Title: 12-Chloracetyl-PPD, a novel dammarane derivative, shows anti-cancer activity via delay the progression of cell cycle G2/M phase and reactive oxygen species-mediate cell apoptosis

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Abstract:

(20R)-Dammarane-3 β , 12 β , 20, 25-tetrol (25-OH-PPD) is a ginsenoside isolated from *Panax ginseng* (C. A. Meyer). This compound exhibits anti-cancer activities on many human cancer cell lines. In this study, we investigated anti-cancer mechanisms of 12 β -O-(L-Chloracetyl)-dammar-20(22)-ene-3 β ,25-diol(12-Chloracetyl-PPD), a modified 25-OH-PPD. We found that compound 12-Chloracetyl-PPD resulted in a concentration-dependent inhibition of viability in prostate, breast, and gastric cancer cells, without affecting the viability of normal cell (human gastric epithelial cell line-GES-1, hair follicle dermal papilla cell line-HHDPC and rat myocardial cell line-H9C2). In MDA-MB-435 and C4-2B cancer cells, 12-Chloracetyl-PPD induced G2/M cell cycle arrest, down-regulated mouse double minute 2 (MDM2) expression, up-regulated p53 expression, triggered apoptosis, and stimulated reactive oxygen species production. Apoptosis can be attenuated by the reactive oxygen species scavenger N-acetylcysteine. Our results suggested that compound 12-Chloracetyl-PPD showed obvious anti-cancer activity based on delaying cell cycle arrest and inducing cell apoptosis by reactive oxygen species production, which supported development of 12-Chloracetyl-PPD as a potential agent for cancer chemotherapy.

Key words: 12-Chloracetyl-PPD, 25-OH-PPD, cell cycle, apoptosis, reactive oxygen species

1. Introduction

Cancer is a major threat of public health with a highly increasing incidence and mortality all over the world. The malignant properties of cancer, especially metastasis, are beyond the potential of surgical removal or local ablation via radiotherapy. Chemotherapy, which is a systemic drug-based method, remains irreplaceable because of the limitations of the two approaches. The development of novel therapeutic agents is still urgently needed to improve treatment outcomes of cancer (Mann, 2002).

Substances derived from plants play an important role in the development of novel anti-cancer drugs. We have recently been interested in evaluating the anti-cancer activity of modified compounds isolated from *P. ginseng*. Ginseng is used in many cultures, especially in China and other Asian countries, for treatment and prevention of various diseases, including cancer (Helms, 2004). People who consume ginseng preparations are at lower risk for cancers of the oral cavity, stomach, lung, liver, pancreas, ovaries, and colon (Yun, 2003). *Panax ginseng* (Korean ginseng), *Panax quinquefolius* (American ginseng), and other related plants, including *Panax notoginseng/pseudoginseng* (*P. notoginseng*, Buck FH Chen), are frequently used for medicinal purposes. In the United States, ginseng is one of the most widely used medicinal herbs (O'Hara et al., 1998). Although only part of the complex mixture of compounds present in these plants, the ginsenosides (saponin triterpene glycosides), are apparently responsible for most of the pharmacological effects of ginseng and

notoginseng. Some ginsenosides assert anti-cancer properties by decreasing DNA synthesis and angiogenesis, reducing host susceptibility to mutation, transformation, and DNA damage, and increasing immunosurveillance and apoptosis (Attele et al., 1999; Kitts and Hu, 2000; Shibata, 2001; Xie et al., 2005). Ginsenosides also enhance the effects of traditional chemotherapeutic agents and protect normal host tissue from damage (Helms, 2004; Nakata et al., 1998; Sato et al., 1994; Wang et al., 2006; Xie et al., 2006).

25-OH-PPD [Fig. 1 A], one of ginsenosides, is a natural anti-cancer compound isolated from *P. ginseng* fruits (Wang et al., 2009), which can inhibit human pancreatic cancer cell (Panc-1 and HPAC cells) proliferation, arrest cell cycle in vitro, and decrease pancreatic tumors weight without side effect in rodent models. 25-OH-PPD has a better anti-cancer activity in pancreatic cancer cells than commercially marketing ginsenosides-related drugs, such as ginsenoside-Rg3, paclitaxel, and methotrexate (Hao et al., 2011; Lee et al., 2009). In order to further improve the anti-cancer activities of 25-OH-PPD, we synthesized a battery of 25-OH-PPD analogs by incorporating chloroacetyl group at C-3-OH or C-12-OH (Qu et al., 2014) since many chemicals combined with halogen groups evidently increase anti-cancer effects (Dias et al., 2013; Huang et al., 2011), and halogens possess strong electron-withdrawing effects, which can influence the charge distribution and enhance the lipophilicity of compounds. We firstly tested anti-cancer activities of 12 synthesized compounds in several human cancer cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.

Among them, 12-Chloracetyl-PPD (Fig. 1B) significantly inhibited the proliferation of various human cancer cell lines. Meantime, the solubility of 25-chlorlacetyl-PPD and AD-2 was tested and the results showed that 25-chlorlacetyl-PPD exhibited better solubility than AD-2 in acetone, methylene chloride and ethyl acetate. In this study, our objective is to investigate the cellular and molecular mechanisms of 12-Chloracetyl-PPD as an anti-cancer agent.

2. Materials and methods

2.1. Reagents

β -D-Glucopyranoside, (3 β ,12 β)-12,20-dihydroxydammar-24-en-3-yl-2-O- β -D-glucopyranosyl (Rg3) (> 98%), 25-OH-PPD (> 95%), 12-Chloracetyl-PPD (> 95%) were obtained as previously described (Qu et al., 2014). Mitomycin C (> 98%) was acquired from Sigma Aldrich (St. Louis, MO, USA). FBS (Fetal Bovine Serum), DMEM medium and penicillin–streptomycin were purchased from Thermo Fisher Scientific Co., Ltd. MTT reagent was acquired from Sigma Aldrich (St. Louis, MO, USA).

2.2. Cell cultures

Human prostate (PC3, C4-2B, DU145, LNCaP), breast (MDA-MB-231, MDA-MB-435, Bcap-37), and gastric (BGC-823, SGC-7901, MGC-803, MKN-28) cancer cell lines and human gastric epithelial cell line-GES-1, hair follicle dermal papilla cell line-HHDPC and rat myocardial cell line-H9C2 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). BGC-823, SGC-7901,

MGC-803, MKN-28 lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and PC3, C4-2B, DU145, LNCaP, MDA-MB-231, MDA-MB-435, Bcap-37, GES-1, HHDPC, H9C2 cell lines were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

2.3. Cell viability assay

Cell growth inhibition caused by Rg3, 25-OH-PPD, 12-Chloracetyl-PPD and Mitomycin C was evaluated by MTT assay. The cells were seeded in 96-well plates (5000 cells/well) and under the conditions described above with varying concentrations (5μM, 10μM, 20μM, 40μM) of Rg3, 25-OH-PPD, 12-Chloracetyl-PPD and Mitomycin C for 24 h. Control cells were exposed to 0.1% DMSO. The cells were incubated with 10 μL MTT (5 mg/mL) for 4 h, and the plates were then read by the plate reader (Bio-Rad iMARK, USA) at 495 nm to determine the percentage of surviving cells. Three replicates were performed for each treatment.

2.4. Cell cycle measurements

To examine the effects of 12-Chloracetyl-PPD on the cell cycle, 2×10^5 MDA-MB-435 and C4-2B cells were seeded in each well of 6-well plate. After 12 h incubation, the cells were treated with 12-Chloracetyl-PPD in the concentrations of 0, 20, and 30 μM. After 12 h treatment, cells were trypsinized, washed with PBS, and fixed in 1.5 mL of 75% ethanol at 4 °C overnight and then incubated in RNase and staining with propidium iodide (Multi Sciences Biotech Co.,Ltd). The cell cycle was

analyzed using Cytomics FC500 Flow Cytometer (Beckman Coulter, USA).

2.5. Detection of apoptosis

Annexin V-FITC apoptosis detection kit (Mountain View, CA) was employed to detect cells in the early and late stages of apoptosis. In brief, MDA-MB-435 and C4-2B cells (2×10^5 cells/well) were seeded in 6-well plate, and after 12 h incubation, treated with the test compound (0, 20, 30 μ M) for 12 h prior to analysis. Cells in early apoptosis were positive for Annexin V-FITC alone and counted by Cytomics FC500 Flow Cytometer (Beckman Coulter, USA).

2.6. Measurement of intracellular reactive oxygen species levels

Intracellular reactive oxygen species levels were examined using 2', 7'-Dichlorofluorescein diacetate (DCFH-DA). MDA-MB-435 cells were seeded in 6-well plates (2×10^5 cells/well) and treated with different concentrations (0, 20, 30 μ M) of 12-Chloracetyl-PPD. The cells were exposed to the 12-Chloracetyl-PPD for 12 h at a concentration of 20 and 30 μ M. DCFH-DA was added to the cells, which were incubated for 30 min at 37 °C. Cytomics FC500 Flow Cytometer (Beckman Coulter, USA) was employed to measure fluorescence intensity at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

2.7. Western blot analysis

RIPA lysis buffer was used to extract total cellular protein and the protein concentration was determined by bicinchoninic acid (BCA) assay. SDS-PAGE was carried out in 10% gel with equal-loading amount of protein per lane (35 μ g/lane). After electrophoresis, the resolved protein bands were transferred to polyvinylidene

fluoride (PVDF) membranes and 5% BSA in TBST buffer was employed to block PVDF membrane for 1 h. After blocking, the membrane was incubated with a 1:1000 dilution of primary antibody against MDM2, (bioworld Technology, Inc), Cl-PRAP, Cl-caspase-3, Cl-caspase-9, Cl-caspase-8, cdc25 and p-cdc25 (cell signaling technology Inc), caspase-3, caspase-8 and p53 (Proteintech Group, Inc), Cyclin B1, cdc2, p-cdc2 (Santa cruz biotechnology, Inc), or β -actin (Ding-Guo Biotech Ltd., Beijing) in 5% BSA and at 4°C for overnight. After incubation, the membrane was washed with TBST containing 0.1% Tween-20, then anti-mouse and anti-rabbit IgG (1:5000 dilution) were incubated with the PVDF membranes at room temperature for 2 h. Signals were developed on X-ray film using an enhanced chemiluminescence system (Eastman Kodak Company, USA).

2.8. Statistical analysis

All data represent the mean $\pm$ S.D. of three independent experiments. The two-way analysis of variance (ANOVA) was employed to determine significance of differences and then Student-Newman-Keuls multiple comparison test (SNK) as post hoc. Differences were considered significant if $P < 0.05$. Western blot band intensity was analyzed using image J and statistical analyses were conducted using SPSS software package (Version 16.0, IBM Corp., Armonk, NY, USA).

3. Results

3.1. 12-Chloracetyl-PPD inhibited cell proliferation

The cytotoxic effects of 12-Chloracetyl-PPD, 25-OH-PPD, Rg3 and Mitomycin C were examined and compared on the survival of human prostate (PC3, C4-2B,

DU145, LNCaP), breast (MDA-MB-231, MDA-MB-435, Bcap-37), and gastric (BGC-823, SGC-7901, MGC-803, MKN-28) cancer cell lines and normal human gastric epithelial cell line-GES-1, hair follicle dermal papilla cell line-HHDPC and rat myocardial cell line-H9C2 (non-malignant). 12-Chloracetyl-PPD exhibited strong inhibition of cell viabilities on various cancer cell lines. As shown in table 1, the IC₅₀ values of 12-Chloracetyl-PPD for the cells ranged from 7.54 to 25.91 μ M, which was significantly more potent than 25-OH-PPD (ranged from 30.07 to 40.41 μ M). The IC₅₀ values of Rg3 for all cells exceeded 100 μ M. Interestingly, compared with Mitomycin C, a well-known anti-cancer drug, 12-Chloracetyl-PPD showed 2- to 4-folds greater inhibition of cell growth in PC3, C4-2B, MDA-MB-435 cell lines. 12-Chloracetyl-PPD barely affected normal cells (GES-1, HHDPC, H9C2). The IC₅₀ values of 12-Chloracetyl-PPD showed 2- to 4-folds and 2- to 20-folds higher than 25-OH-PPD and Mitomycin C, separately.

3.2. 12-Chloracetyl-PPD delayed the progression of cell cycle G2/M phase in MDA-MB-435 and C4-2B cells.

The effect of 12-Chloracetyl-PPD on cell cycle distribution was detected using flow cytometry. As shown in Fig. 2A-a, 12-Chloracetyl-PPD treatment resulted in accumulation of cells in G2/M phases in dose-dependent manner. However, the population of cells in G1 phase was decreased. The results suggested that 12-Chloracetyl-PPD reduced the population of cells in G1 phase and increased the percentage of cells in G2/M phase in both MDA-MB-435 and C4-2B cells (Fig. 2A-a), but 25-OH-PPD did not evidently change cell cycle distribution in

MDA-MB-435 and C4-2B cells (Fig. 2A-b).

The G2/M related protein expression levels were investigated as well and western blot analysis showed that cdc2, cdc25, cyclin B1 and MDM2 were down-regulated in a concentration-dependent manner after treated with 12-Chloroacetyl-PPD for 12 h, whereas the levels of p21, p53, p-cdc2-Tyr15, p-cdc25-Ser216 were increased (Fig. 2B-a), which indicated that 12-Chloroacetyl-PPD arrested the cell cycle at the G2/M phase in both MDA-MB-435 and C4-2B cells, but 25-OH-PPD did not obviously alter those proteins (Fig. 2B-b).

3.3. 12-Chloroacetyl-PPD induced apoptotic cell death in MDA-MB-435 and C4-2B cells

12-Chloroacetyl-PPD also induced cell apoptosis in both MDA-MB-435 and C4-2B cell lines. As observed in Fig. 3A, both of the cancer cell lines exhibited a significantly increase in apoptosis following exposure to 30 μ M by Annexin V-FITC assay.

We further confirmed the apoptosis by examining the expression of the hallmark proteins, such as caspase-9, caspase-8 and caspase-3. As observed in Fig. 3B-a, the levels of cleaved caspase-9, 8, 3 were activated after treatment with 12-Chloroacetyl-PPD in both MDA-MB-435 and C4-2B cells, but the expression levels of those proteins did not change after treatment with 25-OH-PPD in both MDA-MB-435 and C4-2B cells (Fig. 3B-b).

3.4. 12-Chloroacetyl-PPD triggered reactive oxygen species generation, which was

involved in 12-Chloracetyl-PPD-induced apoptosis

The oxidation-sensitive fluorescent dye, DCFH-DA was employed to examine the change of intracellular reactive oxygen species. Our results showed that intracellular reactive oxygen species levels were increased after exposure to 12-Chloracetyl-PPD in MDA-MB-435 cells by DCFH-DA-base assay (Fig. 4A-a, B-a). To investigate whether reactive oxygen species were related with 12-Chloracetyl-PPD-induced apoptosis in MDA-MB-435 cells, cells were exposed to 12-Chloracetyl-PPD with or without reactive oxygen species scavenger, N-acetylcysteine (NAC). The results revealed that pretreatment with NAC reduced the generation of reactive oxygen species induced by 12-Chloracetyl-PPD (Fig. 4A-a, B-a) and the cell viability was inhibited by NAC pretreatment less than by 12-Chloracetyl-PPD only (Fig. 5A), but 25-OH-PPD did not obviously alter the intracellular reactive oxygen species levels in MDA-MB-435 cells (Fig. 4A-b, B-b). Flow cytometric analysis also demonstrated that NAC pretreatment attenuated cell apoptosis compared with 12-Chloracetyl-PPD treatment alone (Fig. 5B). The results suggested that 12-Chloracetyl-PPD-induced cell apoptosis may be regulated by the generation of reactive oxygen species in MDA-MB-435 cells. Furthermore, we investigated the effect of reactive oxygen species on the expression of proteins associated with apoptosis and the data showed that 12-Chloracetyl-PPD (20 μ M and 30 μ M) observably increased the levels of Cl-caspase-9, 8, 3 and Cl-PARP (Fig. 5C) in MDA-MB-435 cells. This data further revealed that reactive oxygen species played crucial role in 12-Chloracetyl-PPD-induced apoptotic cell death in

MDA-MB-435 cells.

4. Discussion

The purpose of this study is to examine whether 12-Chloracetyl-PPD has an anti-cancer activity and investigate its cellular and molecular mechanisms as an anti-cancer agent in human prostate, breast, gastric cancer cells. We found that 12-Chloracetyl-PPD inhibited the proliferation of human prostate, breast, gastric cancer cells and barely affected normal cells (human gastric epithelial cell line-GES-1, hair follicle dermal papilla cell line-HHDPC, rat myocardial cells-H9C2). The anti-cancer activity of 12-Chloracetyl-PPD was related with cell cycle arrest in the G2/M phase and reactive oxygen species-mediated cell apoptosis in MDA-MB-435 and C4-2B cells.

Cell growth is critically regulated by cell cycle, so many anti-cancer drugs were developed by arresting cell cycle at a specific checkpoint to inhibit cell proliferation (Lu et al., 2007; Murray, 2004). During the eukaryotic cell division cycle, cyclin/cyclin-dependent kinase (CDK) complexes are major regulatory factors, which are modulated by CDK inhibitors, such as p21WAF1/Cip1 (Israels and Israels, 2001; Vermeulen et al., 2003). P21, a member of the Cip/Kip family of CDK inhibitors, can induce cell cycle arrest and act as a tumor suppressor (Chen et al., 2010). Furthermore, the classic group of cell cycle regulators and cyclins is essential for the regulation of G2/M transition. The transition from G2 phase to M phase is partly modulated by a mitosis-promoting factor, which was composed of cyclin B1 and cell division cycle 2 (cdc2, a G2-specific CDK). The cdc2-cyclin complex is formed by the interaction of

cdc2 and cyclin B1, and suppressed by the phosphorylation of Tyr15 in cdc2 (Vermeulen et al., 2003). In this study, 12-Chloracetyl-PPD significantly decreased the expression levels of cyclin B1 and cdc2, and increased the expression level of the inactivated p-cdc2. Cdc25c have a great influence in the dephosphorylation of cdc2 on Thr14/Tyr15, which is a major requirement for the onset of mitosis (Takizawa and Morgan, 2000). 12-Chloracetyl-PPD significantly increased the level of p-cdc25c (Ser 216, inactive form) but reduced the level of cdc25c. The expression of functional cdc25c decreased because of the increase in the phosphorylation of cdc25c. The decrease was followed by an increase in phospho-cdc2. Thus, the activation of cdc2/cyclin B was suppressed, and G2/M phrase was arrested. Moreover, the inhibition of the cyclin-cdk complex was possibly amplified by the simultaneous up-regulation of the p21/WAF1 protein, which may facilitate to maintain G2/M arrest by reducing the activity of the cyclin B1/cdc2 complex. In this study, 12-Chloracetyl-PPD significantly increased the expression of p21/WAF1 protein in both MDA-MB-435 and C4-2B cell lines. By contrast, 25-OH-PPD did not exhibit similar profile, did not alter the expression levels of the proteins related with cell cycle in MDA-MB-435 and C4-2B cell lines.

In our previous study, 25-OH-PPD and the analogues of 25-OH-PPD can obviously down-regulate the expression level of MDM2(Wang et al., 2009), meantime MDM2 also affected cell cycle transition by interacting with and regulating proteins related with cell cycle, such as p21 and E2F1, independent of p53 (Zhang and Zhang, 2005). Therefore, we also examined the expression level of oncoprotein MDM2. In

addition, MDM2 was initially identified for its regulating the p53 tumor suppressor. The tumor suppressor protein p53 is a short-lived transcription factor, which was activated to respond to various cellular stresses, such as hyperproliferation and DNA damage (Michael and Oren, 2003). Activated p53 orchestrates the transactivation or transrepression of many target genes and thus triggers apoptosis, senescence or growth arrest. p53 is largely associated with the oncoprotein MDM2, a RING finger-containing E3 ubiquitin ligase that mediates the ubiquitylation and rapid degradation of p53 in proteasome (Toledo and Wahl, 2006). P53 and MDM2 form an auto-regulatory loop, and in which, p53 positively regulates MDM2 expression. In the meantime, MDM2 negatively modulates p53 activity and expression level as well. Anti-cancer agents disrupt p53-MDM2 interaction, as such, p53 degradation is attenuated and MDM2 auto-ubiquitylation is stimulated. In this study, the MDM2 expression level decreased, but the p53 expression increased after the MDA-MB-435 and C4-2B cells were exposed to 12-Chloracetyl-PPD, which indicated that 12-Chloracetyl-PPD possibly disrupted the interaction between p53 and MDM2 by targeting the MDM2 protein, but the parent compound 25-OH-PPD did not alter the expression levels of the MDM2 and p53 in MDA-MB-435 and C4-2B cells.

Apoptosis plays a crucial role in the eradication of cancer cells. The caspase family of cysteine proteases is implicated in the initiation and execution of apoptosis (Green, 2000). Initiator caspases, namely, caspase-8 and caspase-9, mainly activate the death-receptor apoptosis pathway and the mitochondrial apoptosis pathway, respectively. These pathways converge on executioner caspases, including caspase-3,

6, and 7. Caspase-3 is a key molecule that exists in cells as a low-activity zymogen known as procaspase-3 (Green, 2000; Hengartner, 2000). By proteolysis, procaspase-3 is activated to produce caspase-3 (Pop and Salvesen, 2009), which enters the nucleus and cleaves more than 300 substrates, including poly ADP-ribose polymerase (PARP) and MDM2, which induced apoptosis (Hengartner, 2000). Our results demonstrated that 12-Chloracetyl-PPD induced the apoptotic cell death in both MDA-MB-435 and C4-2B cells. We further confirmed cell apoptosis-related proteins expression by Western blot analysis, and found that the apoptosis related protein levels were elevated in MDA-MB-435 and C4-2B cells induced by 12-Chloracetyl-PPD, but the expression of these proteins was slightly affected by 25-OH-PPD.

The role of reactive oxygen species remains controversial because reactive oxygen species are a signal mediator of cell death (Moungjaroen et al., 2006). Increased oxidative stress is involved in the apoptotic response induced by some anti-cancer agents (Luo et al., 2010; Yang et al., 2011). Zhang et al. (Zhang et al., 2013) reported that AD-1, a novel ginsenoside derivative, exhibited anti-lung cancer property by activating the p38 MAPK pathway and by inducing reactive oxygen species generation. Our results demonstrated that 12-Chloracetyl-PPD could increase intracellular reactive oxygen species levels, but 25-OH-PPD did not alter the intracellular reactive oxygen species levels. Furthermore, we observed that the inhibition of the increasing reactive oxygen species levels by N-acetylcysteine (NAC) decreased the intracellular reactive oxygen species levels and the apoptosis induced

by 12-Chloracetyl-PPD. Western blot analysis also revealed that NAC pretreatment inhibited caspase-9, 8, 3, and PARP activation stimulated by 12-Chloracetyl-PPD in MDA-MB-435 cells. Our findings indicated that 12-Chloracetyl-PPD-induced apoptosis was mainly involved in the accumulation of reactive oxygen species in human cancer cells.

In conclusion, our findings showed that 12-Chloracetyl-PPD elicited remarkable anti-cancer activity via down-regulating key oncogenic, pro-growth/pro-proliferation proteins and inducing cell apoptosis. Our study also provided new insights into the mechanisms of 12-Chloracetyl-PPD-induced apoptosis in MDA-MB-435 cells, which may act through generation of reactive oxygen species. Hence, our present data support the further exploration of this compound as a therapy for prostate and breast cancer.

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Figure legends

Fig. 1 (A) 25-OH-PPD (B) 12-Chloracetyl-PPD

Fig. 2 12-Chloracetyl-PPD induces G2/M cell cycle arrest in MDA-MB-435 and C4-2B human cancer cells. (A) Representative DNA fluorescence histograms of PI-stained cells are shown. 12-Chloracetyl-PPD (a) and 25-OH-PPD (b) induce G2/M cell cycle in MDA-MB-435 and C4-2B cells in a concentration-dependent manner 12 h after treatment. Group data analysis is shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control (mean $\pm$ S.D., $n = 3$). (B) Effects of 12-Chloracetyl-PPD (a) and 25-OH-PPD (b) on the expression of various cell cycle-related proteins in human MDA-MB-435 and C4-2B cancer cells. Cells were exposed to various concentrations of 12-Chloracetyl-PPD and 25-OH-PPD for 12 h, followed by Western blot analysis. Representative Western blot results are shown, and protein levels were normalized to actin. Data represent the mean $\pm$ S.D. of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with actin.

Fig. 3 12-Chloracetyl-PPD induces apoptosis in MDA-MB-435 and C4-2B human cancer cells. (A) Cells were exposed to various concentrations of 12-Chloracetyl-PPD for 12 h, followed by flow cytometry-based apoptosis assays. Representative plots of flow cytometry-based apoptosis assays are shown. Group data analysis of the percentage of apoptotic cells is shown. All assays were performed in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the control. (B) Effects of 12-Chloracetyl-PPD (a) and 25-OH-PPD (b) on the expression of apoptosis-related proteins in MDA-MB-435 and C4-2B human cancer cells. Cells were exposed to various concentrations of 12-Chloracetyl-PPD and 25-OH-PPD for 12 h, following by Western blot analysis.

Fig. 4 12-Chloracetyl-PPD induces reactive oxygen species production in MDA-MB-435 cells. The production of intracellular reactive oxygen species levels was detected by using DCFH-DA and pretreatment with NAC reduces 12-Chloracetyl-PPD and 25-OH-PPD-generated reactive oxygen species production in 12-Chloracetyl-PPD and 25-OH-PPD-treated MDA-MB-435 cells. (A) Cells were observed by a fluorescence microscope ($\times 20$) following staining with DCFH-DA. (a) 12-Chloracetyl-PPD, (b) 25-OH-PPD. (B) Intracellular reactive oxygen species levels were examined by using flow cytometry-based reactive oxygen species levels assay. Representative plots of flow cytometry-based reactive oxygen species levels are shown. (a) 12-Chloracetyl-PPD, (b) 25-OH-PPD. Group data analysis of the percentage of reactive oxygen species cells is shown. All assays were performed in triplicate. *** $P < 0.001$ compared with the negative control.

Fig. 5 12-Chloracetyl-PPD inhibits proliferation and induces apoptosis is attenuated by reactive oxygen species scavenger-N-acetylcysteine (NAC). (A) Pretreatment with NAC cells and untreated cells were exposed to various concentrations of 12-Chloracetyl-PPD for 24 h, cell viability was examined by using MTT assay, *** $P < 0.001$ compared with negative control. (B) Pretreatment with NAC cells and untreated cells were exposed to various concentrations of 12-Chloracetyl-PPD for 12 h, followed by flow cytometry-based apoptosis assays. Representative plots of flow cytometry-based apoptosis assays are shown. Group data analysis of the percentage of apoptotic cells is shown. All assays were performed in triplicate. *** $P < 0.001$ compared with the negative control. (C) Pretreatment with NAC cells and untreated cells were exposed to various concentrations of 12-Chloracetyl-PPD for 12 h, followed by Western blot analysis. Representative Western blot results are shown, and protein levels were normalized to actin. Data represent the mean $\pm$ S.D. of three independent experiments. *** $P < 0.001$ compared with negative control.

Table 1. IC₅₀ values of 12-Chloracetyl-PPD, 25-OH-PPD, Rg3 and Mito C in human cells.

Values are the mean of triplicate experiments and the IC₅₀ is with respect to rates of proliferation.

Cell line	IC ₅₀ (μM)			
	12-Chloracetyl-PPD	25-OH-PPD	Rg3	Mito C
PC3	10.44 ± 1.07	39.46 ± 1.38	>100	33.70 ± 1.23
C4-2B	9.85 ± 0.62	30.07 ± 0.49	>100	23.74 ± 0.98
DU145	19.30 ± 0.36	38.74 ± 0.56	>100	31.51 ± 1.09
LNCaP	21.49 ± 0.70	35.54 ± 0.78	>100	30.77 ± 0.99
MDA-MB-231	25.20 ± 0.56	37.51 ± 0.91	>100	46.95 ± 2.34
MDA-MB-435	7.54 ± 0.21	32.74 ± 1.33	>100	25.75 ± 0.56
Bcap-37	16.78 ± 0.08	40.41 ± 2.43	>100	21.66 ± 0.65
BGC-823	17.40 ± 0.63	29.07 ± 0.89	>100	20.51 ± 0.74
SGC-7901	16.65 ± 1.02	30.59 ± 1.67	>100	13.92 ± 0.54
MGC-803	17.96 ± 1.13	30.54 ± 1.56	>100	22.89 ± 0.74
MKN-28	25.91 ± 0.45	32.17 ± 1.27	>100	128.87 ± 4.51
GES-1	120.89 ± 0.92	77.94 ± 3.54	>100	86.17 ± 2.56
H9C2	418.17 ± 3.42	241.60 ± 4.45	>500	24.26 ± 0.93
HHDPC	100.43 ± 2.12	57.25 ± 2.33	>100	20.54 ± 0.67

A

B

Fig. 1 (A) 25-OH-PPD (B) 12-Chloroacetyl-PPD

A

a

b

B

a

b

Fig. 2 12-Chloracetyl-PPD induces G2/M cell cycle arrest in MDA-MB-435 and C4-2B human cancer cells. (A) Representative DNA fluorescence histograms of PI-stained cells are shown. 12-Chloracetyl-PPD (a) and 25-OH-PPD (b) induce G2/M cell cycle arrest in MDA-MB-435 and C4-2B cells in a concentration-dependent manner 12 h after treatment. Group data analysis is shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control (mean $\pm$ S.D., $n = 3$). (B) Effects of 12-Chloracetyl-PPD (a) and 25-OH-PPD (b) on the expression of various cell cycle-related proteins in human MDA-MB-435 and C4-2B cancer cells. Cells were exposed to various concentrations of 12-Chloracetyl-PPD and 25-OH-PPD for 12 h, followed by Western blot analysis. Representative Western blot results are shown, and protein levels were normalized to actin. Data represent the mean $\pm$ S.D. of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with actin.

A

B

a

b

Fig. 3 12-Chloracetyl-PPD induces apoptosis in MDA-MB-435 and C4-2B human cancer cells. (A)

Cells were exposed to various concentrations of 12-Chloracetyl-PPD for 12 h, followed by flow cytometry-based apoptosis assays. Representative plots of flow cytometry-based apoptosis assays

are shown. Group data analysis of the percentage of apoptotic cells is shown. All assays were performed in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the control. (B) Effects of 12-Chloracetyl-PPD (a) and 25-OH-PPD (b) on the expression of apoptosis-related proteins in MDA-MB-435 and C4-2B human cancer cells. Cells were exposed to various concentrations of 12-Chloracetyl-PPD for 12 h, followed by Western blot analysis.

A

B

Fig. 4 12-Chloracetyl-PPD induces reactive oxygen species production in MDA-MB-435 cells.

The production of intracellular reactive oxygen species levels was detected by using DCFH-DA and pretreatment with NAC reduces 12-Chloracetyl-PPD and 25-OH-PPD-generated reactive oxygen species production in 12-Chloracetyl-PPD and 25-OH-PPD-treated MDA-MB-435 cells.

(A) Cells were observed by a fluorescence microscope ($\times 20$) following staining with DCFH-DA.

(a) 12-Chloracetyl-PPD, (b) 25-OH-PPD. (B) Intracellular reactive oxygen species levels were

examined by using flow cytometry-based reactive oxygen species levels assay. Representative

plots of flow cytometry-based reactive oxygen species levels are shown. (a) 12-Chloracetyl-PPD,

(b) 25-OH-PPD. Group data analysis of the percentage of reactive oxygen species cells is shown.

All assays were performed in triplicate. *** $P < 0.001$ compared with the negative control.

A

B

C

Fig. 5 12-Chloracetyl-PPD inhibits proliferation and induces apoptosis is attenuated by reactive oxygen species scavenger-N-acetylcysteine (NAC). (A) Pretreatment with NAC cells and untreated cells were exposed to various concentrations of 12-Chloracetyl-PPD for 24 h, cell

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