Polyphyllin D - A Potential Anti-Cancer Agent to Kill Hepatocarcinoma Cells with Multi-Drug Resistance

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Abstract: To develop drugs to kill cancer cells, we chemically synthesized a number of anti-cancer agents by adding different side chains to the core backbone of saponin. With the use of bioassay-guided methods, we found one agent that possessed a high cytotoxicity to a number of cancer cell lines. Interestingly, this compound was later found to be an active component of a tradition Chinese herb *Paris polyphylla* known as Polyphyllin D (PD) (diosgenyl α-L-rhamnopyranosyl-(1→2)-(β-L-ara-binofuranosyl-(1→4)-β-D-glucopyranoside). In China, the rhizome of *Paris polyphylla* (Chong Lou) has been used as a traditional Chinese medicine to treat a number of cancers including pancreas and liver cancers for a long time. Results from our laboratory demonstrate that PD is a potent anti-cancer agent that bypasses multi-drug resistance (MDR) and induces programmed cell death in R-HepG2 cells over-expressing P-glycoprotein (P-gp). In this paper, we reviewed the mechanisms how PD overcomes the MDR and exhibits a stronger cytotoxicity in the R-HepG2 than its parent line without P-gp through mitochondrial injury.

Keywords: Polyphyllin D, apoptosis, multi-drug resistance, mitochondria, R-HepG2.

INTRODUCTION

Liver cancer is the fifth most common cancer in men and the eighth in women worldwide [1]. Historically, liver cancer has been quite common in the Far East regions; in contrast to the United States where the incidence per hundred thousand lives is about three. The high incidence of liver cancer has been attributed to factors that are unique to the Far East. These include hepatitis infection [2] and the possible contact with hepato-carcinogens such as nitrosamines and aflatoxin [3].

Different kinds of treatment for liver cancers have been attempted. Because of the multifocal nature of hepatocellular carcinoma (HCC), only ~20% of patients with HCC are considered resectable at presentation of the case. Chemotherapy therefore becomes the major treatment for the patients with non-resectable HCC. Unfortunately, no single drug given systematically leads to a satisfactory outcome [4]. Reasons for this are multifold. Resistance to and toxicity of drugs are two general problems in HCC treatment [5, 6].

Resistance to drugs is a crucial factor that limits the effectiveness of chemotherapy. Although resistance can develop through a number of mechanisms, over-expression of drug transporters such as P-gp, a product of the *MDR*1/*ABCB1* gene, seems to be the best-known mediator [7]. As one of the 48 members of the ATP-binding cassette family, P-gp is the most important transporter that effluxes a variety of drugs from cells. In many human cancers, over-expression of P-gp is correlated with decreased survival and poor prognosis [8]. In liver cancers, P-gp is over-expressed in patients with HCC [6]. Therefore, identification of an effective treatment for HCC is urgently needed. Towards this goal we synthesized a number of synthetic agents and developed a drug-resistant cell line (R-HepG2) from a commonly used human HCC HepG2 for drug screening. This paper gives a review on how one of these agents PD, overcomes the MDR and kills the R-HepG2 cells with more efficacy than their parent HepG2 without MDR.

R-HEPG2 CELLS ARE MULTI-DRUG RESISTANT AND EXPRESS P-GLYCOPROTEINS

Well-characterized human HCC HepG2 cells obtained from American Type Culture Collection were cultured in the presence of doxorubicin (Dox) and survival cells were treated stepwise with a higher concentration of Dox from 0.1 to 100 μM during cell passages under standard conditions. After more than 10 rounds of selection, a clone R-HepG2 with Dox resistance was obtained. To maintain the Dox-resistance and the expression of P-gp, R-HepG2 cells were cultured with 1.2 μM Dox during passages [9].

As shown in Table **1**, the IC_{50} values of Dox toxicity in HepG2 and R-HepG2 cells were 7.3 and > 200 μM, respectively. These results indicate that R-HepG2 cells were almost 30 times more resistant to Dox than HepG2 cells. Interestingly, R-HepG2 cells selected from Dox exhibited varying degrees of resistance to taxol, fenretinide and CDDP, ranging from the resistance index of 1.42 to >27 (Table **1**). These results suggest that R-HepG2 cells acquired a broad cross-

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Table 1. Cellular Sensitivity of HepG2 and R-HepG2 Cells to Anti-Cancer Drugs

Drug[1]		IC$_{50}$ (μM)[2]	
	HepG2	R-HepG2	Resistance index[3]
Dox	7.3	>200	>27
CDDP	60	180	3
Taxol	17.2	37.2	2.16
Fenretinide	6.2	8.8	1.42
PD	3.9	2.0	0.50

[1] Cells (1x10^6 /ml) 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. Similar results were reported before [11].
[2] IC$_{50}$, drug concentration causing a 50% decrease in a survival curve.
[3] Resistance index, ratio between the IC$_{50}$ of drug-resistant and -sensitive HepG2 cells.

resistance to mechanistically and structurally unrelated drugs. Expression of P-gp in R-HepG2 cells was demonstrated with immuno-blot analysis. As shown in Fig. (**1a**), P-gp was not detected in HepG2 cells. On the contrary, a high level of P-gp was found in R-HepG2 and the level did not change much when challenged with Dox, Taxol or CDDP for 24 h. These observations suggest that the expression of P-gp was not an innate but acquired feature in R-HepG2 cells. In this regard, R-HepG2 is an ideal *in vitro* model mimicking the acquired clinical MDR for drug screening [10].

To characterize the functionality of P-gp in R-HepG2 cells, flow cytometric analysis was used to determine the intracellular accumulation of Dox since Dox is a P-gp sub-

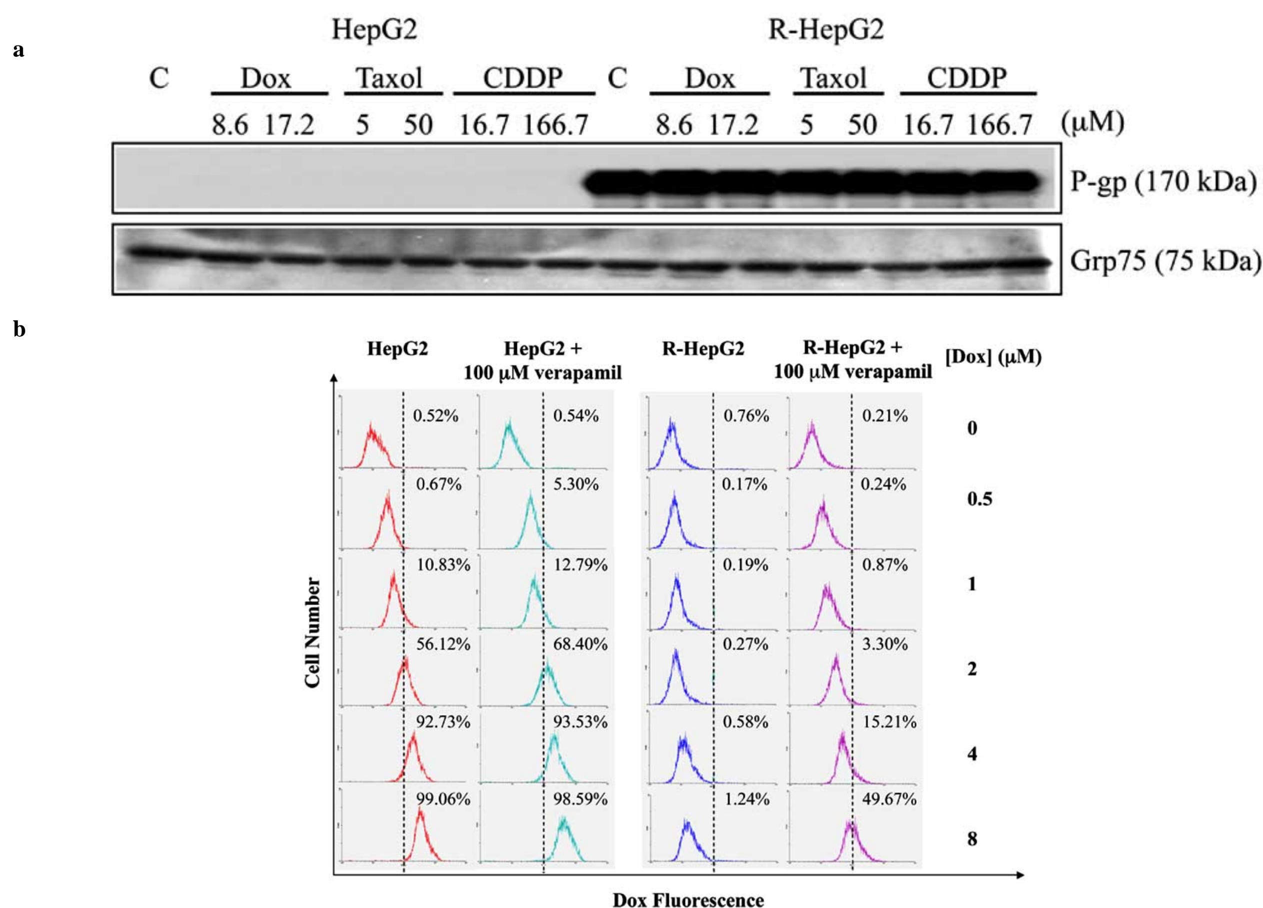


Fig. (1). R-HepG2 cells expresses P-glycoprotein and accumulates less doxorubicin. HepG2 and R-HepG2 cells (1 x 10^6/ml) were treated with various agents at the concentration as indicated for 24 h at 37 °C, 5% CO_2. After treatments, cell lysates, containing equal amount of proteins, were loaded on SDS PAGE. The relative amount of P-gp was determined by Western blot analysis and glucose regulated protein 75 (Grp75) served as an internal control (**a**). Three h after treatment with Dox at the concentration as indicated in the presence or absence of verapamil (100 μM) at 37 °C, 5% CO_2, intracellular accumulation of Dox in HepG2 and R-HepG2 cells were visualized by flow cytometry after washing (**b**). Relative % of cell population with Dox in the cytoplasm fell on the right hand side of the dotted line was calculated. Methods for Western blot and flow cytometric analysis were conducted as previously reported [9].

strate [11] and emits fluorescence when excited at 488nm. As shown in Fig. (**1b**), HepG2 cells accumulated Dox in a dose-dependent manner. When incubated with 8 μM Dox for 3 h, almost all the HepG2 cells were loaded with Dox. On the contrary, only a small population of R-HepG2 (1.24%) accumulated Dox under the same setting. However, when cells were treated with Dox and verapamil (100 μM), an inhibitor of P-gp, a marked increase in Dox accumulation was observed in R-HepG2 cells but not in HepG2 cells (Fig. (**1b**)). These results suggest that R-HepG2 cells express functional P-gp and do not tend to accumulate Dox. Results from our study also show that the DNA sequence of the P-gp promoter in HepG2 and R-HepG2 was identical indicating that the over-expression of P-gp in R-HepG2 cells was not due to mutations in the promoter region (data not shown). However, our recent results indicated that the *MDR1* gene promoter was hypo-methylated in R-HepG2 cells [12]. This hypo-methylation is due to an 8-fold up-regulation of an imprinted *H19* mRNA in R-HepG2 cells, which induced P-gp expression [12].

THE CELL DEATH PROGRAMME OF R-HEPG2 CELLS IS ALTERED

After establishing experimental conditions for P-gp expression, we next tried to find out whether the expression of P-gp was the only mechanism that accounts for the MDR. To address this question, we had incubated the R-HepG2 cells with a very high dose of Dox to achieve an intracellular Dox level which was comparable to that of HepG2 cells and then we evaluated the Dox cytotoxicity in both cell lines. We reasoned that if P-gp was the only mechanism, the cytotoxicity would be comparable in both HepG2 and R-HepG2 cells if they had a similar internal Dox concentration. As shown in Fig. (**2a**), when R-HepG2 cells were incubated with 200 μM Dox, they accumulated an internal Dox level comparable to that of HepG2 cells in the medium with 20 μM Dox. Yet, the viability of R-HepG2 cells under such condition was much higher than that of HepG2 cells in 20 μM Dox (Fig. (**2b**)). These results therefore suggest that the cell death programme of R-HepG2 cells had been altered and P-gp was not the only mechanism accounting for their MDR.

SAPONINS AS ANTI-CANCER DRUG AGENTS

Saponins are a diverse class of glycosides, steroids, and triterpenes, widely distributed in plants. These compounds have a bitter taste and are used routinely as detergents [13]. Increasing evidence suggests that some plant saponins are a unique source of compounds that can induce cell death in many types of cancer [14-16]. Based on the fact that saponins are composed of a lipid-soluble aglycon consisting of either a sterol or more commonly a triterpenoid with different water-soluble sugar residues, we synthesized 27 saponins (HK**1** to HK**13**, HK**15** to HK**28**) with different carbohydrate groups attached to a common steroidal backbone diosgenin by stepwise glycosylations (Fig. (**3**)) [17, 18]. After preliminary screenings, only few synthetic saponins were cytotoxic. Of these 27 saponins, HK18 was found to have the highest toxicity to HepG2 cells (Table **2**) and cell lines MCF-7, MDA-MB-231, and HL-60 (data not shown). HK18 was then selected for further analysis. Interestingly, with the chemical structure from HK**18** (diosgenyl α-L-rhamnopyranosyl-(1→2)-(β-L-arabinofuranosyl)-(1→4)]-[β-D-glucopyranoside), we later found out that HK**18** was actu-

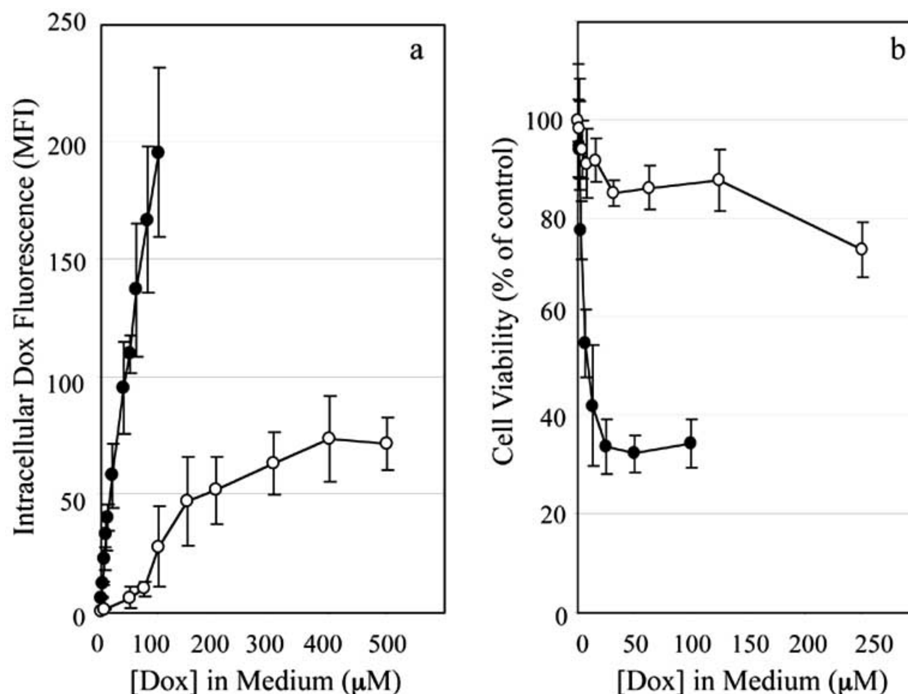
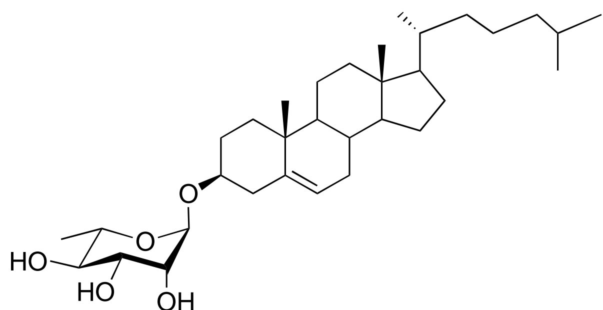
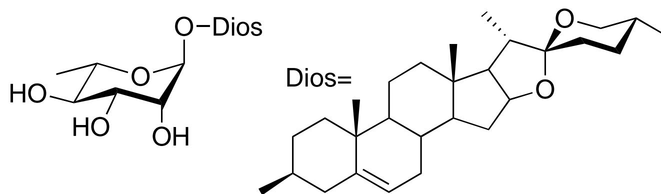


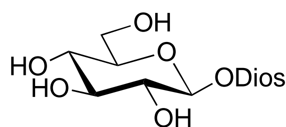
Fig. (2). The cell death programme in R-HepG2 cells is altered. HepG2 (●) and R-HepG2 (❍) cells (1 x 10^6/ml) were treated with various concentrations of Dox as indicated for 4 h at 37 °C, 5% CO_2. Intracellular accumulation of Dox in terms of Dox fluorescence (arbitrary unit) in HepG2 and R-HepG2 cells were visualized by flow cytometry as described in Fig. **1**. Results (mean fluorescence intensity (MFI)) are mean ± SD of 3 determinations (**a**). Twenty-four h after treatment with Dox at the concentration as indicated at 37 °C, 5% CO_2, cell viability was determined by MTT assay. Results are mean ± SD of 4 determinations (**b**). Methods for Western blot and flow cytometric analysis were conducted as previously reported [10].



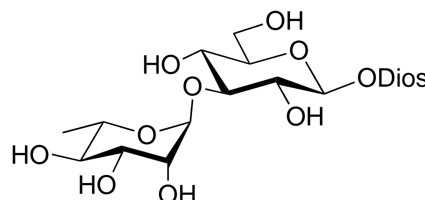
HK-1: Cholesteryl-3-yl α-L-rhamnopyranoside



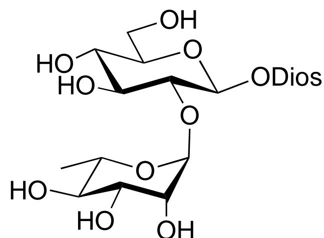
HK-2: Diosgenyl-3-yl-α-L-rhamnopyranoside



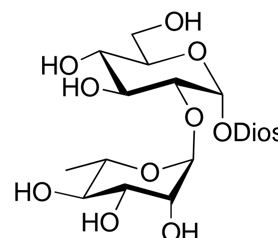
HK-3: Diosgenyl-3-yl β-D-glucopyranoside



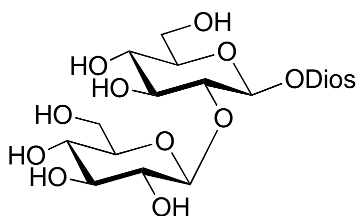
HK-4: Diosgenyl-3-yl 3-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside (Polyphyllin C)



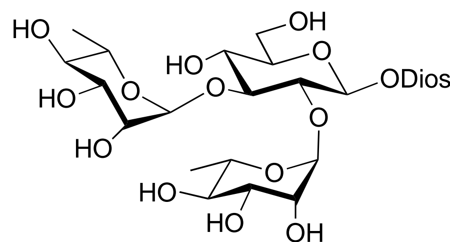
HK-5: Diosgenyl-3-yl 2-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside (Tuberoside C)



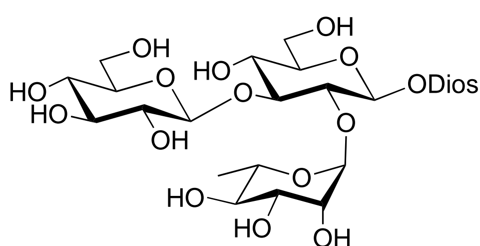
HK-6: Diosgenyl-3-yl 2-*O*-(α-L-rhamnopyranosyl)-α-D-glucopyranoside



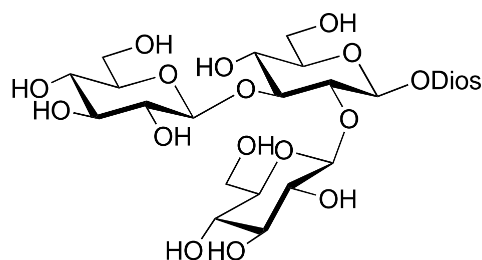
HK-7: Diosgenyl-3-yl 2-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside



HK-8: Diosgenyl-3-yl 2,3-di-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside

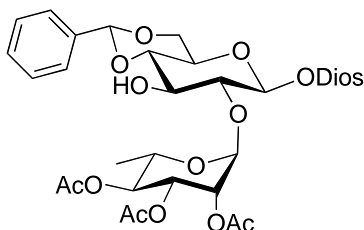


HK-9: Diosgenyl-3-yl 2-*O*-(α-L-rhamnopyranosyl)-3-O-(β-D-glucopyranosyl)-β-D-glucopyranoside

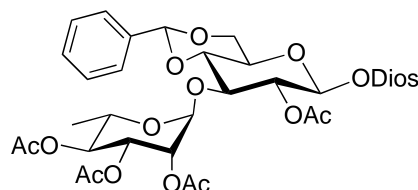


HK-10: Diosgenyl-3-yl 2,3-di-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside

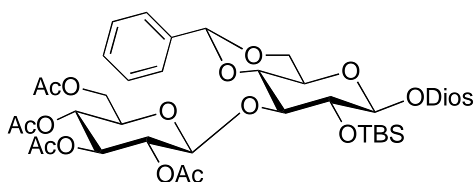
Fig. (3). Contd….



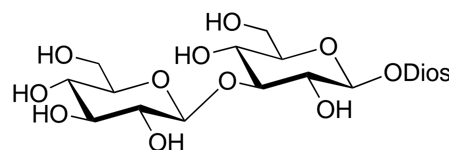
HK-11: Diosgenyl-3-yl 2-*O*-(2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-4,6-*O*-benzylidene-β-D-glucopyranoside



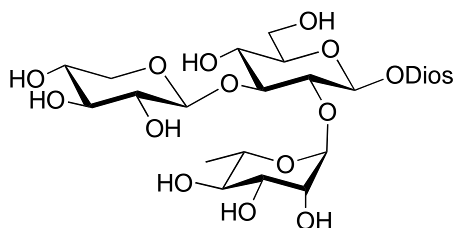
HK-12: Diosgenyl-3-yl 2-*O*-acetyl-3-*O*-(2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-4,6-*O*-benzylidene-β-D-glucopyranoside



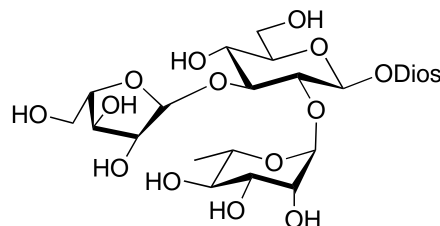
HK-13: Diosgenyl-3-yl 2-*O*-(tert-butyldimethylsilyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-4,6-*O*-benzylidene-β-D-glucopyranoside



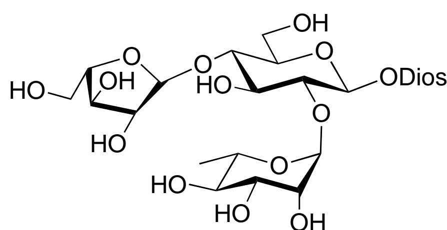
HK-15: Diosgenyl-3-yl 3-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside



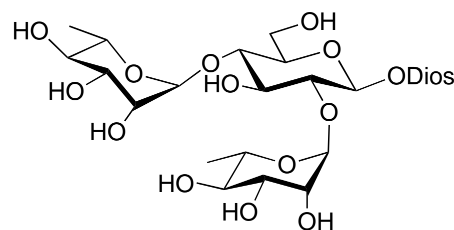
HK-16: Diosgenyl-3-yl 2-*O*-(α-L-rhamnopyranosyl)-3-*O*-(β-D-xylopyranosyl)-β-D-glucopyranoside



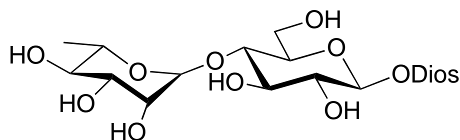
HK-17: Diosgenyl-3-yl 2-*O*-(α-L-rhamnopyranosyl)-3-*O*-(α-L-arabinofuranosyl)-β-D-glucopyranoside



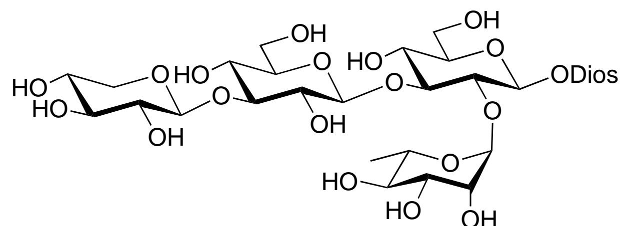
HK-18: Diosgenyl-3-yl 2-*O*-(α-L-rhamnopyranosyl)-4-*O*-(α-L-arabinofuranosyl)-β-D-glucopyranoside



HK-19: Diosgenyl-3-yl 2,4-di-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside



HK-20: Diosgenyl-3-yl 4-di-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside



HK-21: Diosgenyl-3-yl 2-*O*-(α-L-rhamnopyranosyl)-3-*O*-[3-*O*-(β-D-xylopyranosyl)-β-D-glucopyranosyl]-β-D-glucopyranoside

Fig. (3). Contd….

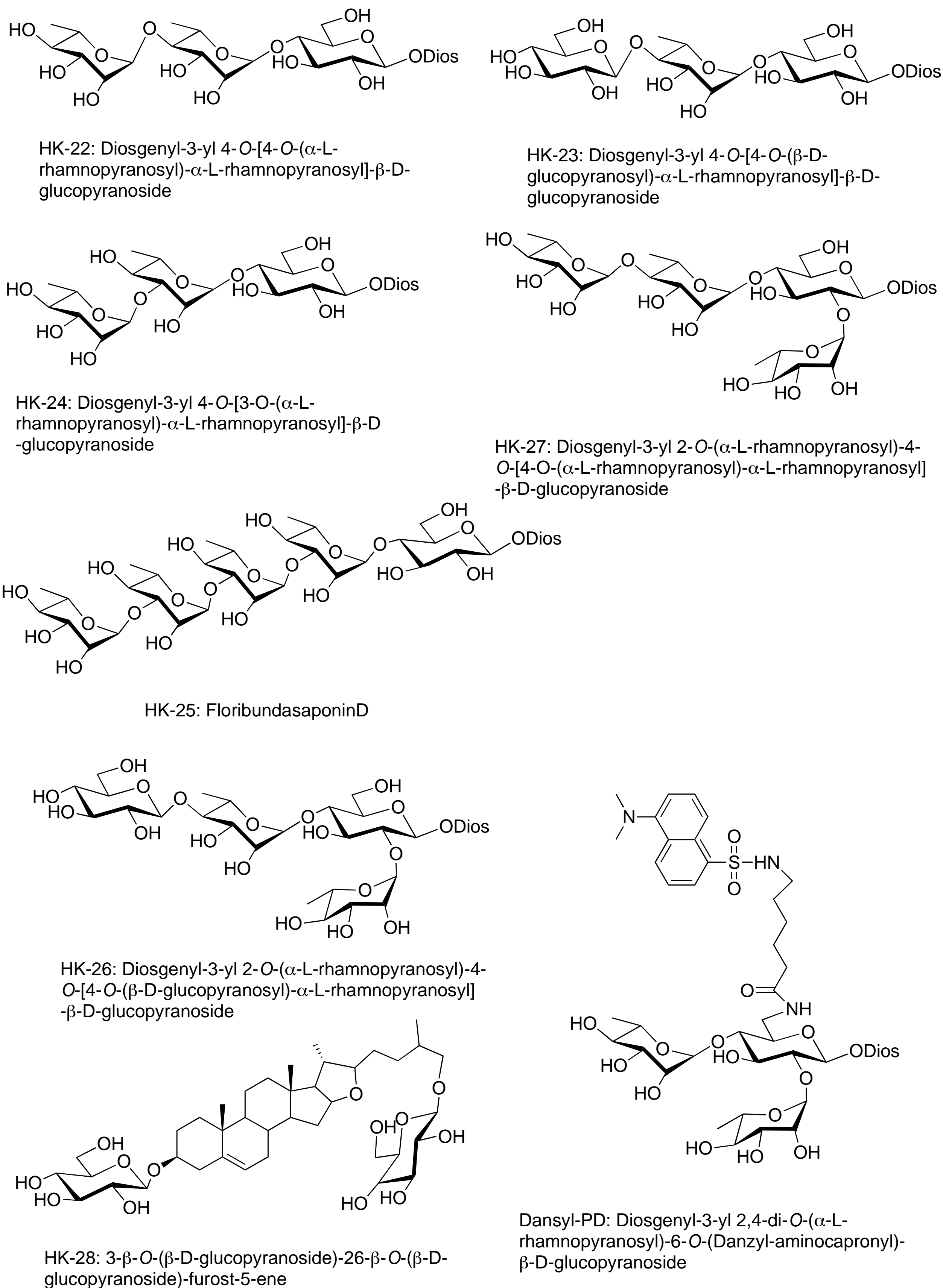


Fig. (3). Chemical structure of different saponin compounds. Synthetic scheme was shown in [17] and [18]. Based on NMR measurements, the compound purity was found to be > 95%.

Table 2. Cellular Sensitivity of HepG2 to HK-Saponins

Drug[1]	Viability	*Drug[1]*	Viability
	(% of control)[2]		(% of control)[2]
HK-1	101.5%	HK-15	96.3%
HK-2	100.5%	HK-16	94.4%
HK-3	97.1%	HK-17	84.2%
HK-4	95.1%	HK-18	49.2%
HK-5	91.9%	HK-19	79.7%
HK-6	99.6%	HK-20	95.1%
HK-7	99.5%	HK-21	89.2%
HK-8	80.9%	HK-22	101.0%
HK-9	103.3%	HK-23	96.2%
HK-10	100.4%	HK-24	96.6%
HK-11	112.2%	HK-25	94.8%
HK-12	86.7%	HK-26	82.3%
HK-13	113.3%	HK-27	58.5%
		HK-28	91.2%

[1] Saponin structures were shown in Fig. **3**.
[2] Cells ($1x10^6$ /ml) were cultured with 2.5 μM of drug at 37 °C, 5% CO_2 for 48 h.
Drug sensitivity was measured by MTT assay after drug exposure.
Viability was compared with control (100%).

ally Polyphyllin D (PD), a major component of a Chinese medicinal herb *Paris polyphylla* (Chong Lou) which had been used to treat liver cancer in China for a long time [19]. Also, results from another research group indicate that the ethanol extract of *Paris polyphylla* gave the lowest IC_{50} (10 μg/ml) in HepG2 cells among 15 traditional Chinese medicines which are usually used in tumor patients in China [20]. Apart from HepG2 cells, PD is able to kill some other cancer cells such as HeLa, MCF-7 and MDA-MB-231 [21, 22]. Taken together, these findings suggest that PD is a potential anti-tumor agent.

PD IS MORE CYTOTOXIC TO R-HEPG2 THAN HEPG2 CELLS

As shown in Table **1**, differently from other conventional anti-cancer agents, HK-**18** is more cytotoxic to R-HepG2 than HepG2 cells. The IC_{50} of PD was 2-fold lower in R-HepG2 cells than that of HepG2. To test whether PD exerted its cytotoxic effects in HepG2 and R-HepG2 cells through apoptosis, analysis of DNA fragmentation by agarose electrophoresis and annexin-V/propidium iodide assay were used. Results from these assays indicate that PD is an apoptosis-inducing agent and again R-HepG2 was more sensitive

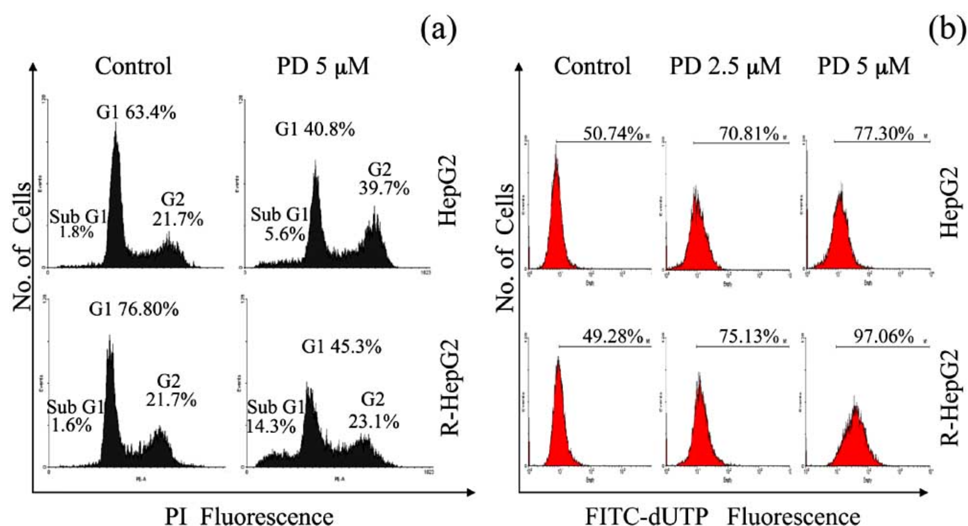


Fig. (4). Polyphyllin D induced more DNA fragmentation in R-HepG2 cells. HepG2 and R-HepG2 cells (1 x 10^6/ml) were treated with PD or medium alone at the concentration as indicated at 37 °C, 5% CO_2 for 24 h. After treatment, cell cycle pattern of the treated and control cells was assessed by propidium iodide with flow cytometry. Percentage of the sub-population at different cell cycle stage was determined (**a**). DNA fragmentation was also determined by TUNEL assays. Figure in each panel represents the % of cells with DNA fragmentation in the selected region (**b**). Methods for flow cytometric analyses were conducted as previously reported [21].

to PD than its parent HepG2 [10]. In this report, we further confirmed these observations by using cell cycle analysis (Fig. (**4a**)) and TUNEL (TdT-mediated dUTP Nick-End Labeling) assay (Fig. (**4b**)). As shown in Fig. (**4a**), under the same experimental conditions, more R-HepG2 cells were found in the sub-G1 peak after PD treatment (14.3% in R-HepG2 versus 5.6% in HepG2). The sub-G1 method we used for apoptosis detection could have underestimated the degree of DNA fragmentation or apoptosis. If cells in the S or G2/M phase of the cell cycle enter apoptosis, they may not be seen in the sub-G1 peak because the cells need to lose a large amount of DNA to appear in the sub-G1 peak. We therefore used TUNEL assay to confirm the stronger apoptotic effect of PD in R-HepG2 cells. As illustrated in Fig. (**4b**), PD indeed induced more DNA fragmentations in R-HepG2 cells than that of HepG2 as evidenced by more transfer of FITC-dUTP to the strand breaks of cleaved DNA by terminal deoxynucleotidyl transferase in the TUNEL assay (97% vs 77% in 5 μM PD-treated groups).

PD INDUCES APOPTOSIS IN R-HEPG2 THROUGH THE ACTIVATION OF INTRINSIC PATHWAY

To verify if the apoptotic effect of PD is mediated through the extrinsic or intrinsic apoptotic pathway, a dansyl-PD was synthesized in which a fluorescent dansyl group was conjugated with the polysaccharide moiety of the PD (Fig. (**3**)). Thereafter, we incubated the cells with dansyl-PD and evaluated its sub-cellular location under a fluorescent microscope. We reasoned that if PD's action was mediated by death receptors, dansyl-PD signals should be found mainly on the cell surface after a short incubation. Results from our study indicate that the fluorescence of dansyl-PD was found evenly distributed in the cytoplasm [23], indicating that dansyl-PD could pass through the plasma membrane and diffused into the cytosol to interact with the molecules and organelles inside. Moreover, incubation of HepG2 or R-HepG2 with PD did not alter the level of pro- caspase-8 but caspase-3 [23], further suggesting that PD was not a death ligand interacting with death receptors but exerted its action through the intrinsic pathway.

To further confirm the effect of PD on the activation of intrinsic apoptotic pathway, changes in the mitochondrial membrane potential ($\Delta\psi_m$) of the PD-treated HepG2 and R-HepG2 cells were studied by using JC-1 and flow cytometric assay. As can be seen in Fig. (**5a**), a dose-dependent collapse of $\Delta\psi_m$ and release of cytochrome *c* and AIF (apoptosis-inducing factor) (Fig. (**5b**)) were observed in both HepG2 and R-HepG2 cells after PD treatment. These results indicate that PD is able to trigger the mitochondrial apoptotic pathway to kill HepG2 and R-HepG2 cells. Consistent with the results from the cell cycle analysis and TUNEL assay, the effects of PD on $\Delta\psi_m$ depolarization and release of mitochondrial proteins were more potent in R-HepG2 cells. For example, ~90% of the R-HepG2 cells lost the $\Delta\psi_m$ when treated with 1.25 μM PD for 24 h. Under the same conditions, only 58% of the HepG2 cells decreased their $\Delta\psi_m$ (Fig. (**5a**)). Also, results from total internal reflection fluorescence microscopic study indicate that challenge of cells with PD caused mitochondrial fragmentation [23]. All these results point to a conclusion that PD was an apoptosis-inducing agent that activated the intrinsic pathway through mitochondrial injury.

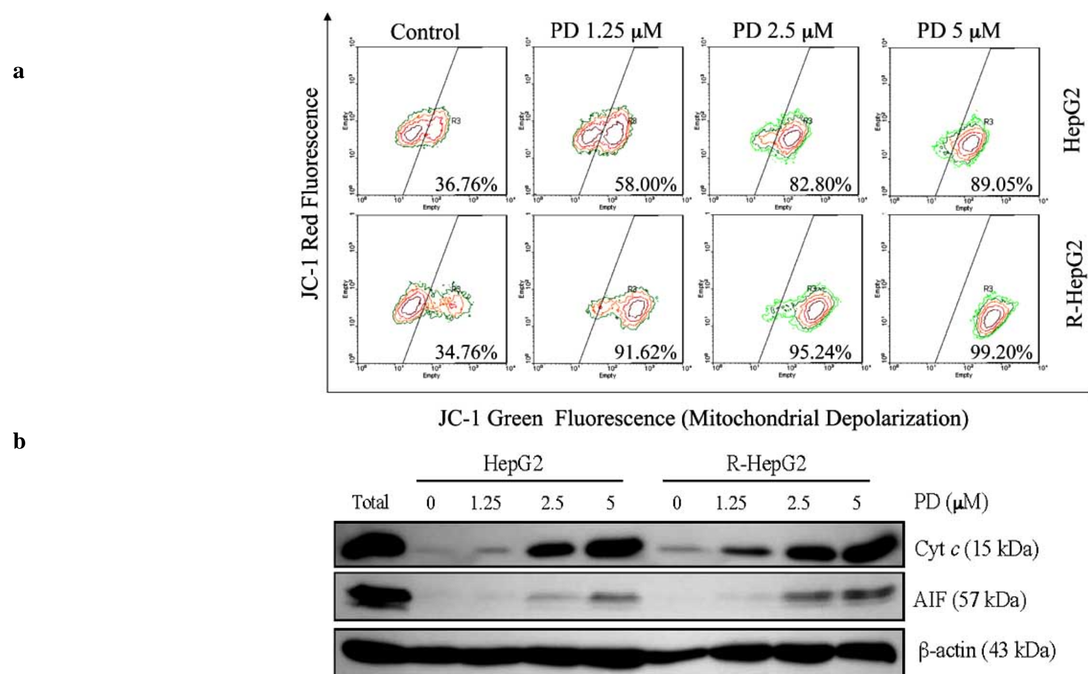


Fig. (5). Effect of Polyphyllin D on the induction of apoptosis in HepG2 and R-HepG2 cells. Cells (1 x 10^6/ml) were treated with PD or medium alone at the concentration as indicated at 37 °C, 5% CO_2 for 24 h. After treatment, changes in $\Delta\psi_m$ were then determined by flow cytometric analysis with JC-1. Numbers at the corners of the two-variant contour plot represent the % of cells with depolarized $\Delta\psi_m$ found in the sub-region (**a**). After PD treatments, cytochrome *c* and AIF released into the cytosol were extracted with selected plasma membrane permeabilization with digitonin (0.8 μg/ml). Supernatants were saved and equal amount of proteins was loaded on SDS PAGE. The relative amounts of cytochrome *c* and AIF were determined by Western blot analysis. β-actin and total cell lysate (Total) were used as an internal control (**b**). Methods for Western blot and flow cytometric analysis were conducted as previously reported [10].

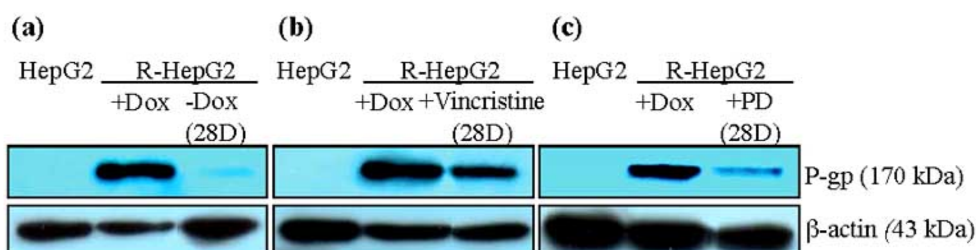


Fig. (6). Effect of PD on the P-gp expression in R-HepG2 Cells. Cells (1 x 10^6/ml) were treated with medium alone or the agent (1.2 μM) as indicated at 37°C, 5% CO_2 for 28 days in the presence (+Dox) or absence (-Dox) of Dox (1.2 μM) with regular passages. After treatments, cell lysates, containing equal amount of proteins, were loaded on SDS PAGE. The relative amount of P-gp was determined by Western blot analysis and β-actin served as an internal control.

PD DID NOT MAINTAIN THE P-GP EXPRESSION IN R-HEPG2 CELLS

To understand the effect of PD on the expression of P-gp, we exposed R-HepG2 cells to PD (1.2 μM) in the absence of Dox for 28 days. As noted before, the P-gp level of R-HepG2 cells was high when compared to that of HepG2 (Fig (**6a**)). When Dox was removed from R-HepG2 cells, originally with 1.2 μM Dox to maintain the P-gp expression, for 28 days, there was a sharp decrease in the P-gp expression (Fig (**6a**)). However when vincristine (1.2 μM), another P-gp substrate, was added back to R-HepG2 cells to replace Dox, the P-gp expression was maintained (Fig. (**6b**)). On the other hand, when PD (1.2 μM) was used to substitute vincristine or Dox also for 28 days, only a weak P-gp band was found (Fig. (**6c**)). This suggests that PD could not maintain a substantial P-gp expression in R-HepG2 cells.

PD ACTED ON ISOLATED MITOCHONDRIA AND RELEASED AIF

At present, little is known about the mechanism of the selective toxicity of PD in R-HepG2 cells. The use of dansyl-PD demonstrated that PD was accumulated evenly in the cytoplasm of HepG2 cells [23]. Results from our previous study from flow cytometry indicated that more mitochondria were found in R-HepG2 than HepG2 cells [9]. Shown in Fig. (**7a**) was the confocal section of HepG-2 and R-HepG2 cells stained with JC-1 for mitochondria labeling. It is clear in these optical sections that R-HepG2 cells contained more mitochondria than HepG2 cells. To verify the occurrence of

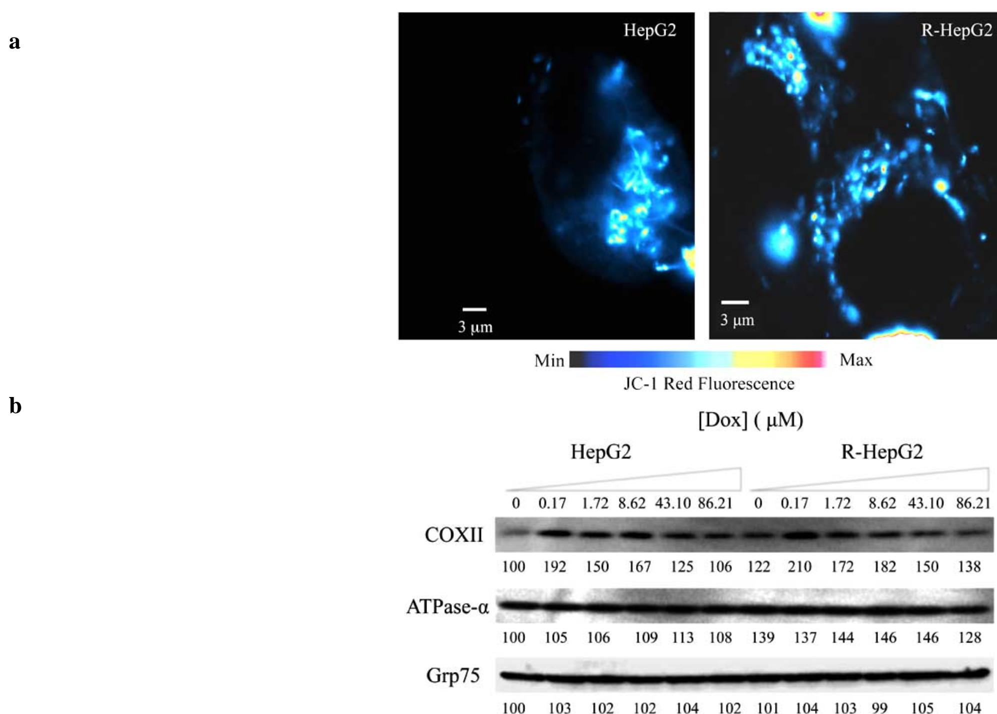


Fig. (7). R-HepG2 cells contained more mitochondria. Cells (1 x 10^5/ml) were loaded with JC-1 (10 μM) at 37 °C, 5% CO_2 for 3 h in the presence verapamil (100 μM) to block the P-gp-mediated efflux of JC-1. After washing, cells were examined under a confocal microscope (Nikon C1 Plus) and the JC-1 red fluorescence was acquired after excitation at 488 nm. The scale bar represents the cell dimension in μm and the pseudo-color palette represents the fluorescence intensity (**a**). R-HepG2 and HepG2 cells were treated with medium or Dox at the concentration as indicated for 24 h at 37 °C, 5% CO_2. After treatments, cell lysates, containing equal amount of proteins, were loaded on SDS PAGE. The relative amount of COXII and ATPase-α were determined by Western blot analysis. Glucose regulated protein 75 (Grp75) served as an internal control. Protein bands were scanned and the relative density was shown below the protein bands (**b**). Data shown in (b) are reproduced from [9] with permission from Elsevier Limited (2008).

different amount of mitochondria in HepG2 and R-HepG2 cells, mitochondrial proteins such as cytochrome c oxidase II (COXII) (encoded by mitochondrial DNA) and ATPase-α (encoded by nuclear DNA) were quantified by using Western blot analysis. As shown in Fig. (**7b**), untreated R-HepG2 cells gave stronger signals in both COXII and ATPase-α than that in parental cells, indicating that more mitochondria were found in R-HepG2 cells. After Dox treatments, the COXII and ATPase-α expression in R-HepG2 and parental cells were altered. Still, more mitochondrial proteins were found in R-HepG2 cells than their counterpart. Taken together, our results clearly demonstrate that R-HepG2 cells produced more mitochondria than HepG2 cells. With these in our mind, we therefore hypothesized that if PD is able to act directly on mitochondria, PD will be more cytotoxic to R-HepG2 cells. To investigate this possibility, mitochondria were isolated from both cell lines by cells grinding and gradient centrifugation. After confirming the purity of the mitochondrial fraction, isolated mitochondria were directly challenged with PD. As expected, PD induced swelling, $\Delta\psi_m$ depolarization, and release of AIF in the mitochondria isolated from R-HepG2 and HepG2 cells (Fig. (**8**)). Interestingly, the effect of PD was still more potent in the mitochondria isolated from R-HepG2 cells. This suggests that the properties of the mitochondrial membrane of HepG2 are different from that of R-HepG2 cells and the mitochondria of R-HepG2 cells are more sensitive to PD. At the moment, the precise mechanism of this action remains unknown. Further studies are needed to elucidate why PD produces a stronger toxic effect on the mitochondria in R-HepG2 cells.

CONCLUSION

Our results demonstrate that R-HepG2 is a good *in vitro* model of acquired MDR. R-HepG2 cells acquired the resistance by expressing a high level of P-gp that extruded many types of drugs such as Dox. However, similar to many clinical cases, expression of P-gp is not the solely mechanism accounting for the acquired MDR [24]. To develop a better anti-cancer drug, we characterized a number of saponin derivatives. Among the 27 saponins synthesized by our group, the effect of PD on R-HepG2 cells was particularly encouraging because (i) it overcomes the P-gp-mediated drug resistance in R-HepG2 cells; (ii) PD is more cytotoxic to R-HepG2 cells. These occur by two mechanisms. First, PD does not seem to be a substrate of P-gp. Second, the cytotoxicity of PD is mediated by the activation of intrinsic apoptotic pathway (Fig. (**9**)). As R-HepG2 cells have more mitochondria, they are therefore more sensitive to PD. Moreover, PD causes stronger $\Delta\psi_m$ depolarizations and releases more AIF from the mitochondria isolated from R-HepG2 cells. Although the mechanism is as yet to be defined, this observation indicates that the property of the mitochondrial membrane of HepG2 and R-HepG2 cells is different and is a good target for killing cancer. Also, in view of the results from our animal study that administration of PD (2.73 mg/kg body weight) in tumor-bearing nude mice through intravenous injection for 10 days reduced tumor size by 50% without any hepatic and cardiac toxicity [21], our results suggest that PD is a promising agent for treatment of liver cancer with MDR.

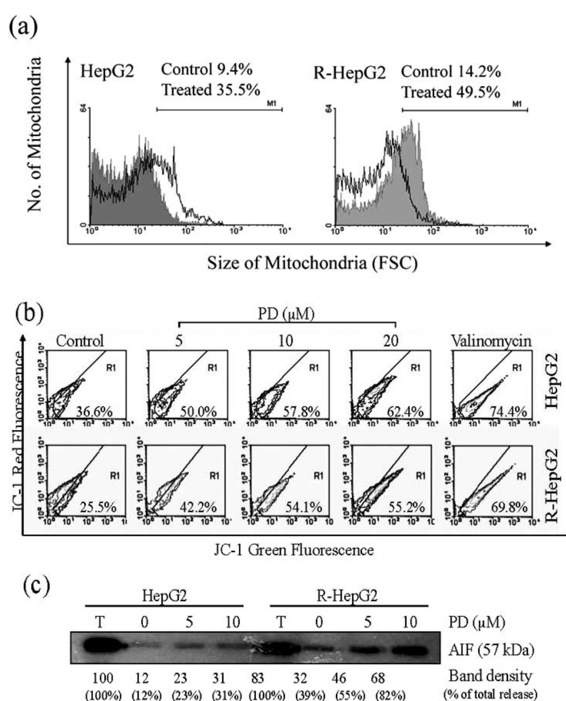


Fig. (8). Polyphyllin D induced injury in isolated mitochondria. Mitochondria from HepG2 cells and R-HepG2 were isolated with mitochondrial isolation kits. The swelling and $\Delta\psi_m$ of isolated mitochondria were analyzed by using flow cytometry with the measurement of forward scatter signals (**a**), and the red and green JC-1 fluorescence (**b**). For the $\Delta\psi_m$ determination, isolated mitochondria with JC-1 were treated with valinomycin (500 nM) for 30 min as positive controls (**b**). Mitochondrial protein AIF released from the isolated mitochondria to the buffer after PD treatments was examined by Western blot analysis. AIF in total lysates (T) were also prepared from the isolated mitochondria with digitonin (8 μg/ml) for comparison (**c**). Figures below the protein bands are the relative density measured by software ImageJ and % of total release of each protein band were normalized by corresponding control. Data shown here are reproduced from [23] with permission from Elsevier Limited (2008).

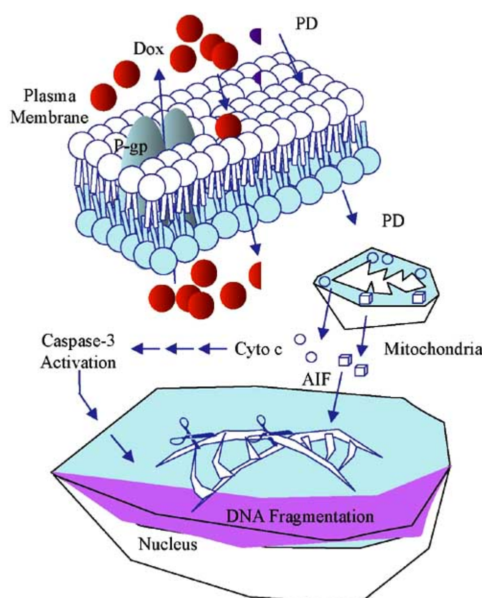


Fig. (9). Action mechanism of Polyphyllin D in the induction of cell death in R-HepG2 Cells. P-gp extrudes Dox from R-HepG2 cells and the expression of P-gp is not the solely mechanism accounting for the acquired MDR in R-HepG2 cells. Our work demonstrates that PD kills more R-HepG2 cells than their parental line HepG2 without P-gp. This occurs by two mechanisms. First, the toxicity of PD like DNA fragmentation is mediated largely by the activation of intrinsic apoptotic pathway through the release of cytochrome *c*, AIF and activation of caspase-3. As R-HepG2 cells contain more mitochondria, they therefore have a stronger sensitivity to PD. Second, PD induces more $\Delta\psi_m$ depolarization and releases more AIF from the mitochondria isolated from R-HepG2 cells, with an unknown mechanism, than that of HepG2.

ACKNOWLEDGEMENTS

This study was supported by a direct grant and a Scheme C support (RAC/2006/136-7) from CUHK and grants from Shanghai-Hong Kong Anson Research Foundation and Shun Hing Institute of Advanced Engineering.

ABBREVIATIONS

AIF	=	Apoptosis-inducing factor
Dox	=	Doxorubicin
Grp	=	Glucose regulatory protein
HCC	=	Hepatocellular cacinoma
JC-1	=	5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethyl-benzimidazolcarbocyanine iodide
MDR	=	Multi-drug resistance
PD	=	Polyphyllin D
P-gp	=	P-glycoprotein
TUNEL	=	TdT-mediated dUTP Nick-End Labeling
$\Delta\psi_m$	=	Mitochondrial transmembrane potential

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Received: May 28, 2008 Revised: July 23, 2008 Accepted: July 31, 2008