

Protopanaxadiol Promotes Apoptosis in Human Melanoma Cells through the JNK Signaling Pathway by Targeting MLK3

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ABSTRACT

Melanoma is an aggressive form of skin cancer that often shows limited response to existing therapies because of frequent drug resistance and disease recurrence. Protopanaxadiol (PPD), a key bioactive metabolite obtained from *Panax ginseng*, has shown notable anticancer potential in various studies. However, its specific impact on melanoma cells and the underlying molecular mechanisms are not yet well understood. This study explores the anticancer activity of PPD against melanoma and clarifies the pathways involved. The anti-melanoma effects of PPD and its mechanism of action were examined using MTT and crystal violet staining assays, GO enrichment analysis, KEGG enrichment analysis, flow cytometry, Western blot analysis, and CETSA experiments. PPD markedly suppressed the viability of melanoma cells in both dose- and time-dependent ways, triggering morphological alterations typical of apoptosis. Network pharmacology analysis pointed to the MAPK signaling pathway as a key target for PPD, which was subsequently validated experimentally. Blocking JNK with the inhibitor SP600125 attenuated PPD-induced apoptosis, highlighting the essential role of JNK signaling. Moreover, PPD selectively activated MLK3 in a ROS-independent manner, without affecting ASK1 or TAK1. These findings suggest that PPD triggers the JNK pathway primarily by targeting and activating MLK3, thereby promoting apoptosis. CETSA assays confirmed direct binding between PPD and MLK3. Molecular docking combined with site-directed mutagenesis indicated that the binding site likely involves residue LEU248. These results demonstrate that PPD possesses strong anti-melanoma activity by directly engaging MLK3 and stimulating the MLK3-JNK signaling cascade, which leads to apoptotic cell death. The study offers new understanding of PPD's molecular actions and supports its potential development as a therapeutic option for melanoma.

Keywords: Melanoma, Protopanaxadiol, MLK3, JNK pathway

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Introduction

Melanoma ranks among the most aggressive and lethal skin cancers, largely due to its strong tendency to metastasize and its resistance to many treatments. Its onset is influenced by multiple risk factors, such as ultraviolet (UV) radiation exposure, the presence of atypical moles or dysplastic nevi, and specific genetic mutations. Research has established that mutations in genes including BRAF, NRAS, and TP53 play central roles in driving melanoma development and progression by enhancing uncontrolled cell growth and survival [1]. Current treatment approaches for melanoma include surgical removal, targeted therapies, immunotherapy, and combined regimens. While these strategies have improved patient outcomes, issues like drug resistance and tumor recurrence continue to pose significant challenges [2]. Consequently, ongoing efforts focus on uncovering the molecular basis of melanoma advancement and identifying more effective therapeutic agents.

Ginsenosides, the primary pharmacologically active constituents of *Panax ginseng*, are triterpenoid saponins known for a wide range of biological effects, including antioxidant, anti-inflammatory, antitumor,

can additionally trigger apoptosis via reactive oxygen species (ROS) production. These attributes suggest MLK3 as a viable candidate for targeted anticancer interventions [7].

This investigation demonstrates that PPD exerts robust antitumor effects in melanoma primarily by triggering apoptosis. Network pharmacology approaches helped identify MLK3 as a direct molecular target. Experimental data confirmed that PPD binds to MLK3 and promotes its phosphorylation. Taken together, the anticancer action of PPD in melanoma occurs through direct engagement of MLK3, subsequent activation of the JNK pathway, and induction of apoptotic processes.

Materials and Methods

Materials and reagents

PPD with 99% purity was generously supplied by Prof. Weicheng Hu from Yangzhou University. Melanoma lines SK-MEL-28, MALME-3M, SK-MEL-2, and the HEK293T human embryonic kidney line were obtained from the American Type Culture Collection (Rockville, MD, USA). Reagents including dimethyl sulfoxide, MTT, inhibitors U0126, SP600125, SB202190, N-acetylcysteine (NAC), and polyethyleneimine (PEI) were acquired from Sigma-Aldrich (St. Louis, MO, USA). The FITC-Annexin V Apoptosis Detection Kit I came from BD Bioscience (San Jose, CA, USA). Primary antibodies targeting caspase-3, caspase-8, cleaved caspase-8, Bax, Bcl-2, caspase-9, PARP, and the total/phosphorylated forms of ERK, JNK, p38, MLK3, ASK1, TAK1, MKK7, MKK4, and c-Jun were purchased from Cell Signaling Technology (Beverly, MA, USA). β -actin antibody was sourced from Santa Cruz Biotechnology (Dallas, TX, USA).

Target prediction and bioinformatics analysis

Candidate targets for PPD were gathered from PharmMapper and Swiss Target Prediction platforms [11, 12]. Melanoma-associated genes were sourced from the GeneCards Human Gene Database. Overlapping targets were determined with Venny software. The protein-protein interaction (PPI) network was built using STRING and visualized in Cytoscape software (version 3.9.1). GO and KEGG enrichment analyses were executed on the OmicShare bioinformatics tool [13].

Cell culture and drug treatment

MALME-3M and SK-MEL-2 cells were grown in RPMI-1640, SK-MEL-28 cells in MEM, and HEK293T cells in DMEM. All culture media contained 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were maintained at 37°C with 5% CO₂.

Assessment of cell viability

Melanoma cells were plated at 1×10^6 cells/mL density in 96-well plates, allowed to settle for roughly 18 h, and then exposed to varying PPD concentrations for different time intervals. Viability was quantified using standard MTT assays.

Cell morphology and crystal violet staining

SK-MEL-28 cells were seeded at 5×10^6 cells/mL in 12-well plates, cultured for ~18 h, and treated with different PPD doses for 12 h to observe morphology or 24 h for crystal violet staining.

Annexin V-FITC/PI staining and flow cytometry

SK-MEL-28 cells were plated at 6×10^6 cells/mL in 6-well plates and incubated for ~18 h prior to 12 h PPD treatment. Both detached and adherent cells (collected via mild trypsinization) were washed with cold PBS, stained per the manufacturer's protocol with Annexin V-FITC/PI, and analyzed on a FACScan flow cytometer (Becton Dickinson).

Site-directed mutagenesis

Using the MLK3 sequence retrieved from NCBI, specific primers were designed to generate MLK3 mutants.

Cellular thermal shift assay (CETSA)

HEK293T cells overexpressing either wild-type or mutant MLK3 were used for CETSA. Lysates treated or untreated with PPD were heated across a temperature gradient. MLK3 protein stability was assessed by Western blot, with quantification performed using ImageJ.

Western blot analysis

Total proteins from SK-MEL-28 and HEK293T cells were extracted following a previously reported protocol [14]. Concentrations were measured with the PROMEASURE kit (iNtRON). Bands were detected using an Enhanced Peroxidase kit on a Chemidoc imaging system (ATTO, Tokyo, Japan).

Molecular docking analysis

The MLK3 3D structure was retrieved from the AlphaFold Protein Structure Database [15], while the PPD structure was downloaded from PubChem. Docking was performed with CB-DOCK2, and the resulting binding modes and interactions were examined in Discovery Studio Visualizer [16].

Statistical analysis

All statistical computations were conducted using GraphPad Prism 9.3.0. Data from a minimum of three independent experiments were evaluated by one-way ANOVA with Tukey's multiple comparison test. Results are reported as mean $\pm$ SEM, with $p < 0.05$ considered statistically significant.

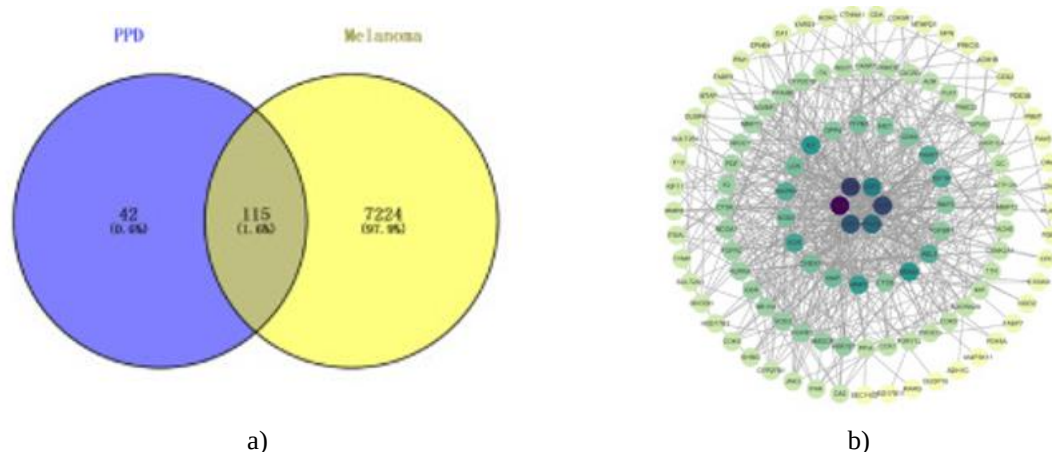
Results and Discussion

PPD suppresses proliferation of melanoma cells

MTT assays revealed that PPD decreased viability of various melanoma cell lines in a concentration-dependent manner (**Figure 1b**). SK-MEL-28 cells were especially responsive, showing clear reductions in viability that depended on both PPD dose and treatment duration (**Figure 1c**). Crystal violet staining corroborated the dose-dependent loss of cell viability in SK-MEL-28 cultures (**Figure 1d**). Microscopic examination after 12 h of PPD exposure showed classic apoptotic changes such as cell rounding, shrinkage, and blebbing (**Figure 1e**).

Network pharmacology analysis of PPD's potential mechanisms in melanoma

Potential mechanisms were explored by predicting 157 targets for PPD via Swiss Target Prediction and PharmMapper, and collecting 7,339 melanoma-related genes from GeneCards. Intersection analysis identified 115 common targets (**Figure 2a**). The PPI network constructed from these targets comprised 114 nodes connected by 626 edges (**Figure 2b**). GO enrichment analysis strongly associated these targets with apoptotic regulation and programmed cell death processes (**Figure 2c**). KEGG analysis highlighted enrichment in multiple cancer pathways, the Ras signaling route, and the MAPK signaling pathway (**Figure 2d**).



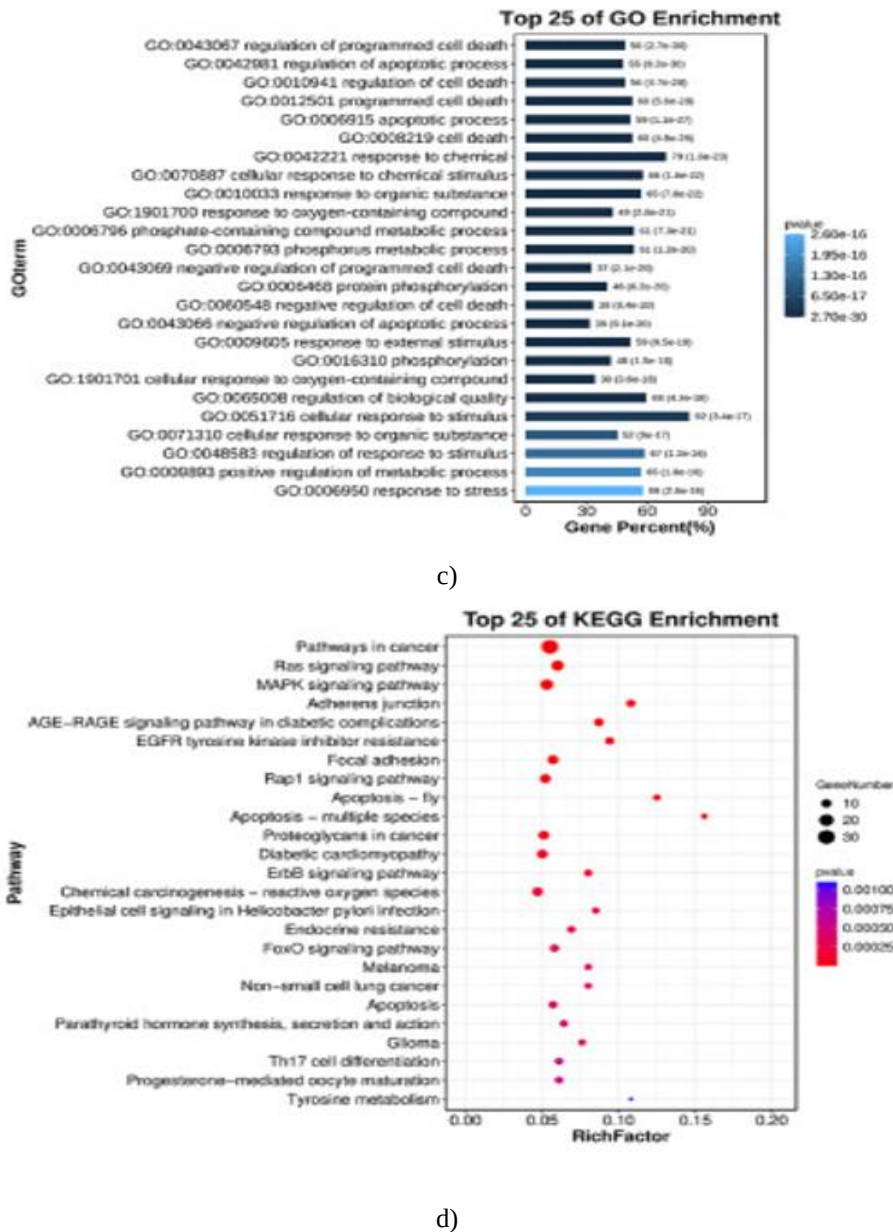


Figure 2. Network pharmacology study of PPD in the context of melanoma. (a) Venn diagram illustrating the intersection between predicted PPD targets and melanoma-associated genes. (b) Protein-protein interaction network showing the interconnected potential targets of PPD relevant to melanoma treatment. (c) GO enrichment analysis revealing the main biological processes through which PPD may influence melanoma cells. (d) KEGG pathway enrichment analysis identifying the key signaling routes potentially modulated by PPD in melanoma.

PPD promotes apoptotic cell death in SK-MEL-28 cells

Drawing from the GO analysis outcomes, experiments were performed to evaluate whether PPD could stimulate apoptosis in SK-MEL-28 melanoma cells. Annexin V-FITC/PI-based flow cytometry demonstrated a progressive, concentration-related rise in the proportion of apoptotic cells after a 12-hour exposure to PPD (**Figure 3a**). Supporting evidence came from Western blot experiments that tracked alterations in apoptosis regulators. Treatment with PPD substantially upregulated multiple pro-apoptotic factors—such as cleaved forms of caspase-8, caspase-9, PARP, and caspase-3, along with Bax—while downregulating the anti-apoptotic protein Bcl-2 (**Figure 3b**). A notable observation was the early appearance of cleaved-caspase 8 within 3 hours of PPD addition, which occurred prior to detectable caspase-9 processing. This sequence strongly suggests that the extrinsic apoptosis pathway is the dominant route activated by PPD. These protein expression shifts occurred in a dose-dependent fashion, matching the patterns observed in the flow cytometry experiments (**Figure 3c**).

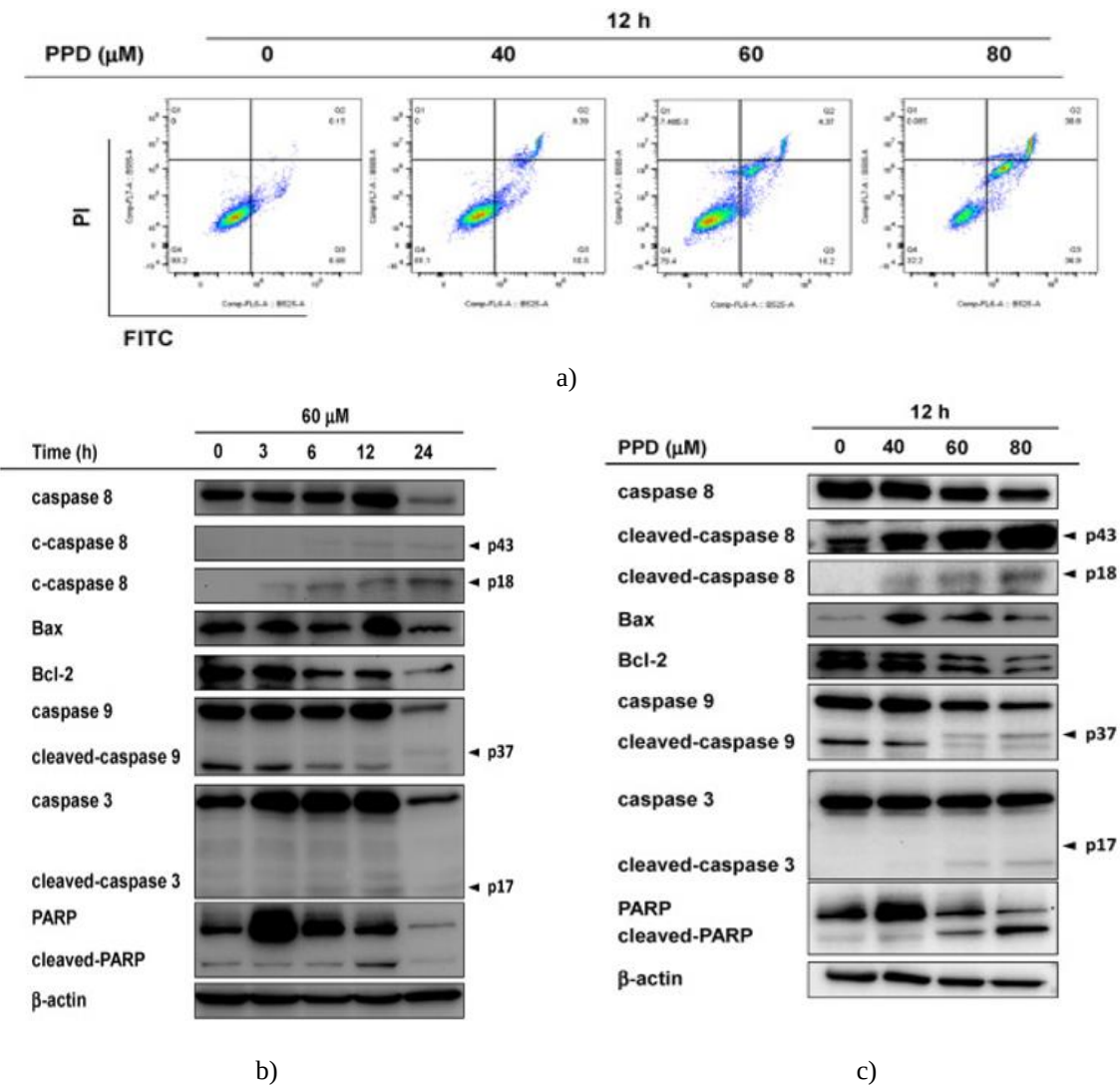


Figure 3. PPD triggers apoptosis in melanoma cells. (a) SK-MEL-28 cells were labeled with Annexin V and PI, then subjected to flow cytometric analysis. (b–c) SK-MEL-28 cells received 60 μM PPD for various durations or were exposed to different concentrations of PPD for 12 h. Levels of apoptosis-related proteins were examined through Western blotting.

PPD drives apoptosis through the JNK signaling pathway

Results from KEGG pathway enrichment pointed to the MAPK cascade as a major route through which PPD exerts its influence. To explore this in greater depth, the activation states of the principal MAPKs—ERK, JNK, and p38—were examined via their phosphorylation levels. PPD treatment led to rapid and pronounced phosphorylation of these kinases at early time points (**Figure 4a**).

To pinpoint the critical pathway, melanoma cells were pretreated with selective inhibitors before PPD exposure, and cell viability was subsequently measured. Only the JNK inhibitor SP600125 substantially blocked PPD’s cytotoxic impact, while the ERK inhibitor U0126 and the p38 inhibitor SB202190 showed no significant protective effect (**Figure 4b**). Consistent with this, SP600125 pretreatment largely prevented the alterations in apoptosis-related proteins normally induced by PPD (**Figure 4c**). These findings collectively establish that PPD stimulates apoptosis in melanoma cells primarily by activating the JNK signaling pathway.

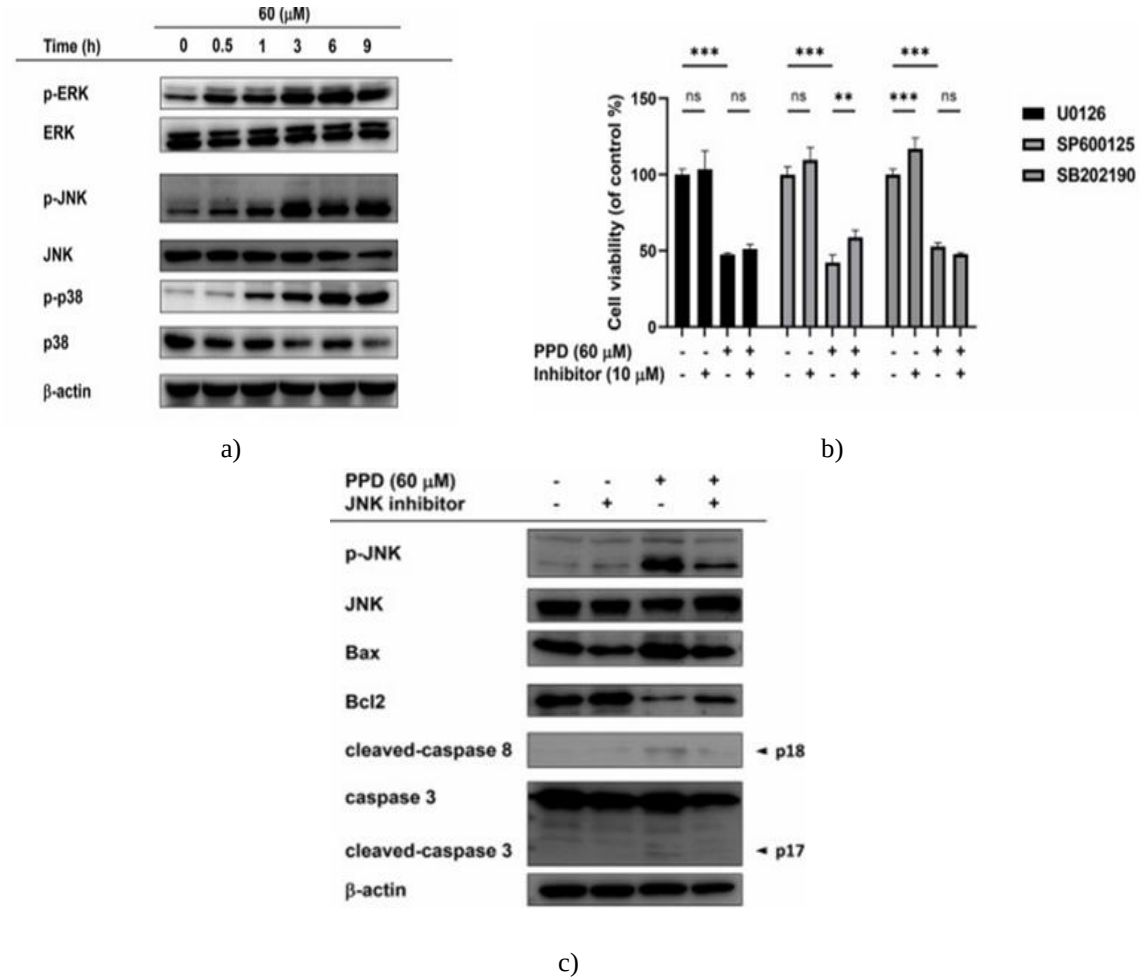


Figure 4. PPD promotes apoptosis in melanoma cells by engaging the JNK pathway. (a) SK-MEL-28 cells were exposed to 60 μ M PPD for varying time periods, after which the phosphorylation status of MAPK proteins was analyzed by Western blot. (b) SK-MEL-28 cells were first incubated with 10 μ M of specific MAPK pathway inhibitors for 1 h, followed by treatment with 60 μ M PPD for 24 h. Cell viability was then measured using MTT assays. (c) SK-MEL-28 cells were pretreated with 10 μ M SP600125 for 1 h and subsequently exposed to 60 μ M PPD for 12 h. Expression levels of apoptotic markers and JNK-related proteins were assessed by Western blotting.

PPD elicits apoptosis by directly engaging MLK3

To determine which upstream kinase was responsible for JNK activation, several key components of the JNK signaling module were evaluated. Western blot results revealed that PPD rapidly stimulated MLK3 phosphorylation within 30 minutes, while leaving the activation status of ASK1 and TAK1 unchanged (**Figure 5a**). This selective effect implies that PPD stimulates the JNK cascade specifically through MLK3. In addition, PPD enhanced JNK pathway activity in a concentration-dependent manner (**Figure 5b**).

To rule out involvement of reactive oxygen species (ROS), cells were pretreated with the antioxidant N-acetyl-L-cysteine (NAC) prior to PPD exposure. NAC pretreatment failed to mitigate PPD's impact on cell viability (**Figure 5c**), demonstrating that the pro-apoptotic action of PPD occurs independently of ROS and is instead mediated through direct activation of MLK3.

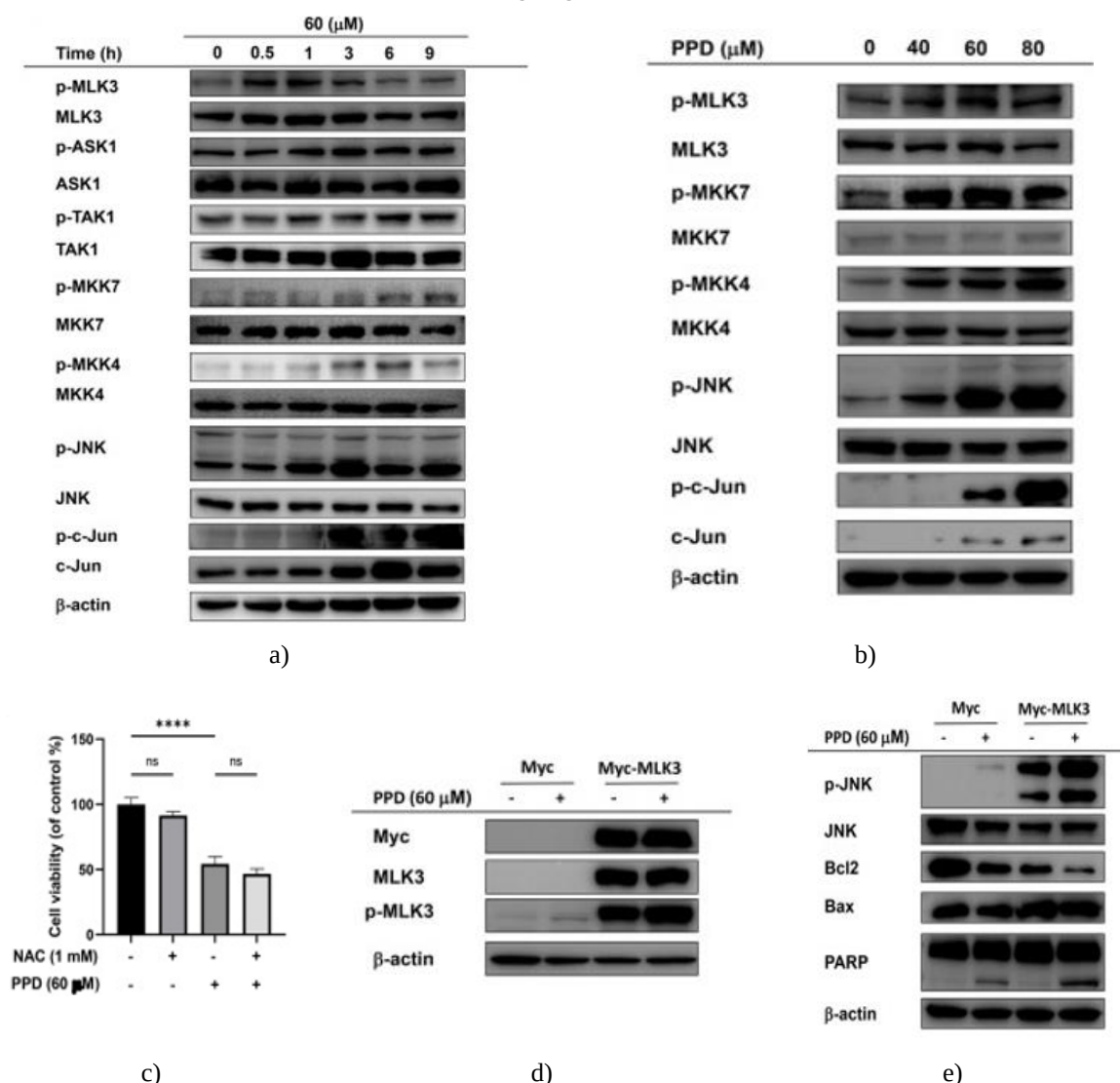


Figure 5. PPD triggered apoptosis in melanoma cells by activating the JNK signaling cascade through its action on MLK3. (a–b) SK-MEL-28 cells received 60 μ M PPD for varying durations or were exposed to different concentrations of PPD for a set period. Western blotting was used to examine the main components of the JNK pathway. (c) SK-MEL-28 cells were first incubated with 1 mM NAC for 1 hour and subsequently exposed to 60 μ M PPD for 24 hours. Cell survival rates were measured via MTT assays. (d) HEK293T cells overexpressing exogenous MLK3 were treated with 60 μ M PPD for 30 minutes. Levels of MLK3, phosphorylated MLK3, Myc, and β -actin were assessed by western blot. (e) SK-MEL-28 cells with overexpressed exogenous MLK3 were treated with 60 μ M PPD for 30 minutes. Expression of JNK, phosphorylated JNK, and apoptosis-related proteins was analyzed by western blot.

To verify that MLK3 serves as a direct target of PPD, researchers introduced overexpressed MLK3 into HEK293T cells. Western blot results showed that PPD increased phosphorylation of both the native and introduced forms of MLK3 (**Figure 5d**), pointing to direct interaction and activation of MLK3 by PPD. In SK-MEL-28 cells engineered to overexpress MLK3, PPD treatment led to stronger JNK pathway activation and greater apoptotic response (**Figure 5e**). These observations reinforce the idea that MLK3 stimulation plays a central role in PPD-driven cell death.

PPD directly interacts with MLK3 to stimulate JNK signaling

To establish whether PPD physically binds to MLK3, cellular thermal shift assays (CETSA) were conducted. PPD exposure markedly enhanced the thermal stability of MLK3 (**Figure 6c**), providing evidence of direct binding. Computational molecular docking simulations predicted an interaction between PPD and MLK3 with a binding affinity of $pK_i = 6.22$ (**Figure 6a**), reflecting a moderate strength of association. The modeling

highlighted seven key amino acid residues potentially involved in binding: ILE123, ALA142, TYR195, ALA196, PRO200, ASN245, and LEU248 (**Figure 6b**). Multiple sequence alignment of MLK proteins from various species demonstrated that four of these sites (ILE123, ALA142, ALA196, and LEU248) are highly conserved (**Figure 6c**). To test the importance of these sites, site-directed mutagenesis was performed on the four conserved residues. The resulting mutant MLK3 constructs were introduced into HEK293T cells, after which CETSA evaluated the thermal stability of the altered proteins in response to PPD. Results indicated that the LEU248 mutation abolished the PPD-induced stabilization of MLK3 (**Figure 6b**), demonstrating that PPD loses its ability to bind MLK3 when this residue is altered. Quantitative analysis of band intensities from **Figure 6d** is presented.

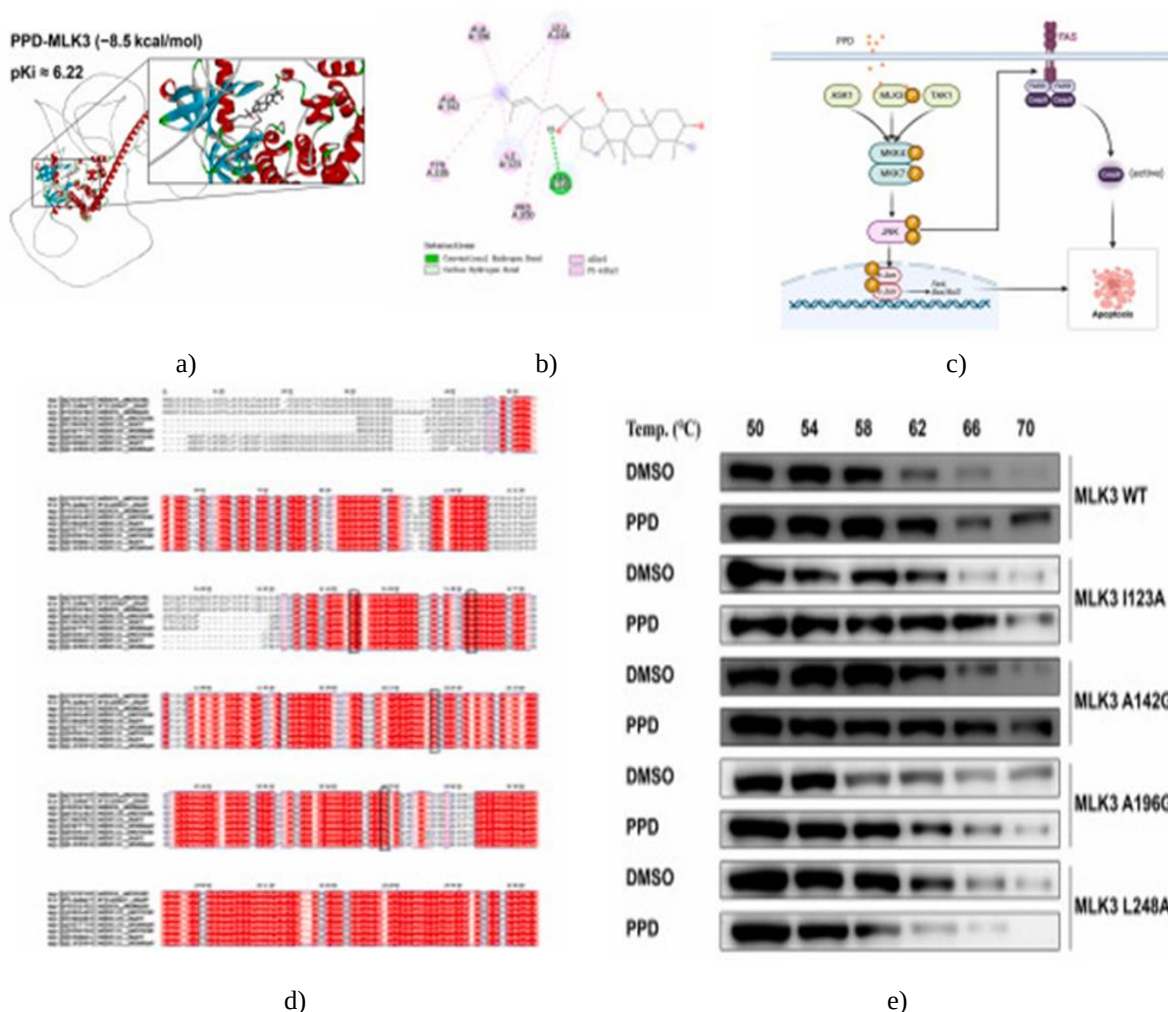


Figure 6. PPD binds to MLK3, leading to its activation. (a–b) Molecular docking was employed to model the binding conformation and identify potential interaction sites on MLK3. (c) Protein sequences of MLK family members from multiple species were aligned using ESPrpt 3.0. (d) Cellular thermal shift assays (CETSA) were carried out on wild-type MLK3 and its mutants in the presence or absence of PPD, with temperatures ranging from 50 °C to 70 °C. (e) Schematic overview illustrating the anti-melanoma effects of PPD.

Melanoma accounts for the majority of skin cancer fatalities because of its strong tendency to metastasize. Existing therapies encompass surgical removal, immunotherapy, targeted agents, and chemotherapy; however, outcomes remain unsatisfactory for individuals with advanced or metastatic disease owing to issues like drug resistance and disease relapse [2]. PPD, an active aglycone produced through the hydrolysis of ginsenosides, has demonstrated multiple pharmacological properties such as anti-inflammatory [17], neuroprotective [18], and antioxidant activities [19]. In particular, recent studies have emphasized its promising anti-tumor capabilities, including suppression of cell growth, promotion of programmed cell death, and inhibition of cancer spread in different malignancies [20]. Nevertheless, its specific influence on melanoma remains incompletely understood. The present investigation demonstrates that PPD suppresses melanoma cell growth and promotes apoptosis by

directly interacting with MLK3, thereby stimulating the JNK signaling route. This work provides new insights into PPD's anti-cancer actions and supports its potential application as a therapeutic option for melanoma. Apoptosis represents a precisely controlled process crucial for eliminating injured or superfluous cells and preserving tissue balance. It mainly operates through two routes: the intrinsic pathway, activated by internal stressors or genomic damage that causes mitochondrial impairment and caspase-9 engagement; and the extrinsic pathway, started by death receptor ligation (such as FasL or TNF) followed by caspase-8 activation. These routes ultimately merge to activate executioner caspases that carry out cell demise [21]. Abnormal control of apoptotic mechanisms is a defining feature of cancer, enabling tumor cells to avoid death and maintain persistent expansion [22]. Consequently, approaches designed to reactivate apoptosis have emerged as a major strategy in oncology. Numerous anti-cancer agents exert their influence by stimulating apoptosis via diverse pathways, exemplified by compounds like physapubenolide [23], butyrate [24], curcumin [25], and pimozide [26]. In this study, network pharmacology combined with GO enrichment analysis forecasted that PPD modulates programmed cell death and apoptosis processes in melanoma cells (**Figure 2c**). Supporting this, flow cytometry experiments revealed that PPD markedly promotes apoptosis in SK-MEL-28 cells in a concentration-dependent fashion (**Figure 3a**). Additionally, levels of apoptosis-associated proteins rose in a dose-dependent manner following PPD exposure, reaching maximum activation at 12 hours (**Figure 3a**). Importantly, caspase-8 activation preceded that of caspase-9 and caspase-3, suggesting that PPD primarily drives apoptosis via the extrinsic route. KEGG pathway enrichment further highlighted several relevant cascades, with the most prominent being pathways in cancer, Ras signaling, and MAPK signaling (**Figure 2d**).

The MAPK cascade is a pivotal regulator of essential cellular activities including growth, differentiation, viability, and regulated cell death. It includes three primary arms—ERK, p38 MAPK, and JNK—each responding to unique triggers and producing distinct biological effects [27]. Of these, JNK stands out for its strong involvement in apoptosis control. Upon stimulation, JNK phosphorylates c-Jun to upregulate pro-apoptotic genes such as Bax and Bim, modulates Bcl-2 family members through phosphorylation, and initiates the caspase cascade [28]. JNK can also directly contribute to the extrinsic pathway by affecting cell surface receptors like Fas and TNF receptors, thereby triggering caspase-8 and subsequent apoptotic events [29]. Our bioinformatics analysis suggested that PPD might elicit apoptosis in SK-MEL-28 cells via the MAPK pathway. Experimental validation involved assessing phosphorylation levels of major MAPKs (ERK, JNK, and p38). PPD notably stimulated MAPK phosphorylation (**Figure 4a**), with particularly robust JNK activation. In contrast, inhibitors targeting ERK or p38 did not block PPD's effects (**Figure 4b**). These findings establish JNK as the dominant MAPK route responsible for PPD-mediated apoptosis in these cells. Further investigation pinpointed MLK3 as the key upstream kinase for JNK under PPD treatment. As a MAP3K family member, MLK3 functions as an important activator of the JNK pathway. When stimulated, MLK3 phosphorylates MKK4/7, which subsequently activates JNK and promotes apoptosis [30]. Our data showed that PPD swiftly activates MLK3 while leaving other MAP3Ks such as ASK1 and TAK1 unaffected (**Figure 5a**). Experiments using ROS scavengers confirmed that PPD-triggered apoptosis does not rely on oxidative stress, reinforcing the direct targeting of MLK3 by PPD to initiate JNK signaling (**Figure 5c**). Overexpression of MLK3 in SK-MEL-28 cells amplified both JNK activation and apoptotic responses to PPD, underscoring MLK3's essential contribution (**Figure 5e**). Direct interaction between PPD and MLK3 was verified through CETSA, which demonstrated increased thermal stability of MLK3 upon PPD exposure (**Figure 6c**). Complementary molecular docking analyses illustrated the binding orientation and identified seven candidate contact residues on MLK3, four of which exhibit high conservation across species (**Figure 6**). Mutagenesis experiments targeting these residues established that PPD interacts with MLK3 specifically at LEU248 (**Figure 6c**).

Conclusion

Our study positions PPD as a promising candidate for melanoma therapy. However, its pharmacokinetic profile indicates limited oral bioavailability in humans, primarily resulting from inadequate absorption, highlighting the importance of developing improved formulations to boost clinical efficacy [31, 32]. In summary, the results indicate that PPD promotes apoptosis in melanoma cells via direct binding to and activation of MLK3, which in turn stimulates the JNK pathway, as depicted in **Figure 6e**. These discoveries broaden knowledge of PPD's anti-tumor properties and identify MLK3 as a viable molecular target for melanoma treatment.

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