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Phellopterin drives apoptosis in A549 lung cancer cells via Caspase-3 activation and bcl2 suppression while activating NRF2/NF-κB cytoprotective pathways in BEAS-2B normal cells

Yinyu Li[1], Hao Xu[2], Weiru Xiao[3] & Xiaoyu Chen[4]*

[1]Department of Cardiothoracic Surgery, Minda Hospital of Hubei Minzu University, Enshi Hubei, 445 000, China
[2]Department of Oncology, Ezhou Central Hospital, Ezhou Hubei, 436 000, China
[3]Department of Medical Oncology, Chifeng Tumor Hospital, Chifeng, Inner Mongolia, 024 000, China
[4]Department of Respiratory Medicine, Yiwu Second People's Hospital Yiwu, Zhejiang, 322 000, China

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Phellopterin, a naturally occurring furanocoumarin, has attracted interest as a potential anticancer compound because it can differentially modulate redox and apoptotic signaling pathways. This study investigated the tumor-selective effects of phellopterin in human lung adenocarcinoma A549 cells compared with normal human bronchial epithelial BEAS-2B cells using a focused mechanistic approach. Cells were treated with 25 µM phellopterin for 24 h to reflect early apoptosis and stress signaling while minimizing cytotoxic effects. Control cells received the vehicle only. Pathway specificity was determined using the caspase-3 inhibitor Z-DEVD-FMK (20 µM) in A549 cells and the NRF2 inhibitor ML385 (5 µM) in BEAS-2B cells for 24 h. BCL2, NRF2, and HO-1 expression levels were measured by reverse transcription-polymerase chain reaction (RT-PCR), while NF-κB P65, cleaved caspase-3, interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-alpha (TNF-α) levels were measured by enzyme-linked immunosorbent assay (ELISA).In A549 cells, phellopterin induced cleaved caspase-3 and decreased BCL2 mRNA expression, activating caspase-dependent apoptosis, which corresponded with reduced TNF-α and IL-6. In BEAS-2B cells, phellopterin did not activate caspase-3 or inhibit BCL2 but enhanced NRF2 activation and increased HO-1 expression. Moderate NF-κB P65 inhibition was observed in BEAS-2B cells, correlating with decreased IL-8. NRF2 inhibition blocked HO-1 upregulation, confirming NRF2-mediated cytoprotective signaling.Phellopterin exerts tumor-selective activity by inducing apoptosis and inflammatory modulation in lung cancer cells while activating antioxidant and anti-inflammatory pathways in normal bronchial epithelial cells, highlighting its potential as a mechanistically targeted therapeutic candidate for lung cancer.

Keywords: Apoptosis, Immunomodulation, Lung cancer

Lung cancer remains one of the most prevalent and deadly cancers across the globe. Even with advancements in detection and treatment, the outlook for those diagnosed is still far too often discouraging[1]. Non-small cell lung carcinoma (NSCLC) is currently the most common type of lung cancer, accounting for 85-90% of lung cancer cases. There is still insufficient evidence to recommend a single treatment for patients diagnosed with NSCLC. There has been immense progress made towards the use of molecular targeted therapy and immune checkpoint inhibitors to treat patients with NSCLC. Still, the number of patients diagnosed with advanced cases (metastatic), the associated resistance to treatments, and the toxicity to surrounding normal tissues from the drugs being used continue to impede long-term survival rates[2]. Consequently, there is an urgent need for new agents that can selectively kill cancer cells while preserving the function of normal lung epithelium.

Apoptosis is a highly orchestrated form of programmed cell death that removes damaged or abnormal cells. Dysregulation of apoptotic signaling is a hallmark of cancer and contributes to tumor progression and therapeutic resistance. B-cell leukemia/lymphoma 2 protein (BCL2) is a negative regulator of intrinsic apoptosis, and the anti-apoptotic protein BCL2 is most commonly overexpressed in NSCLC and associated with poor prognosis and treatment resistance[2,3].

In addition to apoptosis, redox signaling also plays a critical role in regulating the survival of cancerous cells. Cancerous cells tend to have higher baseline levels of reactive oxygen species (ROS) compared to

*Correspondence:
Phone: +86 15958917818
E-mail: cccxy789@outlook.com

normal cells because of their altered metabolism and oncogene activation, which ultimately makes them more susceptible to additional oxidative stress than normal cells[4]. Normal cells sustain redox homeostasis by strong antioxidant defense systems. Cellular redox homeostasis is controlled at least in part by nuclear factor erythroid 2-related factor 2 (NRF2), a transcription factor that regulates the expression of antioxidant and cytoprotective genes, such as heme oxygenase-1 (HO-1)[5]. However, sustained NRF2 activity can support survival and therapy resistance in cancer cells, whereas transient activation in normal tissues is predominantly considered as protective during oxidative stress and inflammation[6].

The nuclear factor-kappa B pathway (NF-κB), a redox-sensitive transcription factor, is involved in inflammation, stress responses, and cell survival. Constitutive activation of NF-κB has been associated with tumor development and resistance to apoptosis, while its tightly regulated activation in normal tissues is involved in tissue homeostasis. The interplay between NRF2 and NF-κB signaling may therefore influence the therapeutic selectivity of anticancer agents[7,8].

Historically, natural products have been an excellent source of anticancer therapeutics due to their wide range of chemical structure and their ability to target numerous cellular signaling pathways. Several types of furanocoumarins, including psoralen, bergapten, and imperatorin, have been shown to have the ability to inhibit proliferation and induce apoptosis in models of cancer via modulating oxidative stress and mitochondrial signaling [9,10]. Phellopterin is a compound found in Angelica dahurica, a functional food and herb, and is widely regarded for its therapeutic effects. Phellopterin has a long history of use as an antispasmodic and anti-inflammatory agent; moreover, studies have shown that it may have beneficial effects on the nervous system in animal studies[11].

Accumulating evidence shows that phellopterin likely has anticancer effects through multiple tumor types, by influencing cell cycle control, causing apoptosis, and promoting survival in cancer cells through disrupting important biochemical pathways. Recent evidence suggests that phellopterin exerts anticancer activity in ovarian cancer models. By causing DNA damage, halting the growth of cancer cell lines, and promoting apoptosis, it appears to reduce both the proliferation of cancer cells and the response to chemotherapy. Furthermore, it seems to interfere with the positive feedback loop of PU.1/CLEC5A/PI3K/AKT to help reduce tumor cell growth and treatment resistance. Therefore, because it inhibits PI3K/AKT signaling and reduces the production of CLEC5A, phellopterin could potentially be an effective treatment with predictive capability [12]. Phellopterin has also been shown to have an anti-cancer effect by regulating inflammation and promoting a non-tumor supportive environment through regulating immune function. In a study of a colon cancer model of colitis, the administration of Phellopterin decreased both the rate and the severity of tumor formation in accordance with a defined dose response. Decreased levels of pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α), increased levels of anti-inflammatory IL-10, and inhibition of TLR4/NF-κB signaling were associated with the effects of Phellopterin on reducing the incidence of tumors. In addition to its role in modulating pro-inflammatory cytokines, Phellopterin inhibited the activation of M2 Macrophage polarized cells. Both of these effects provide evidence of the potential of Phellopterin to inhibit the progression of colon tumors due to inflammation through immunomodulatory mechanisms[13].

The purpose of this study was to find out how phellopterin affects the mechanism underlying apoptosis, inflammation, and redox signaling in A549 lung cancer cells (a well-established *in vitro* model of NSCLC), as compared to BEAS-2B normal bronchial epithelial cells.

Materials and Methods

Cell culture and treatment

Human lung adenocarcinoma A549 cells (obtained from ATCC® CCL-185™) and immortalized normal human bronchial epithelial BEAS-2B cells (obtained from ATCC® CRL-9609™) were cultured at 37°C in humidified 5% CO_2 conditions using RPMI-1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Phellopterin (Sigma-Aldrich) was diluted in dimethyl sulfoxide (DMSO) to prepare a 10 mM stock solution. Cells were treated with an optimized concentration of 25 μM phellopterin for 24 h during the experiments due to the findings of preliminary viability assays, which indicated it would provide an accurate representation of early mechanistic signaling while also decreasing the amount of unrelated cytotoxicity. Vehicle control was treated with an equal volume of DMSO

(final concentration ≤0.25%). To determine the role of caspase-3 and NRF2 pathways, separate groups of A549 cells were pre-treated for 1 h with Z-DEVD-FMK (Sellekchem, final concentration 20 μM), while the BEAS-2B cells were pre-treated for 1 h with ML385 (Sellekchem, final concentration 5 μM), followed by co-treatment for 24 h with phellopterin.

Enzyme-Linked Immunosorbent Assay (ELISA)

A quantitative approach was taken to determine the levels of the secreted cytokines interleukin-6 (IL-6), interleukin-8 (IL-8) (R&D Systems, USA), tumor necrosis factor-α (TNF-α) (R&D Systems, USA), and NRF2 (Abcam, USA) present in the supernatants collected from cell cultures, as well as phospho-NF-κB p65 (R&D Systems, USA), cleaved Caspase-3 (R&D Systems, USA), BCL2 (R&D Systems, USA), and HO-1 (R&D Systems, USA) present in cell lysate, using commercially available ELISA Kits in accordance with the protocols outlined in the manufacturer's handbook. Cell lysates were prepared using IP lysis buffer containing protease and phosphatase inhibitors.

Quantitative real-time PCR (RT-qPCR) analysis of gene expression

TRIzol reagent (Invitrogen) was used to extract total RNA from control and treated cells according to the manufacturer's directions. Total RNA was assessed for purity and concentration via spectrophotometry, measuring absorbance at 260 nm to 280 nm. 1 μg of total RNA was used to synthesize complementary DNA (cDNA) with a reverse transcriptase kit (Applied Biosystems). Quantitative real-time PCR (RT-qPCR) was performed using SYBR Green Master Mix (Applied Biosystems) on the StepOnePlus System (Applied Biosystems). Premade gene-specific primers were obtained to evaluate levels of mRNA expression for genes that encode BCL2, NRF2, HO-1, and all other target mRNAs, and GAPDH served as the internal housekeeping mRNA control. The thermal cycling temperature profile included an initial denaturation period, followed by 40 cycles of denaturation, annealing, and extension for each PCR. Quantification of relative gene expression levels was calculated utilizing the formula, $2^{-\Delta\Delta Ct}$, and reported as fold-change compared to vehicle control. All reactions were done in triplicate, and all experiments were repeated a minimum of three times.

Statistical analysis

All experiments were carried out in five independent biological replicates (n = 5). Results are expressed as mean ± standard deviation (SD). Statistical analyses between groups were conducted using one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test. The significance of differences was defined as $P< 0.05$.

Results

Optimization of Phellopterin concentration and treatment duration

Before mechanistic studies, we assessed the cytotoxicity of phellopterin in A549 lung cancer cells and BEAS-2B normal bronchial epithelial cells by dose response and time response viability assays. Phellopterin decreased the cell viability in both cell lines in the time- and concentration-dependent manner, however, sensitivity of BEAS-2B cells to phellopterin was substantially lower than that of A549 cells. In A549 cells, the viability was reduced from 100% in untreated cells to 76%, 58% and 39% after exposure to 25 μM phellopterin for 24, 48 and 72 h, respectively. At the higher concentrations more pronounced effects were found, with the viability estimated at 55%, 34% and 18%, at 50 μM and 28%, 12% and 5%, at 100 μM after 24, 48 and 72 h, respectively. On the other hand, BEAS-2B cells showed lower sensitivity to the cytotoxicity of phellopterin, with 25 μM phellopterin maintaining 91%, 82%, and 70% of their viability after 24, 48, and 72 h, respectively. At the maximum dose of 100 μM, BEAS-2B survival was 53%, 39%, and 28% after 24, 48, and 72 h incubation, respectively. These results indicated that A549 cells are more sensitive to phellopterin than human bronchial epithelial cells. The condition of treatment with 25 μM phellopterin for 24 h was selected for further studies for elucidation of mechanisms, because this treatment caused a modest decrease in A549 cell viability with minimal effect on BEAS-2B cells, allowing us to evaluate early apoptotic and cytoprotective signaling events, as well as secondary cytotoxic effects.

Cytokine secretion profile demonstrates differential inflammatory modulation in A549 and BEAS-2B cells

ELISA analysis of cytokine secretion showed that the response of cell types to phellopterin differed by cell type. The A549 lung cancer cell type responded

with a significant decrease in the secretion of the proinflammatory cytokines TNF-α and IL-6, as well as IL-8, when phellopterin (25 μM, 24 h) treatment was compared with vehicle controls (compared to control; p= 0.0057, p= 0.0003, p= 0.0008, respectively). When caspase-3 was inhibited using Z-DEVD-FMK (20 μM), the phellopterin-induced suppression of TNF-α, IL-6, and IL-8 was not reversed to control (compared to control; p= 0.0136, p= 0.0056, 0.0011, respectively) (Fig. 1a).

In contrast, treatment of BEAS-2B (normal bronchial epithelial) cells with phellopterin leads to a moderate decrease in the release of pro-inflammatory cytokine, TNF-α and IL-6, but rather reduces IL-8 secretion (compared to control; p= 0.0223, p= 0.0255, p= 0.0208, respectively). Furthermore, pretreatment of cells with the NRF2 inhibitor ML385 (5 μM) also partially reversed the phellopterin-mediated suppression of TNF-α, IL-6, and IL-8 to control, suggesting a link between NRF2 pathway activation and the modulation of inflammatory signaling in normal epithelial cells (Fig. 1b).

These results indicate that phellopterin regulates cytokine secretion in a dependent on cell type. In both cell types, treatment reduced the secretion of inflammatory cytokines, although the underlying

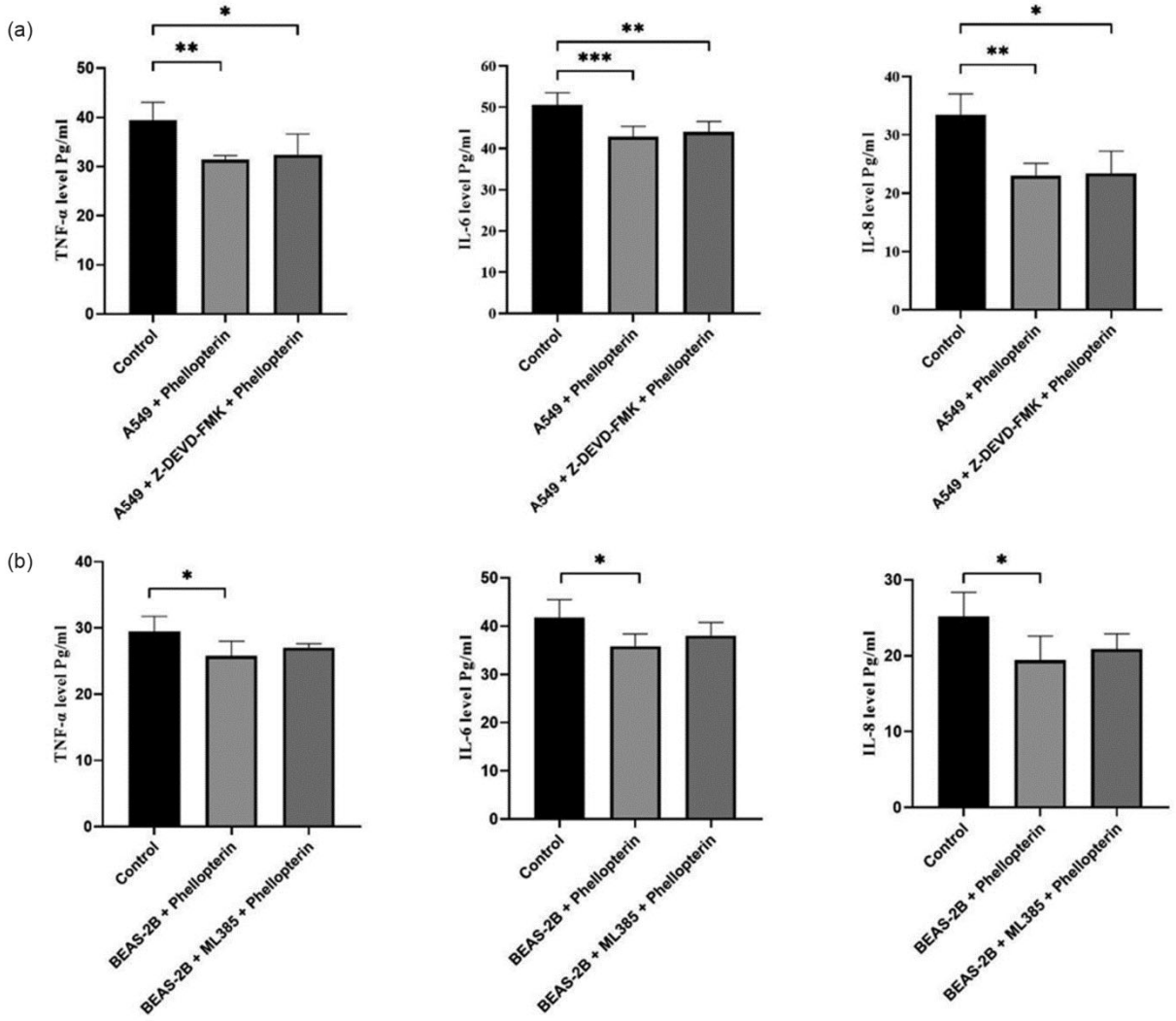


Fig. 1 — Phellopterin differentially modulates cytokine secretion in A549 lung cancer cells and BEAS-2B normal bronchial epithelial cells. (a) In A549 cells, phellopterin treatment (25 μM, 24 h) significantly decreases secretion of TNF-α, IL-6, and IL-8, which was not reversed by caspase-3 inhibition with Z-DEVD-FMK (20 μM); and (b) In BEAS-2B cells, phellopterin reduces TNF-α, IL-6, and IL-8 secretion, the effect partially reversed by NRF2 inhibition with ML385 (5 μM). Data are presented as mean ± SD from five independent biological experiments (n = 5). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. **P*< 0.05, ***P*< 0.01, ****P*< 0.001

mechanisms appeared distinct. In A549 cells, cytokine suppression occurred alongside activation of apoptotic signaling, whereas in BEAS-2B cells, it was associated with NRF2 activation and reduced NF-κB activity.

Phellopterin induces Caspase-3 activation selectively in A549 lung cancer cells

Active, cleaved caspase-3 levels were measured via ELISA to evaluate the induction of apoptosis. The treatment with phellopterin (25 μM, 24 h) caused a specific activation of caspase-3, depending on the type of cell.

The administration of phellopterin to A549 lung cancer cells produced a markedly elevated level of cleaved caspase-3 compared to control (vehicle) treated cells ($P< 0.001$). Pre-treatment of A549 cells with the specific caspase-3 inhibitor Z-DEVD-FMK (20 μM) abolished this substantial increase (Fig. 2a). This illustrates that the effects observed were specific and confirms that phellopterin has its pro-apoptotic effect through activation of caspase-3 in this particular cancer cell line.

In stark contrast, phellopterin treatment of normal BEAS-2B human bronchial epithelial cells did not show a significant change in the levels of cleaved caspase-3 compared to the untreated control. The cleaved caspase-3 levels were still considered to be at baseline, indicating that caspase-3-mediated apoptotic activation is not present in normal cells when treated with phellopterin (Fig. 2b).

Thus, the results indicate that phellopterin specifically activates the effector phase of apoptosis through caspase-3 activation in malignant A549 cells, but not through this pathway in normal BEAS-2B cells.

Gene expression analysis via RT-qPCR reveals differential regulation of apoptotic and cytoprotective pathways

Gene expression changes in response to phellopterin treatment were quantified using RT-qPCR to elucidate the transcriptional mechanisms underlying its selective effects.

In A549 lung cancer cells, phellopterin treatment (25 μM, 24 h) altered the expression of several major regulators of apoptosis. Specifically, phellopterin

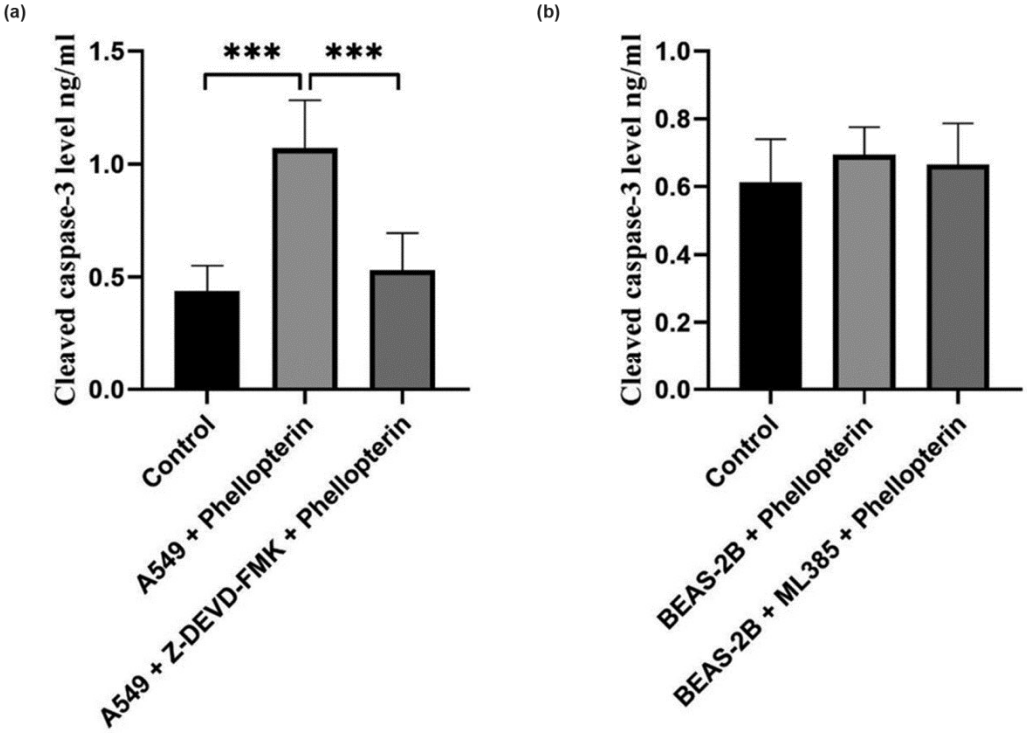


Fig. 2 — Phellopterin selectively activates caspase-3-dependent apoptosis in A549 cells but not in BEAS-2B cells. (a) Cleaved caspase-3 levels are significantly increased in A549 cells after phellopterin treatment, an effect abolished by Z-DEVD-FMK; and (b) No significant change in cleaved caspase-3 is observed in BEAS-2B cells treated with phellopterin. Data are presented as mean ± SD from five independent biological experiments (n = 5). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. *$P< 0.05$, **$P< 0.01$, ***$P< 0.001$

caused a significant reduction in expression of the anti-apoptotic factor BCL2 (p =0.0206). In contrast to this apparent distinct response that can likely be attributed to phellopterin treatment, there is the absence of significant alteration of NRF2 and its target (HO-1) at the level of mRNA under phellopterin treatment conditions in A549 cells. Furthermore, Z-DEVD-FMK, the caspase-3 inhibitor, did not reverse the ability of phellopterin to decrease expression of BCL2, NRF2, and HO-1. This indicates that downregulations occur before or independently of the cascade leading to caspase-3 activation (Fig. 3a).

In BEAS-2B normal bronchial epithelial cells, phellopterin showed a completely different transcriptional response than with no change in the BCL2 expression level of mRNA. Instead, phellopterin treatment induced a marked increase in the cytoprotective pathway. NRF2 levels were elevated when treated with phellopterin (p = 0.0002), along with an increase in the canonical downstream antioxidant gene HO-1 (p = 0.0022). This coordinated increase in both NRF2 and HO-1 expression was shown to be completely inhibited by the NRF2 inhibitor ML385 (5 μM) treatment before phellopterin, returning HO-1 expression levels to close to baseline, confirming the NRF2-dependence of this transcriptional response (Fig. 3b).

Together, the results of RT-qPCR show that phellopterin's effect on gene expression varies depending on the type of cell it is acting upon; in cancer cells, it inhibits BCL2, the gene responsible for preventing apoptosis, while in healthy cells, it activates the NRF2/HO-1 antioxidant system. This provides evidence at the molecular level of how phellopterin can preferentially target tumors.

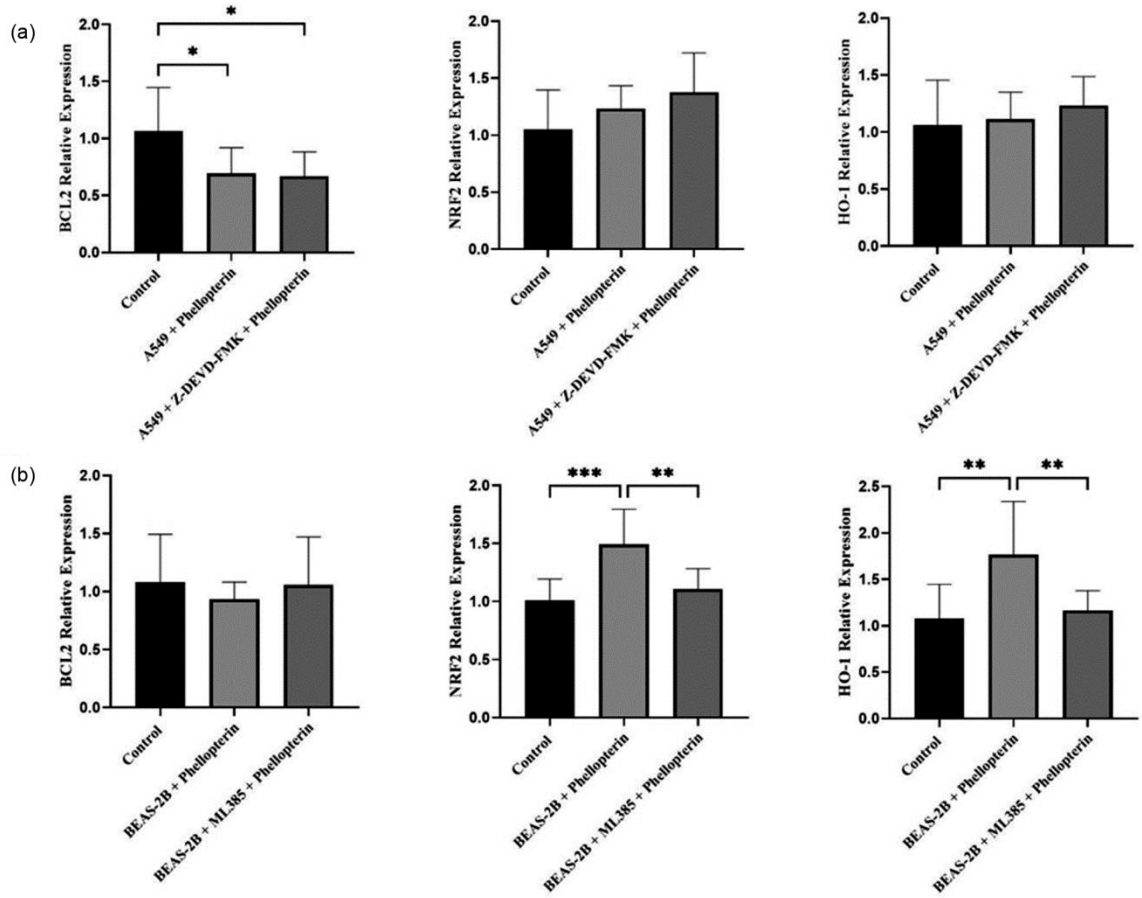


Fig. 3 — Gene expression analysis reveals cell-type-specific regulation of apoptotic and cytoprotective pathways by phellopterin. (a) In A549 cells, phellopterin downregulates BCL2 mRNA expression without affecting NRF2 or HO-1. Caspase-3 inhibition does not reverse these suppressions; and (b) In BEAS-2B cells, phellopterin upregulates NRF2 and its downstream target HO-1, effects blocked by NRF2 inhibition with ML385. Data are presented as mean ± SD from five independent biological experiments (n = 5). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. **P*< 0.05, ***P*< 0.01, ****P*< 0.001

Protein expression analysis confirms differential regulation of apoptotic and cytoprotective pathways by phellopterin

In A549 lung cancer cells, the BCL2 protein level was decreased by the phellopterin treatment (25 μM, 24 h) as compared to the vehicle-treated control group ($p = 0.0473$), in line with the reduction of BCL2 mRNA level. Pretreatment with the caspase-3 inhibitor Z-DEVD-FMK also failed to restore BCL2 protein expression, implying BCL2 down-regulation takes place either upstream or independent of caspase-3 activation. However, there were no significant alterations in the expression of NRF2 or HO-1 protein after phellopterin treatment, further supporting the notion that induction of the NRF2/HO-1 antioxidant response is not a major event in A549 cells (Fig. 4a).

In BEAS-2B normal bronchial epithelial cells, there was no significant effect of phellopterin on the BCL2 protein expression. However, NRF2 protein levels were significantly increased compared with controls ($p = 0.0242$), accompanied by a marked elevation in HO-1 protein expression ($p = 0.0080$). Pretreatment with NRF2 inhibitor ML385 potently inhibited the expression of NRF2 and HO-1, and recovered protein expression levels to control ones (Fig. 4b). These data demonstrate that phellopterin induces NRF2-mediated cyto-protective signaling cascade in normal bronchial epithelial cells.

Overall, protein expression levels were consistent

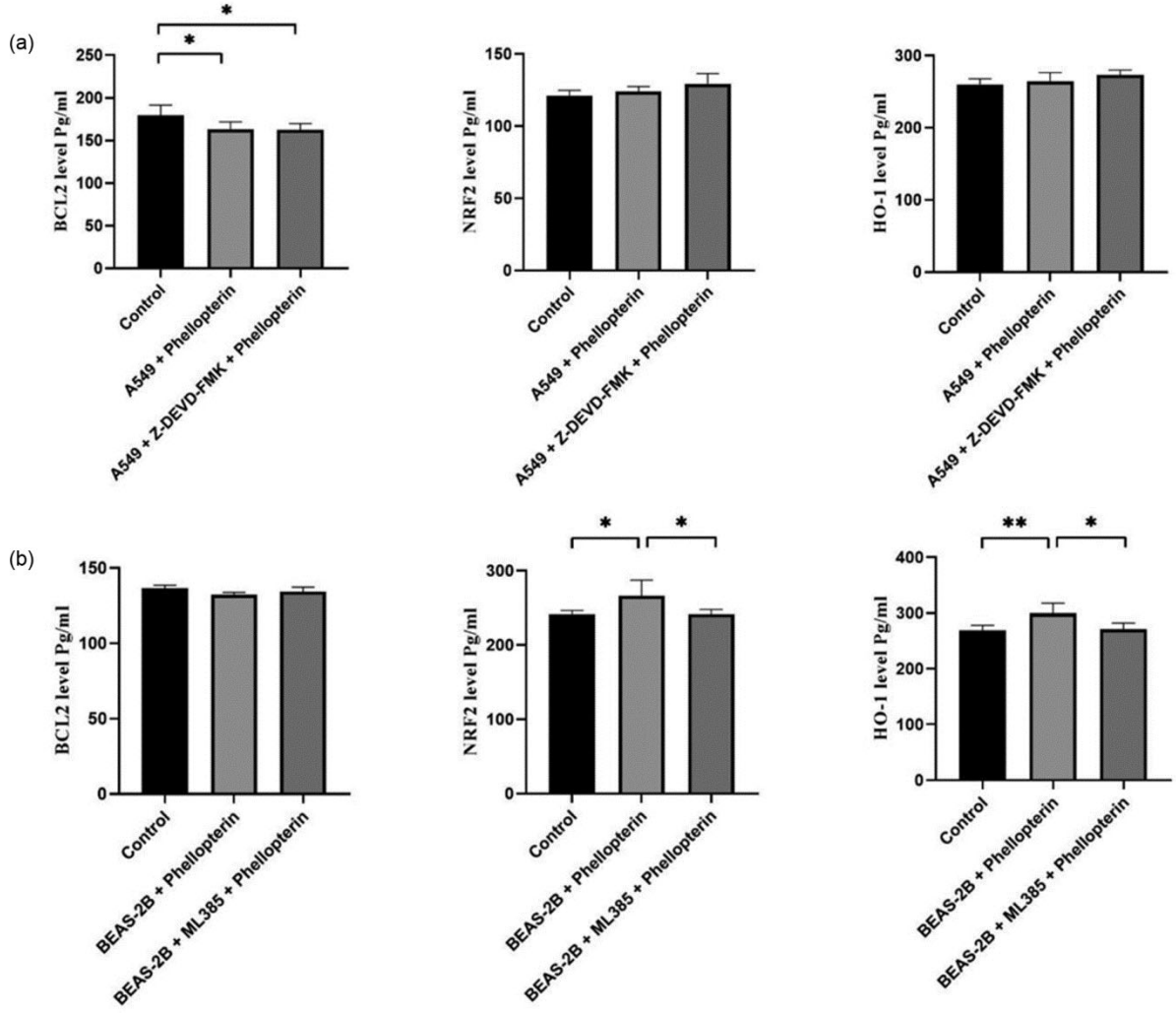


Fig. 4 — Protein expression analysis confirms differential regulation of apoptotic and cytoprotective pathways by phellopterin in A549 and BEAS-2B cells. (a) In A549 cells, phellopterin treatment significantly decreases BCL2 protein expression without significantly affecting NRF2 or HO-1 levels. Pretreatment with the caspase-3 inhibitor Z-DEVD-FMK does not restore BCL2 expression; (b) In BEAS-2B cells, phellopterin treatment significantly increases NRF2 and HO-1 protein expression without altering BCL2 levels. Data are presented as mean ± SD from five independent biological experiments (n = 5). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. *$P < 0.05$, **$P < 0.01$

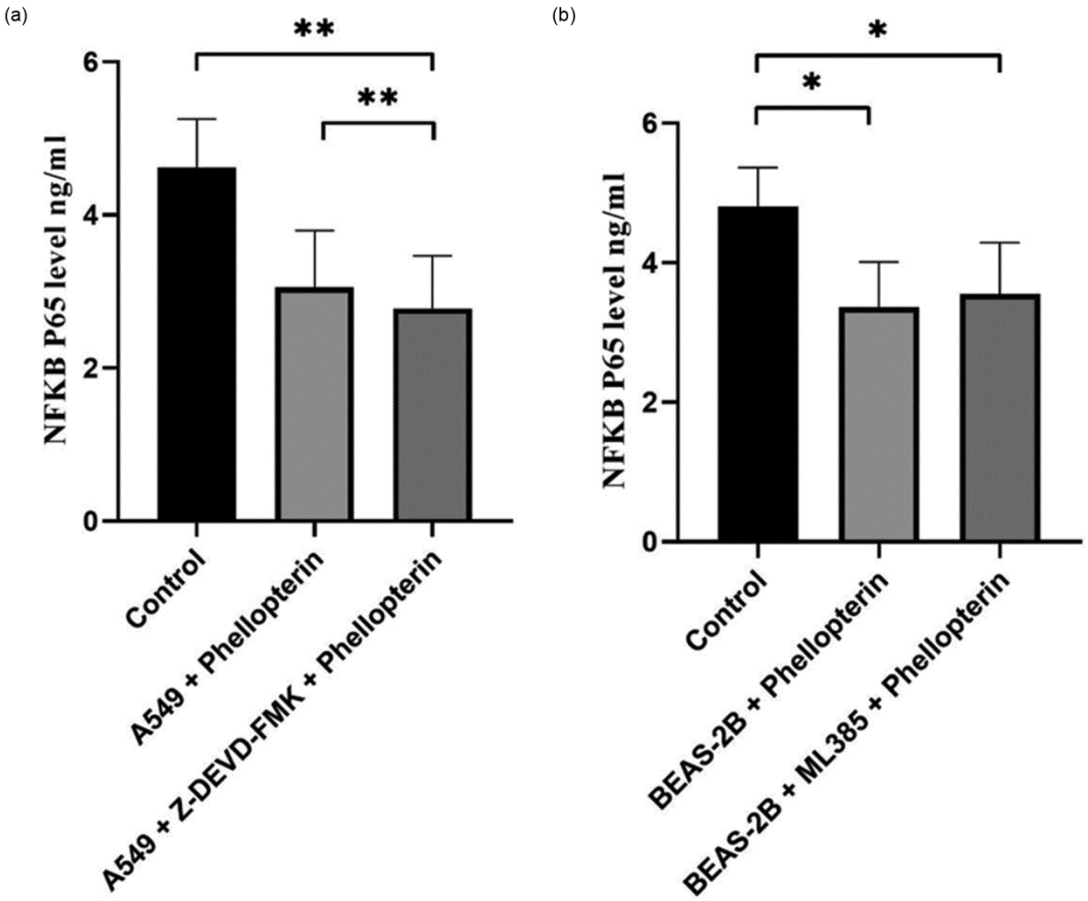


Fig 5 — Phellopterin differentially modulates NF-κB activation in A549 and BEAS-2B cells. (A) In A549 cells, phellopterin treatment significantly reduces phosphorylated NF-κB p65 levels, indicating inhibition of NF-κB activity. (B) In BEAS-2B cells, phellopterin also moderately decreases phospho-p65 levels, consistent with its cytoprotective and anti-inflammatory effects. Inhibition of caspase-3 or NRF2 does not significantly reverse the changes in phospho-p65 levels in either cell type. Data are presented as mean ± SD from five independent biological experiments (n = 5). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. *P< 0.05, **P< 0.01.

with the results of RT-qPCR and further indicated that phellopterin inhibited the anti-apoptotic signal in A549 cells, but activated the NRF2/HO-1 dependent antioxidant response in BEAS-2B cells.

Phellopterin differentially modulates NF-κB activation in A549 and BEAS-2B cells

We also investigated how phellopterin affects the NF-kB pathway by measuring phosphorylated NF-κB p65 levels by ELISA. When treated for 24 h with 25 μM phellopterin, there was a significant decrease in the amount of phospho-p65 levels in A549 cells. This indicates that phellopterin inhibits NF-kB activity in A549 lung cancer cells. However, in BEAS-2B cells, phellopterin treatment also leads to a moderate decrease in the amount of phosphorylated p65; therefore, it appears as though there may be cytoprotective properties associated with phellopterin treatment in BEAS-2B cells. Similar to the changes in the levels of phosphorylated p65, the changes observed in the cytokine response were consistent: A549 had decreased pro-inflammatory cytokine secretion due to inhibited NF-kB activity, while BEAS-2B had decreased IL-8 and improved NRF2-mediated antioxidant responses due to inactive NF-kB. Inhibitors have no significant effects on reversing the level of phosphorylated p65 (Fig. 5a & b).

Discussion

The current study shows that phellopterin activity induces different outcomes between malignant and non-malignant lung epithelial cells. In A549 cells, phellopterin prompted an elevation of cleaved

caspase-3 and a decrease of BCL2, consistent with the induction of apoptotic signaling. On the contrary, BEAS-2B cells showed upregulation of NRF2 and HO-1 without signs of caspase-3 cleavage with implication of a cytoprotective antioxidant response.

Phellopterin induced an intrinsic apoptotic pathway in A549 NSCLC cells through the robust activation of cleaved caspase-3 and the transcriptional suppression of the anti-apoptotic protein BCL2. Furthermore, inhibition of the caspase-3 pathway did not restore BCL2 expression, indicating that phellopterin acts upstream of caspase-3 in the apoptotic cascade, likely through either transcriptional regulation of BCL2 or by modulating the protein stability of BCL2-family proteins. Persistent overexpression of BCL2 is established as a mechanism by which cancer cells evade apoptosis and develop resistance to chemotherapy[14,15]. Hence, by inhibiting expression of the BCL2 protein, phellopterin may enhance the sensitivity of cancer cells to apoptotic signals. Also, the caspase-3-dependent decrease in the pro-inflammatory cytokines TNFα and IL-6 indicates that phellopterin modulates inflammatory signaling in cancer cells, possibly contributing to its pro-apoptotic effect.

The study also demonstrates that the triggering of the apoptotic pathway by phellopterin leads to decreased production and release of the pro-inflammatory cytokines TNF-α and IL-6 by A549 cells. Suppression of these cytokines was reversed moderately by inhibiting the activity of caspase-3 with pharmacological agents, suggesting a partial role for caspase-3 in modulating the expression of these cytokines. While caspase-3 is regarded as the executor of apoptosis, several recent studies have indicated that caspase-3 may also play a role in modulating inflammatory signaling pathways, either through the cleavage of the substrates responsible for cytokine production or by interacting with transcription factors such as NF-κB[16]. However, the decrease in the levels of TNF-α and IL-6 may be because apoptosis is occurring; during the apoptotic phase, dying cells will express lower levels of synthesis and secretion of these pro-inflammatory cytokines. To clarify if phellopterin affects TNF-α and IL-6 levels directly or as a result of cellular death, studies will be performed using caspase-3 knockdown models, along with measuring the levels of TNF-α and IL-6 mRNA. Overall, this area of research provides insight into how phellopterin may affect the inflammatory tumor microenvironment, thereby connecting the pro-apoptotic activity of phellopterin with the modulation of the inflammatory tumor microenvironment in the lung.

In contrast, BEAS-2B normal cells exhibited an opposite response that was protective. Phellopterin did not trigger the pro-apoptotic pathway, but instead activated a strong cytoprotective response involving the antioxidant NRF2 pathway. The coordinated upregulation of NRF2 and its canonical target HO-1 was abolished completely by treatment with the NRF2 inhibitor ML385. NRF2 is known to be the most important cellular defense against electrophilic and oxidative stress[17]. In normal cells, the temporary activation of NRF2 enhances the cells' ability to withstand stress; however, in several cancers, the continuous activation of NRF2 promotes the survival, proliferation, and drug resistance of tumor cells[6]. Additionally, phellopterin demonstrated anti-inflammatory activity towards BEAS-2B cells by reducing IL-8 production. The ability to suppress IL-8 levels appeared to be partially dependent on NRF2, suggesting that there may be crosstalk between antioxidant and anti-inflammatory signaling pathways. NRF2 antagonizes the pro-inflammatory NF-κB pathway by competing for the same transcriptional coactivators, and through up-regulating antioxidant genes to eliminate ROS that activate NF-κB[18].

Our results from prior research have greatly improved the pharmacology of phellopterin. Previous reports demonstrated the ability of phellopterin to inhibit the growth of ovarian cancers through the disruption of the PI3K/AKT/CLEC5A pathway and colitis-associated colon cancers through modulation of the immune system and inhibition of TLR4/NF-κB [12,13]. This investigation is unique in that we have identified the regulatory mechanisms of phellopterin in different types of cells, demonstrating a very specific molecular mechanism involved in the selection of cell types. This research supports the role of phellopterin as a multi-functional compound capable of overcoming the apoptotic resistance and abnormal redox signaling characteristic of aggressive NSCLC.

Notably, the protein expression results were consistent with the transcriptional data and further supported the hypothesized mechanisms. In A549 cells, phellopterin decreased BCL2 protein level but not NRF2 and HO-1 protein level, which further supports the notion that apoptotic signaling but not

antioxidant pathway activation dominates in these cells. On the other hand, the elevated expression values of NRF2 and HO-1 proteins in BEAS-2B cells, as well as their downregulation by ML385, validates an NRF2-mediated cytoprotective response. Since protein expression is a more immediate indicator of biological function than mRNA levels alone, these results provide further mechanistic support for the contrasting responses to phellopterin in cancerous versus normal lung epithelial cells.

There are several limitations to this study. The findings come from an *in vitro* study done with two immortalized cell lines, A549 and BEAS-2B, which cannot reproduce the full complexities of the tumor microenvironment *in vivo* or the function of primary normal cells. Using only one concentration and time point leaves uncertainty about the full range of pharmacological actions of phellopterin. Specific inhibitors were used to demonstrate the proposed mechanisms; however, additional validation through complementary genetic techniques (*e.g.*, siRNA knockdown) will provide additional confirmation. The use of a single NSCLC cell line limits the generalizability of the findings. Future studies should evaluate phellopterin in additional lung cancer models, including EGFR-mutant and KRAS-mutant NSCLC cell lines, to determine whether the observed responses are broadly applicable. Additionally, phellopterin's upstream molecular target(s) and the specific effects it has on protein levels and the functional activity of the pathways studied have not yet been elucidated, and further *in vivo* investigations and identification of phellopterin's targets will be necessary for therapeutic translation.

Conclusion

In the present study the results suggest that phellopterin has a differential effect on apoptotic, antioxidant, and inflammation-associated signaling pathways in A549 and BEAS-2B cells. Phellopterin induced pro-apoptotic signaling via caspase-3 activation and BCL2 inhibition in A549 cells, but it did not result in apoptosis in BEAS-2B cells, where it stimulated cytoprotective NRF2/HO-1 signaling. Phellopterin also attenuated inflammatory cytokine secretion and NF-κB activation, albeit through different manners in tumor versus normal cells. These data indicate that the biological activity of phellopterin might be tumor-selective. However, additional studies, including protein level confirmation and additional lung cancer models, as well as *in vivo* studies, are necessary to validate the mechanistic underpinnings and therapeutic implications of these findings.

Acknowledgement

All authors participated in the study's design and conceptualization. (Yinyu Li) and (Hao Xu) carried out material preparation and data collection. (Weiru Xiao) edited and analyzed the data. (Xiaoyu Chen) edited and supervised the study. They have reviewed and approved the final manuscript.

Conflict of interest

All authors declare no conflicts of interest.

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