [8]. The ratio between green and red fluorescence provides an estimate of $\Delta\Psi$m that is independent of the mitochondrial mass. Briefly, cells (5×10^5 ml^{-1}) were incubated with 10 μM JC-1 for 15 min at room temperature in darkness. Subsequently, cells were analyzed on a flow cytometer (FACSort, Becton Dickinson). Data acquisition was similar to that of the annexin-V/PI assay. Valinomycin (500 nM) was used as a positive control to depolarize the $\Delta\Psi$m for the calculation of the relative change of $\Delta\Psi$m.

2.8. Measurement of intracellular H_2O_2

The generation of H_2O_2 from cells was analyzed by flow cytometry as previously described [9]. Briefly, cells (5×10^5 ml^{-1}) after treatment were incubated with 10 μM DCFH-DA for 30 min at 37 °C. Subsequently, cells were then analyzed on a flow cytometer. The esterified form of DCFH-DA can permeate cell membranes and be deacetylated by intracellular esterases. The resulting compound, dichlorodihydrofluorescein (DCFH), is reactive with H_2O_2 to produce an oxidized fluorescent compound, dichlorofluorescein (DCF), which can be detected by flow cytometry with excitation and emission settings of 488 and 530 nm.

2.9. Statistics

Data are expressed as the mean ± SD from at least three determinations. Statistical analysis was performed using Student's *t*-test, with $P<0.001$ as a criterion of significance.

3. Results and discussion

3.1. Polyphyllin D induces DNA fragmentation in HepG2 cells and drug resistant R-HepG2 cells

A drug-resistant cell line (R-HepG2) was developed by exposure of a human HCC line HepG2 to increasing concentrations of Dox in a stepwise manner. The degree of resistance to different anticancer drugs such as cisplatin (CDDP) and taxol in R-HepG2 cells was analyzed by the MTT assay. The concentration of each drug including PD and Dox that reduced cell survival by 50% (IC_{50}) was determined from cell survival curves and the results are presented in Table 1.

As indicated in Table 1, treatment of HepG2 cells with conventional anticancer drugs including PD at various concentrations resulted in loss of cell viability. However, R-HepG2 cells are >50-, 3.3- and 2.5-fold more resistant to Dox, CDDP and taxol, respectively, when compared to their corresponding IC_{50} of parental HepG2 cells (Table 1). These results suggest that R-HepG2 cells expressed drug resistance to a variety of functionally and structurally unrelated chemotherapeutic agents. Interestingly, the IC_{50} of PD in HepG2 and R-HepG2 was more or less the same and the resistance index was found below 1 (Table 1). These observations suggest that PD is potent in cell killing, especially in R-HepG2.

Many anticancer agents function by inducing apoptosis in tumor cells [10]. To test whether PD induced cytotoxic effects in HepG2 and R-HepG2 cells via apoptosis, we tried to detect intranucleosomal DNA fragmentation, an apoptosis Hallmark [11], by agarose gel electrophoresis. As shown in Fig. 2a, ladder-like DNA fragmentation was observed in a dose-dependent manner in both cell lines 24 h after PD treatment (Fig. 2a). To confirm these results, cells treated with PD were analysed for the presence of early apoptotic events such as PS externalization and no loss of membrane integrity upon annexin-V and PI staining. As reported in Fig. 2b, a very low percentage of annexin-V positive cells (less than 6%) was observed in the untreated population. On the contrary, 5 μM PD induced apoptosis in the two cell lines although a different degree of apoptosis was observed. In particular, about 34 and 16% of R-HepG2 and HepG2 cells, respectively, were annexin-V positive and PI negative after PD treatment. These results provided further evidence that PD induces apoptosis in both cell lines while R-HepG2 cells seemed to be more sensitive to PD. A similar observation was made in the DNA fragmentation assay (Fig. 2a).

Table 1
Cellular sensitivity of HepG2 and R-HepG2 cells to anticancer drugs

Drug[a]	IC_{50} (μM)[b]		
	HepG2	R-HepG2	RI[c]
DOX	4	>200	>50
CDDP	50	167	3.3
Taxol	20	50	2.5
Polyphyllin D	7	5	0.7

[a] Cells (1×10^6 ml^{-1}) were cultured with various concentrations of drug at 37 °C, 5% CO_2 for 24 h. Drug sensitivity was measured by MTT assay after drug exposure.
[b] IC_{50}, drug concentration causing a 50% decrease in a survival curve.
[c] RI, resistance index, ratio between the IC_{50} of drug-resistant and -sensitive HepG2 cells.

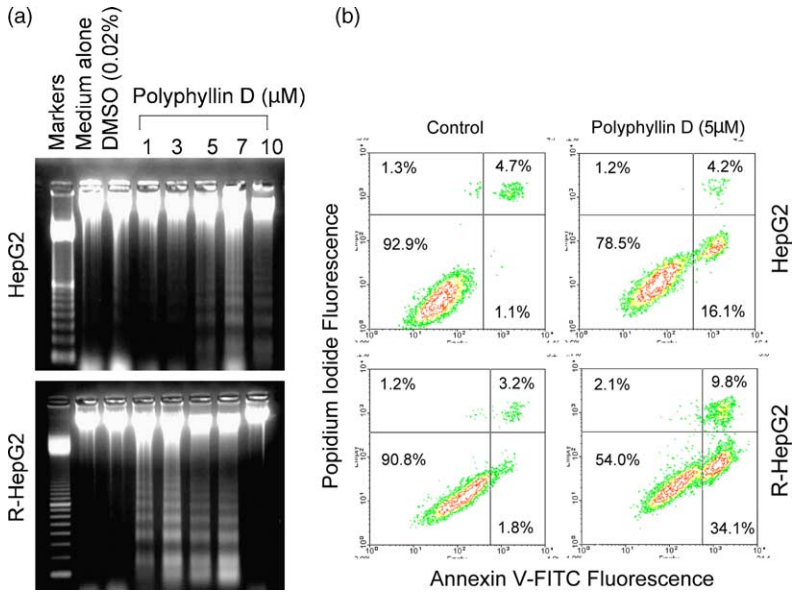

Fig. 2. Effect of polyphyllin D on the induction of apoptosis in HepG2 and R-HepG2 cells. Cells (2×10^5 ml^{-1}) were treated with medium alone, DMSO (0.02%) or polyphyllin D at the concentration as indicated at 37 °C, 5% CO_2 for 24 h. After treatment, DNA was extracted and analyzed by agarose gel electrophoresis (a). In panel (b), cells after treatment were incubated with FITC labeled annexin-V and propidium iodide. After washing, cells were submitted to flow cytometric analysis. Numbers at the corners represent the percentage of cells found in each quadrant.

3.2. Polyphyllin D disrupted mitochondrial membrane potential in both HepG2 and R-HepG2 cells

Increasing evidence indicates that mitochondrial dysfunction often participates in the induction of apoptosis and in many systems, decrease in $\Delta\Psi$m is an early requirement for apoptosis [12]. In an attempt to identify the molecular basis of the apoptosis mediated by PD, we determined the changes in $\Delta\Psi$m in HepG2 and R-HepG2 cells with JC-1. As shown in Fig. 3a, a dose-dependent loss of $\Delta\Psi$m was observed in HepG2 and R-HepG2 cells after exposure to PD as evidenced by the extension of a budding region with high green fluorescence. The percentage of cells with depolarized $\Delta\Psi$m (right sub-region) increased from 11.3% of control cells to 86.2% of PD-treated HepG2 within 24 h and 21.7–92.5% in R-HepG2 cells.

To test whether collapse of $\Delta\Psi$m participates in cell death induced by PD, bongkrekic acid (BA) was used to analysis the PD-elicited cell death. BA is an inhibitor of the adenine nucleotide translocators on the mitochondrial membrane that stabilizes $\Delta\Psi$m [13]. As can be seen in Fig. 3b, although BA by itself elicited a small cytotoxic effect, BA prevented cells from PD-induced cell death in a dose-dependent manner. These observations suggest that decreased $\Delta\Psi$m is a cause of PD-induced cell death in both HepG2 and R-HepG2 cells.

3.3. Polyphyllin D released cytochrome c, AIF and ROS in HepG2 and R-HepG2 cells

It has been reported that decrease in $\Delta\Psi$m results in a variety of deleterious outcomes including generation of reactive oxygen species (ROS) and release of cytochrome *c* and AIF from mitochondria. Like cytochrome *c*, AIF is localized to mitochondria and released in response to death stimuli [14]. Cytochrome *c* and AIF can activate caspase-dependent and -independent (e.g. DNases) pathways, respectively, resulting in cleavage of essential macromolecules in the final execution phase of apoptosis [15,16]. To determine whether PD elicited the release of these mitochondrial proteins to the cytosol, we carefully permeabilized the plasma membrane with digitonin at

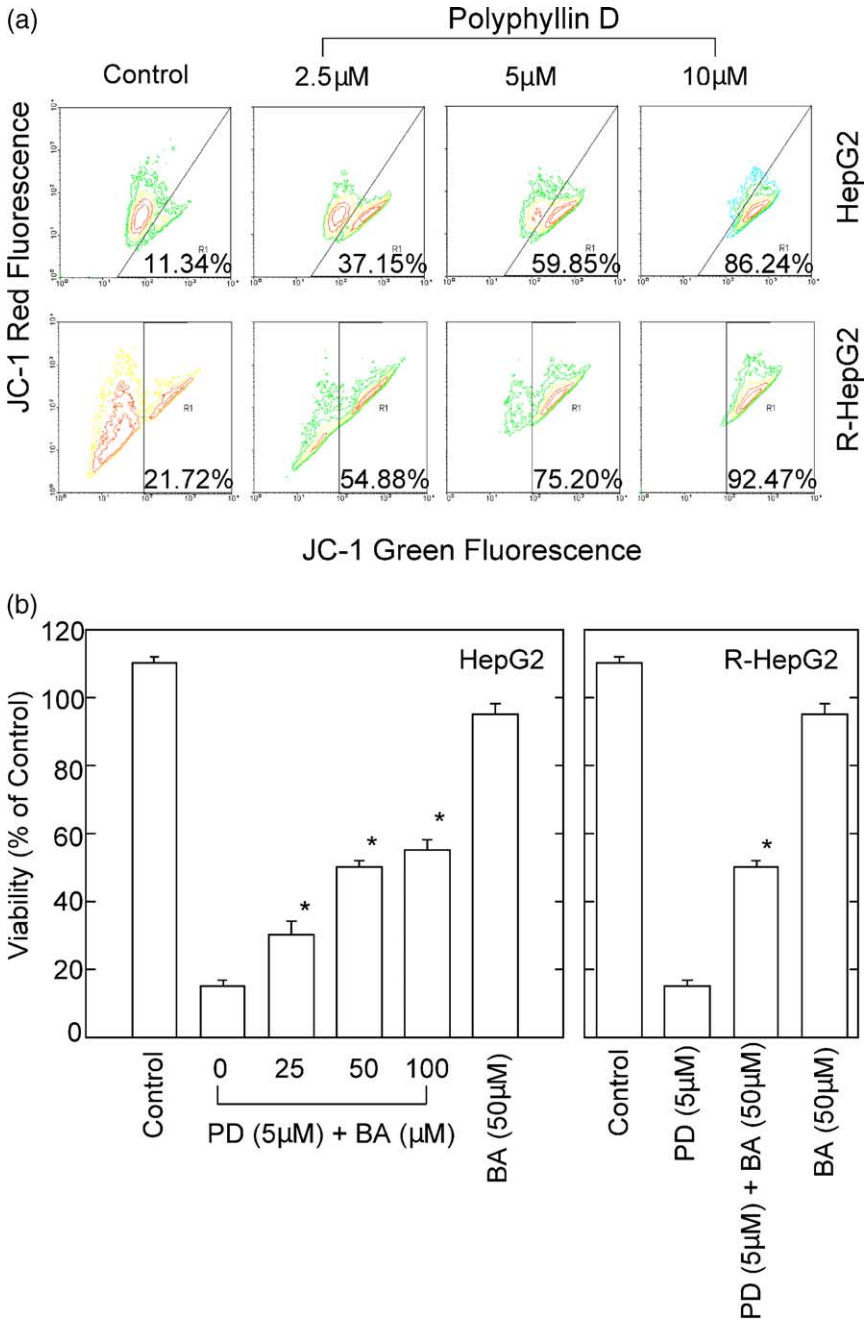

Fig. 3. Polyphyllin D induced $\Delta\Psi$m depolarization and cell death in HepG2 and R-HepG2 cells. Cells (2×10^5 ml^{-1}) were treated with polyphyllin D at the concentration as indicated or medium with 0.02% DMSO at 37 °C, 5% CO_2 for 24 h. Change in $\Delta\Psi$m (a) and viability (b) were then determined, respectively, by flow cytometric analysis with JC-1 and MTT assay. Numbers at the corners of the two-variant plot (a) represent the percentage of cells with depolarized $\Delta\Psi$m found in the sub-region. For the viability determination (b), cells were treated with polyphyllin D (5 μM) or medium with 0.01% (v/v) DMSO in the presence or absence of bongkrekic acid (BA) at the concentration as indicated. Viability of cells was compared with that with medium alone (normalized as 100%). Results are mean ± SD of five determinations. Asterisks express a significant difference compared with control ($^*P<0.001$).

a concentration not affecting the mitochondrial membranes so that any mitochondrial proteins released to the cytosol could be determined in the supernatant by Western blotting. As illustrated in Fig. 4a, treatment of cells with PD but not DMSO caused a dose-dependent release of cytochrome *c* and AIF to the cytosol. These results indicate that PD was able to depolarize $\Delta\Psi$m and release apoptogenic proteins from mitochondria in HepG2 and R-HepG2 cells.

As the generation of ROS has been shown to accelerate cell death, we therefore examined the generation of H_2O_2 using flow cytometry with DCFH-DA. As shown in Fig. 4b, fluorescence intensity increased in both cell lines after exposure to PD in a time-dependent manner. After 12 and 24 h treatment, the percentage of cells within M1 region increased from the basal level of 8.0 to 43.8 and 34.3%, respectively, in R-HepG2 cells and from 11.0 to 23.9 and 35.3% in HepG2 cells. Similar to our previous observations, R-HepG2 cells seemed to be more sensitive to PD when compared to the parental cells in terms of the response amplitude and time.

Next, we analyzed the protective effect of *N*-acetyl-cysteine (NAC) on the PD-induced cell death. NAC is a thiol antioxidant that can increase intracellular glutathione levels and thereby protect cells from the effects of ROS [17]. As shown in Fig. 4c, ROS scavenger NAC (25 mM) clearly prevented cells from PD-induced cell death. Our results also indicate that incubation of cells with NAC alone (25 mM) did not cause any cell death (data not shown). These results therefore suggest that induction of H_2O_2 is involved in the PD-induced apoptotic response.

4. Conclusion

Developing novel and effective anticancer agents that induce apoptosis in tumor cells, especially in those cancer cells with drug resistance, has long been a goal of cancer drug discovery research. One of the mechanisms by which cancer cells survive in the presence of chemotherapeutic drugs is by increasing antiapoptotic activities [18]. Since mitochondria are crucial regulator to the apoptosis process, development of cytotoxic drugs that target mitochondria may provide a new strategy to induce apoptosis in drug-sensitive and -resistant cells [18]. In the present study, we show for the first time that PD induces apoptosis in both HepG2 and R-HepG2 cells. Mechanistically, our data suggest that PD acts on mitochondria and elicits dissipation of $\Delta\Psi$m, generation of ROS, and release of

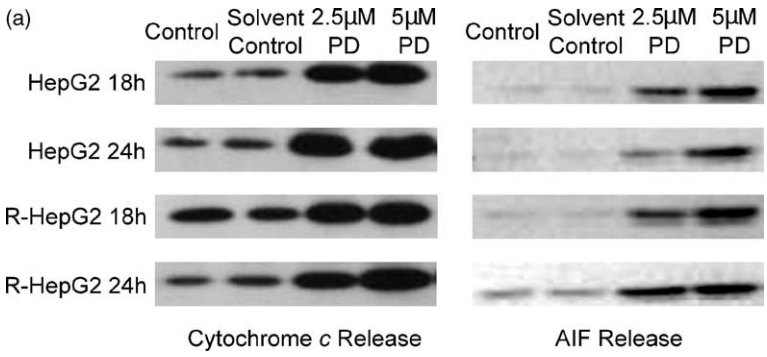

Fig. 4. Polyphyllin D induced cytochrome *c*, AIF and H_2O_2 release in HepG2 and R-HepG2 cells. HepG2 and R-HepG2 cells (1×10^6 ml^{-1}) were treated with or without polyphyllin D at the concentration as indicated at 37 °C, 5% CO_2 for 24 h. The relative amount of cytochrome *c* and AIF released into the cytosol were determined by selective membrane permeabilization and Western blot analysis (a). After treatment, cytochrome *c* and AIF released in the cytosol were extracted with digitonin as described in Section 2. Supernatants were saved and equal amount of proteins was loaded on SDS-PAGE. The protein bands of 15 and 57 kDa, respectively, for cytochrome *c* and AIF were probed with specific antibody. For the release of H_2O_2, cells after treatment with polyphyllin D (5 μM) for 12 or 24 h were loaded with DCFH-DA and the DCF fluorescence was determined by flow cytometry. Numbers in each panel represent the percentage of cells in the region of interest of the treated and control population (b). Viability of cells (2×10^5 ml^{-1}) after treatment with polyphyllin D (5 or 10 μM) or medium alone in the presence or absence of antioxidant NAC (25 mM) for 24 h was determined by alamar blue assay (c). Data are expressed as mean ± SD for 10 determinations. Statistical significance of the differences between PD-treated and PD + NAC-treated cells was analyzed ($^*P<0.001$).

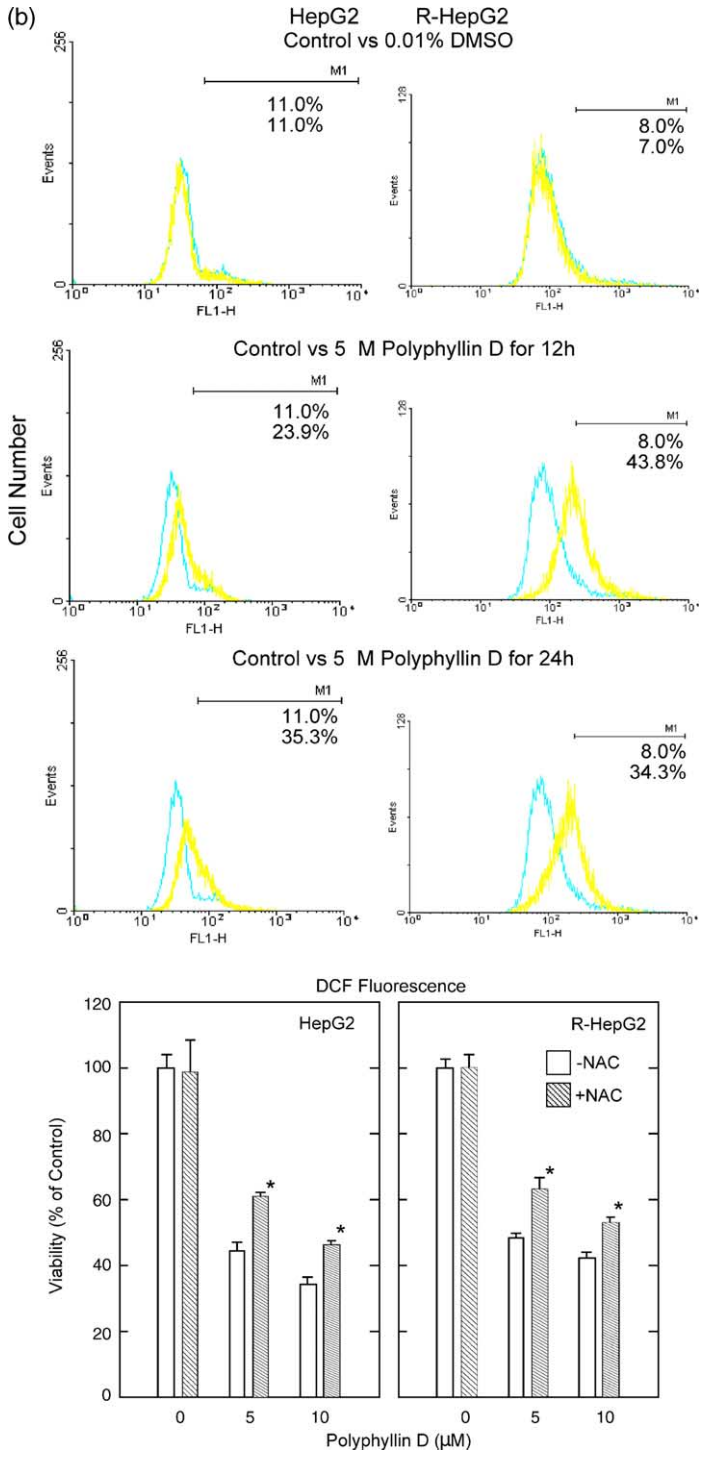

Fig. 4. (*continued*).

cytochrome *c* and AIF. Also, we provide evidence that suppression of these mitochondrial dysfunctions will alleviate the cytotoxic action of PD, further supporting our notion that PD induces apoptosis in cancer cells via mitochondrial damages. In view of the potent apoptogenic activity in R-HepG2 cells, PD is a promising anticancer agent that can overcome drug resistance.

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