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Dietary Luteolin Exerts Anticancer Effects in Models of Acquired BRAF Inhibitor-Resistant Cutaneous Melanoma via Dual Inhibition of RAF/MEK/ERK and PI3K/AKT Signaling

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ABSTRACT: Approximately half of cutaneous melanoma patients harbor the BRAF (V600E) mutation. Although BRAF inhibitors (BRAFi), such as vemurafenib and dabrafenib, initially demonstrate clinical efficacy, resistance frequently develops within eight months, limiting their long-term benefit. Luteolin, a flavonoid widely present in foods and vegetables, exhibits anti-melanoma activity and inhibits signaling pathways implicated in BRAFi resistance, including the AKT, STAT3, and ERK pathways. However, its impact on BRAFi-resistant melanoma has not been explored. In this study, we show that luteolin significantly reduced the viability of vemurafenib- and dabrafenib-resistant cutaneous melanoma cells, with minimal toxicity to normal melanocytes. Luteolin also suppressed colony formation, inhibited tumor spheroid growth, and induced apoptosis in resistant cells. *In vivo*, luteolin dose-dependently suppressed BRAFi-resistant cutaneous melanoma growth in mice without observable toxicity. Network pharmacology analysis predicted involvement of the PI3K/AKT and MAPK signaling cascades in mediating luteolin's effects. Western blotting confirmed that luteolin decreased phospho-AKT1 (Ser473) and phospho-ERK1/2 levels in both cells and tumors, and reduced the expression of PI3K-p110 α , Bcl-2, Mcl-1, B-Raf (V600E), C-Raf, and phospho-MEK1/2 in resistant cells. Notably, the suppressive effects of luteolin on cell viability were partially reversed by the AKT activator SC-79 and the ERK activator Ro 67-7476. Taken together, these findings demonstrate for the first time that luteolin exerts anticancer effects in BRAFi-resistant cutaneous melanoma models, at least in part through concurrent inhibition of the RAF/MEK/ERK and PI3K/AKT pathways. Owing to its efficacy, safety, and dietary origin, luteolin holds promise as a functional food component or nutraceutical candidate for the adjunct management of drug-resistant cutaneous melanoma.

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1. Introduction

Cutaneous melanoma is an aggressive form of skin cancer, with metastatic cases exhibiting a poor 5-year survival rate of less than 35%^[1,2]. Approximately 50% of patients harbor the BRAF(V600E) mutation, which constitutively activates the MAPK pathway and drives tumor progression^[3,4]. Selective BRAF inhibitors (BRAFi), such as vemurafenib and dabrafenib, demonstrate notable initial efficacy; however, their long-term clinical benefits are limited by the rapid emergence of resistance, often leading to relapse within 5–8 months^[5]. Mechanisms of resistance include MAPK pathway reactivation and engagement of alternative survival pathways, with the PI3K/AKT signaling axis being particularly important for promoting cell survival and evasion of apoptosis^[6].

To address resistance and relapse, combination therapies have been explored. Clinically approved regimens combining BRAFi and MEK inhibitors (MEKi), including dabrafenib–trametinib, vemurafenib–cobimetinib, and encorafenib–binimetinib, have demonstrated improved progression-free survival in patients with BRAF(V600E)-mutant melanoma^[7]. Nevertheless, approximately 50% of patients develop resistance within 12 months^[8,9]. In addition to targeted combination therapies, immunotherapies—particularly immune checkpoint inhibitors—are widely used as first-line treatments for cutaneous melanoma. These include agents such as ipilimumab (anti-CTLA-4), nivolumab and pembrolizumab (anti-PD-1), and atezolizumab (anti-PD-L1)^[10,11]. Despite their established clinical efficacy, durable responses are achieved only in a subset of patients, and immune-related adverse events are frequent and occasionally severe. Additional strategies, such as small molecule agents targeting resistance pathways (e.g., hydroxychloroquine [autophagy inhibitor], ganetespib [Hsp90 inhibitor])^[12,13], have been tested in clinical trials. Unfortunately, many of these trials have been discontinued due to dose-limiting toxicities or insufficient efficacy. Collectively, these limitations underscore the unmet need for novel therapeutic approaches for BRAFi-resistant melanoma.

Luteolin (3',4',5,7-tetrahydroxyflavone), a flavonoid present in a variety of fruits and vegetables, has attracted interest due to its broad pharmacological properties, including anti-inflammatory and anticancer effects^[14,15]. Our previous work has demonstrated its anti-melanoma activity both *in vitro* and *in vivo*^[16]. Studies indicate that luteolin targets key mediators of BRAFi resistance, such as AKT, STAT3, and ERK^[16,17,18], and has been shown to sensitize ovarian and gastric cancer cells to chemotherapy^[19,20]. Despite these promising findings, the effects of luteolin in BRAFi-resistant melanoma remain unexplored.

In this study, we investigated the effects of luteolin in models of BRAFi-resistant cutaneous melanoma, utilizing acquired vemurafenib- and dabrafenib-resistant cell models as well as a xenograft mouse model. We further employed network pharmacology and experimental approaches to elucidate the molecular mechanisms underlying luteolin's actions. To our knowledge, this is the first study to assess the efficacy of naturally derived, food-based luteolin in models of BRAFi-resistant melanoma. Our findings not only highlight the potential development of luteolin as a safe and effective therapeutic candidate for patients with BRAFi-

resistant cutaneous melanoma, but also underscore its promise as a functional food ingredient or nutraceutical for adjunct cancer therapy.

2. Materials and methods

2.1. Reagents

Luteolin (purity $\geq 98\%$) was purchased from Chengdu Alfa biotech Co., Ltd (Chengdu, China). Vemurafenib, dabrafenib, ganetespib, trametinib, SC-79, and Ro 67-7476 were obtained from MedChemExpress (MCE, USA).

2.2. Cell culture

The human cutaneous melanoma cell lines A375 and RPMI-7951 were purchased from the American Type Culture Collection (ATCC, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin-streptomycin-amphotericin B solution (Gibco, USA). The immortalized human melanocyte line PIG1 was obtained from Shanghai Guan & Dao Biological Engineering Co., Ltd (Shanghai, China), and cultured in DMEM (Gibco, USA) supplemented with 10% FBS (Gibco, USA), 1% penicillin-streptomycin-amphotericin B solution (Gibco, USA), and 10 ng/mL tetradecanoyl phorbol 13-acetate (TPA; MCE, USA). The cells were maintained at 37 °C in a humidified incubator with 5% CO₂. Mycoplasma contamination was routinely screened every three months using the MycoAlert™ PLUS Mycoplasma Detection Kit (Lonza, Switzerland).

2.3. Establishment of acquired BRAFi-resistant cells

To establish vemurafenib-resistant (A375-VR) and dabrafenib-resistant (A375-DR) subpopulations, parental A375 cells were treated with sublethal BRAFi concentrations (50% viability inhibition at 48 hours). Resistant clones emerged during a 3-month continuous culture with BRAFi-containing medium refreshed every 3–4 days. Drug doses were gradually increased to counteract adaptive resistance until stable proliferation was observed^[21,22]. Resistance was confirmed by observing an IC₅₀ value ≥ 10 -fold higher than in parental cells. Resistant cells were cultured in complete medium supplemented with 5 μ mol/L vemurafenib or dabrafenib for further analyses^[23].

2.4. Cytotoxicity analysis

Cell viability of A375-VR, A375-DR, RPMI-7951, and PIG1 cells was evaluated using the MTT assay^[16]. Cells were seeded (3×10^3 /well) in 96-well plates, allowed to attach for 24 hours, and then treated with 0.1% DMSO (vehicle), luteolin (10 μ mol/L –100 μ mol/L), or ganetespib (100 nmol/L) for 24 or 48 hours. After treatment, 10 μ L of MTT solution (5 mg/mL) was added and incubated for 4 hours at 37°C. Formazan crystals were dissolved in 100 μ L DMSO, and absorbance at 570 nm was measured using a Multiskan SkyHigh microplate reader (Thermo Fisher Scientific, USA).

2.5. Colony formation assay

A375-VR and A375-DR cells (500 cells/well) were plated in 60-mm dishes and exposed to luteolin (10, 20, 40 $\mu\text{mol/L}$) or ganetespib (100 nmol/L). The treatment-containing medium was replaced every 3 days. After 10 days of incubation, the cell colonies were washed with PBS, fixed with 10% neutral buffered formalin for 30 minutes, and stained with a 2% crystal violet solution for another 30 minutes. After staining, the colonies were washed and allowed to air dry before being photographed. Then, the crystal violet was dissolved in 2% sodium dodecyl sulfate. The absorbance at 570 nm was quantified^[24].

2.6. Tumor spheroid formation assay

A375-VR and A375-DR cells were plated in ultra-low attachment 96-well plates at a density of 5×10^3 cells per well to initiate spheroid formation. After 48 hours of incubation, mature spheroids were treated with luteolin (10, 20, 40 $\mu\text{mol/L}$) or ganetespib (100 nmol/L) for 7 consecutive days. The morphology of the spheroids was evaluated on post-treatment days 1, 4, and 7. The spheroid diameter was measured using Fiji software^[25].

2.7. Apoptosis assay

Apoptosis was assessed using the Annexin V-FITC/PI Apoptosis Detection Kit (Abcam, #ab14085) according to the manufacturer's protocol. Briefly, A375-VR and A375-DR cells were seeded in 6-well plates and treated with luteolin (10, 20, or 40 $\mu\text{mol/L}$) or ganetespib (100 nM). After 48 hours, the cells were harvested by trypsinization, centrifuged at $300 \times g$ for 5 minutes, and rinsed with PBS. The harvested cells were then incubated in 500 μL of binding buffer with 5 μL of Annexin V-FITC and 5 μL of PI, and kept in the dark at room temperature for 5 minutes. In total, 10,000 events were recorded using a BD Accuri™ C6 Plus Flow Cytometer (BD Biosciences, USA) for the flow cytometry analysis. Data were analyzed using FlowJo software (version 10.4).

2.8. Network pharmacology analysis

To elucidate the mechanisms underlying luteolin's action, we performed network pharmacology analysis following established protocols^[26], with minor modifications. Luteolin targets were identified using the TCMSP, SwissTargetPrediction, and PharmMapper databases. Genes associated with BRAFi-resistant melanoma were obtained from the GEO dataset GSE50509^[27], which contains transcriptomic profiles of paired pre-treatment and post-progression melanoma samples from patients harboring BRAF(V600E) mutations treated with dabrafenib or vemurafenib. Data normalization and differential expression analysis were conducted using GEO2R, with p values adjusted for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) method. Differentially expressed genes (DEGs) were defined as those with adjusted P value (FDR) < 0.05 and $|\log_2 \text{fold change} (\log_2\text{FC})| \geq 1$. Additional BRAFi-resistant melanoma-related genes were retrieved from GeneCards and OMIM databases. For GeneCards, the keyword BRAF inhibitor-resistant melanoma was used; only protein-coding genes and RNA genes were retained, and entries were further filtered by selecting genes with relevance scores above the median of the retrieved gene set^[28]. For OMIM, the same keyword was queried in the Gene Map module, and only genes with approved symbols were

extracted. After merging and removing duplicate entries, the overlapping targets between luteolin targets and BRAFi-resistant melanoma-related targets were identified using Venny 2.1. The common targets were then subjected to protein-protein interaction (PPI) analysis using STRING and pathway enrichment analysis via Reactome. Significantly enriched pathways were defined as those with FDR-adjusted $P < 0.05$. Pathways were ranked according to the signal value in Reactome, calculated as a weighted harmonic mean of the observed-to-expected gene ratio and $-\log(\text{FDR})$, offering a balanced and intuitive metric for enrichment significance^[29]. The resulting PPI network was visualized using Cytoscape v3.9.0, and node degree was calculated to identify key hub targets. All databases and analytical tools used in this study are summarized in Table S1.

2.9. Western blot analysis

Cells and tumor samples were collected, with tumor tissue ground to a powder under liquid nitrogen, and then lysed for Western blotting following previously described methods^[30]. Band intensities were quantified using Fiji software, and protein levels were normalized to endogenous controls, including GAPDH, α -tubulin, or α -actinin. The primary antibodies used for detection were as follows: PARP (CST, #9532S), PI3K-p110 α (CST, #4255), AKT1 (CST, #2938), phospho-AKT1 (Ser473) (CST, #9018S), B-Raf (V600E) (Abcam, #ab200535), C-Raf (CST, #53745S), MEK1/2 (CST, #9122), phospho-MEK1/2 (Ser217/221) (CST, #9121), ERK1/2 (CST, #4695S), phospho-ERK1/2 (Thr202/Tyr204) (CST, #4370S), Mcl-1 (CST, #94296S), Bcl-2 (CST, #3498S), GAPDH (Santa Cruz, #sc-32233), α -tubulin (CST, #3873), and α -actinin (CST, #6487S).

2.10. Animal experiments

All animal procedures were conducted in accordance with the guidelines of the Hong Kong Department of Health and were approved by the Committee on the Use of Human and Animal Subjects at Hong Kong Baptist University (REC/20-21/0166). Male BALB/c nude mice (6 weeks old, 25 ± 2 g) were obtained from the Laboratory Animal Services Centre, Chinese University of Hong Kong. A375-VR cells (2.5×10^6 cells in 100 μL PBS) were subcutaneously injected into the right flank of each mouse to establish xenograft tumors. Once tumors became palpable, mice were randomly assigned to five groups ($n = 7$ per group) and treated once daily for three consecutive weeks. Luteolin was administered intraperitoneally owing to its poor oral bioavailability in rodents^[31]. To match the route of administration, the positive control, trametinib, was likewise administered intraperitoneally, as previously described for subcutaneous melanoma xenograft models^[32]. Treatment regimens included vehicle (PBS with 2% PEG 400 and 2% Tween 80), luteolin at 10, 15, or 20 mg/kg, and trametinib (0.3 mg/kg). Tumor size, body weight, and food intake were recorded daily, and tumor volume was calculated as $(\text{length} \times \text{width}^2) / 2$. At the end of the experiment, blood samples were collected under isoflurane anesthesia (induction: 3% at 1 L/min; maintenance: 1% at 0.3 L/min). Mice were then euthanized by overdose of isoflurane (5%). Xenografts and major organs (heart, liver, spleen, lung, and kidney) were excised and weighed for subsequent analysis.

2.11. Biochemical assay

To evaluate potential organ toxicities associated with luteolin, blood samples were centrifuged at 3,500 × g for 10 minutes at room temperature, and the serum was subsequently stored at -80°C. Biochemical assays were conducted using kits sourced from the Jiancheng Bioengineering Institute (Nanjing, China) to measure levels of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (Cre), and blood urea nitrogen (BUN).

2.12. Statistical analysis

Data were presented as mean ± standard deviation (SD). Statistical analyses were performed utilizing Prism 10.0 software (GraphPad Software, USA). One-way or two-way analysis of variance (ANOVA) was employed to compare multiple groups, followed by Tukey's test for post-hoc analysis. A *p* value below 0.05 is deemed statistically significant.

3. Results

3.1. Luteolin reduces cell viability, suppresses colony formation, and inhibits tumor spheroid growth in BRAFi-resistant cutaneous melanoma cells

Our previous study demonstrated that luteolin significantly reduces cell viability of several human cutaneous melanoma cell lines harboring the oncogenic BRAF(V600E) mutation (including A375 and IGR-1)^[16]. To further assess its therapeutic relevance, we investigated whether luteolin retains activity against BRAFi-resistant cutaneous melanoma cells. We established vemurafenib-resistant (A375-VR) and dabrafenib-resistant (A375-DR) cutaneous melanoma sublines, using a stepwise selection procedure illustrated in Fig. 1A. Resistance was validated by comparing the 48-hour viability of parental and BRAFi-adapted A375 cells upon inhibitor exposure. As shown in Fig. S1, parental cells displayed markedly greater reduction in cell viability under BRAFi treatment compared to their resistant counterparts, confirming successful acquisition of resistance. Specifically, the 48-hour IC₅₀ for vemurafenib increased from 0.57 μmol/L in parental cells to 19.83 μmol/L in A375-VR cells (Fig. S1A), while the IC₅₀ for dabrafenib increased from 0.045 μmol/L in parental cells to > 40 μmol/L in A375-DR cells (Fig. S1B). These results confirm stable, significant BRAFi resistance in both lines, with IC₅₀ values elevated by ~35-fold for vemurafenib and > 889-fold for dabrafenib. In addition, the RPMI-7951 cutaneous melanoma cell line, which also carries the BRAF(V600E) mutation but exhibits intrinsic resistance to BRAFi, was included as a comparator. Consistent with previous reports^[33,34], RPMI-7951 cells maintained over 90% viability in the presence of 40 μmol/L vemurafenib or dabrafenib (Figs. S1C–D), confirming this phenotype.

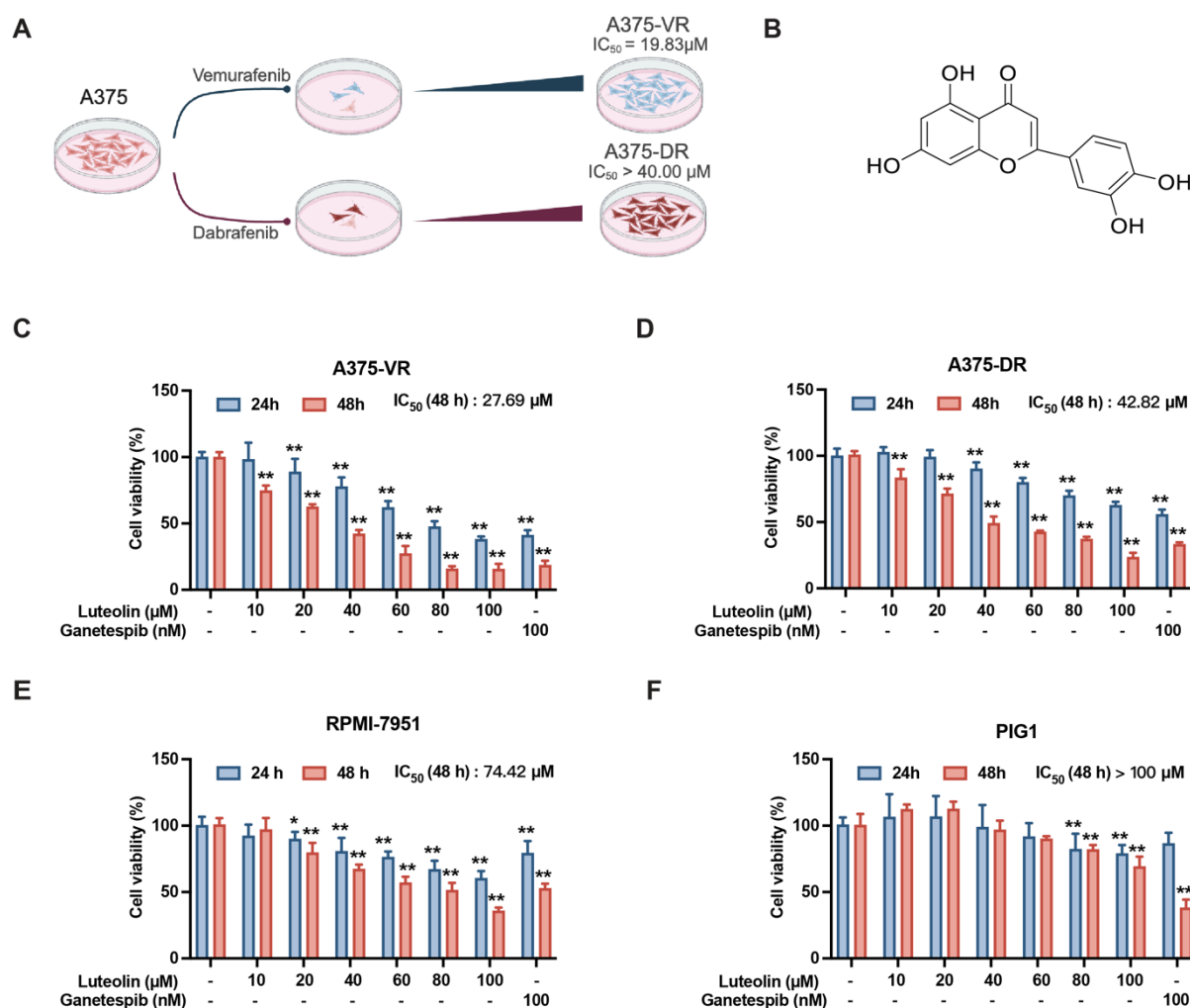


Fig. 1. Luteolin reduces the viability of BRAFi-resistant cutaneous melanoma cells. (A) Schematic diagram of the process for establishing BRAFi-resistant cells. (B) Chemical structure of luteolin. (C–F) Inhibitory effects of luteolin on the viability of A375-VR (C), A375-DR (D), RPMI-7951 (E) and PIG1 (F) cells. Cells were treated with the indicated concentrations of luteolin for 24 or 48 hours, followed by MTT assay. Data are shown as mean ± SD from three independent experiments, * $P < 0.05$, ** $P < 0.01$, vs. the control group.

Luteolin's effects on cell viability were evaluated using MTT assays in A375-VR, A375-DR, and RPMI-7951 cutaneous melanoma cells, as well as in normal human melanocytes (PIG1 cells). Luteolin (chemical structure shown in Fig. 1B) markedly reduced the viability of resistant melanoma cells in a concentration- and time-dependent manner. The 48-hour IC₅₀ values were 27.69 μmol/L and 42.82 μmol/L for A375-VR and A375-DR cells, respectively (Figs. 1C–D), while RPMI-7951 cells exhibited a higher IC₅₀ of 74.42 μmol/L (Fig. 1E), indicating that A375 sublines with acquired BRAFi resistance were more sensitive to luteolin than the intrinsically BRAFi-resistant RPMI-7951 cells. Notably, luteolin had minimal effects on the viability of PIG1 melanocytes (Fig. 1F) compared with BRAFi-resistant cutaneous melanoma cells.

Colony formation assays further supported the anti-proliferative effects of luteolin, demonstrating dose-dependent inhibition of colony formation in both A375-VR and A375-DR cells (Fig. 2A). Given that three-dimensional (3D) tumor spheroids closely recapitulate the *in vivo* tumor microenvironments^[35], we also evaluated luteolin in this context. Luteolin significantly inhibited spheroid growth by day 7 (Fig. 2B), further validating its efficacy against BRAFi-resistant cutaneous melanoma cells under physiologically relevant conditions.

Ganetespiib, a selective Hsp90 inhibitor effective in BRAFi-resistant melanoma^[36], served as a positive control and, as expected, reduced cell viability, suppressed colony formation, and inhibited spheroid growth in resistant cells (Figs. 1C–E). Notably, it also reduced the viability of PIG1 melanocytes to below 50% at 48 hours (Fig. 1F).

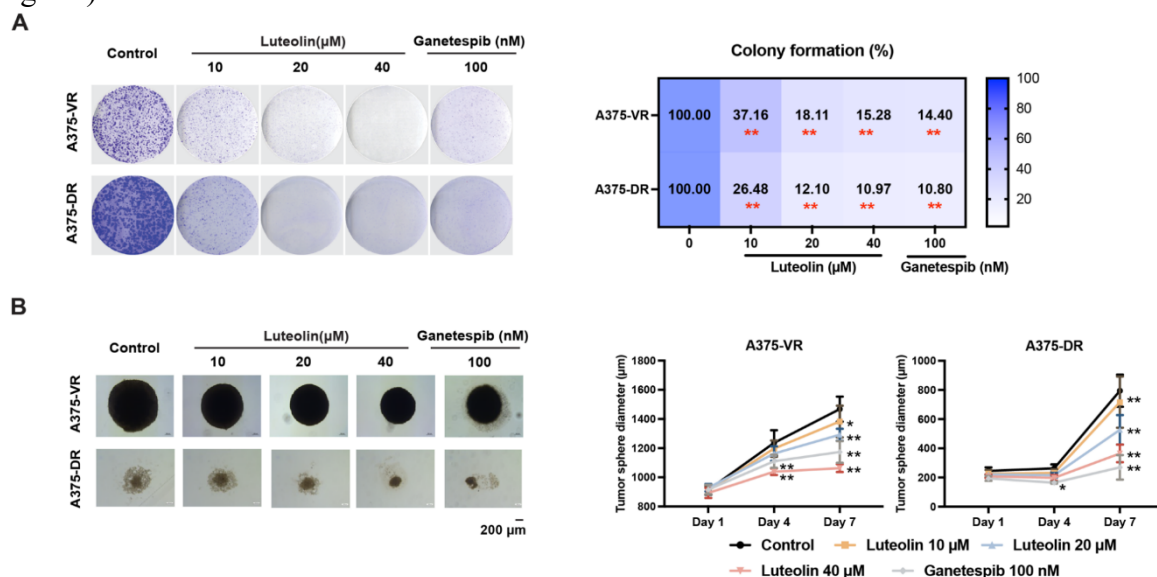


Fig. 2. Luteolin suppresses colony formation and inhibits tumor spheroid growth in BRAFi-resistant cutaneous melanoma cells. (A) Representative images of colony formation. A375-VR and A375-DR cells were treated with 0.1% DMSO (control), luteolin or the positive control ganetespiib for 10 days. (B) Representative images of tumor spheroid formation. A375-VR and A375-DR cell spheroids were treated with 0.1% DMSO (control), luteolin or the positive control ganetespiib for 7 days. Statistical analyses of colony formation rates and tumor spheroid diameters are shown in the right panels. Data are shown as mean $\pm$ SD from three independent experiments, * P < 0.05, ** P < 0.01, vs. the control group.

Overall, these findings demonstrate that luteolin exerts anti-proliferative effects in BRAFi-resistant cutaneous melanoma cells, with enhanced selectivity over normal melanocytes.

3.2. Luteolin induces apoptosis in BRAFi-resistant cutaneous melanoma cells

To determine whether luteolin induces apoptosis in BRAFi-resistant cutaneous melanoma cells, we performed flow cytometry with Annexin V-FITC/PI staining. As shown in Fig. 3A, 48-hour luteolin treatment markedly increased the proportion of apoptotic cells in both A375-VR and A375-DR lines. In A375-VR cells, the apoptotic rate increased from 8.4% in controls to 20.3% and 37.3% following treatment with 20 μ mol/L and 40 μ mol/L luteolin, respectively. Similarly, A375-DR cells showed increased apoptosis from 9.9% (control) to 24.4% (20 μ mol/L) and 39.0% (40 μ mol/L). The positive control, ganetespiib, also robustly induced apoptosis, reaching 52.5% in A375-VR and 64.8% in A375-DR cells. Western blotting corroborated these findings, revealing elevated levels of cleaved PARP (Fig. 3B), a hallmark of apoptosis^[37]. Overall, these findings indicate that luteolin induces apoptosis in BRAFi-resistant cutaneous melanoma cells.

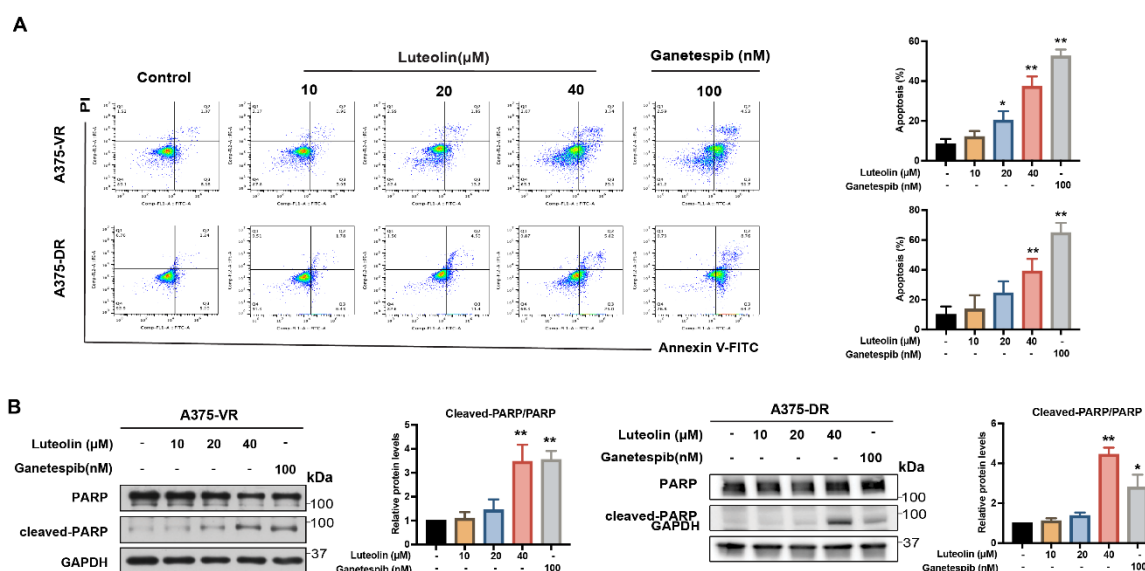


Fig. 3. Luteolin induces apoptosis in BRAFi-resistant cutaneous melanoma cells. (A) Luteolin induced apoptosis in A375-VR and A375-DR cells. Cells were treated with the indicated concentrations of luteolin for 48 hours, stained with Annexin V/PI and then analyzed by flow cytometry. Quantification of the apoptotic rates is represented as a bar chart. (B) Representative Western blot images (left) and corresponding densitometric quantification (right) of PARP and cleaved-PARP protein levels after luteolin treatment. GAPDH served as a loading control. Data are shown as mean $\pm$ SD from three independent experiments, * P < 0.05, ** P < 0.01, vs. control group.

3.3. Luteolin suppresses tumor growth in A375-VR bearing mice

To further evaluate the *in vivo* anti-tumor activity of luteolin in BRAFi-resistant melanoma, we established a xenograft model by subcutaneously inoculating A375-VR cells—chosen for their higher sensitivity to luteolin compared to A375-DR cells—into BALB/c-nu/nu mice. The experimental design is depicted in Fig. 4A, and representative images of excised tumors clearly demonstrate luteolin's inhibitory effect on tumor growth (Fig. 4B). After 21 days of treatment, luteolin induced dose-dependent reductions in tumor weight of 61.8%, 73.3%, and 85.1% at 10, 15, and 20 mg/kg, respectively (Fig. 4C). Correspondingly, tumor volume was suppressed by 72.2%, 85.0%, and 87.0% (Fig. 4D). The positive control, trametinib, also significantly inhibited tumor growth, resulting in reductions of 91.6% (volume) and 93.8% (weight) (Figs. 4C–D).

Throughout the treatment period, luteolin exhibited good tolerability, with no significant changes in body weight (Fig. 4E), food or water intake, or clinical observations (including eye condition, gait, and posture). Organ index analysis revealed no significant differences in organ-to-body weight ratios between luteolin-treated and control groups (Fig. S2). Moreover, serum biochemical analyses indicated no signs of hepatic (ALP, AST, ALT) or renal (Cre, BUN) toxicity in luteolin-treated mice (Fig. 4F). In contrast, trametinib treatment caused diarrhea (data not shown; patients receiving oral trametinib also commonly experience this side effect), increased kidney index (Fig. S2E), and elevated serum ALP (Fig. 4F), suggesting renal and hepatic toxicity. These findings indicate that luteolin was better tolerated than trametinib under the experimental conditions used in this mouse model.

Collectively, these data demonstrate that therapeutic administration of luteolin significantly suppresses tumor growth in A375-VR bearing mice without causing overt toxicity.

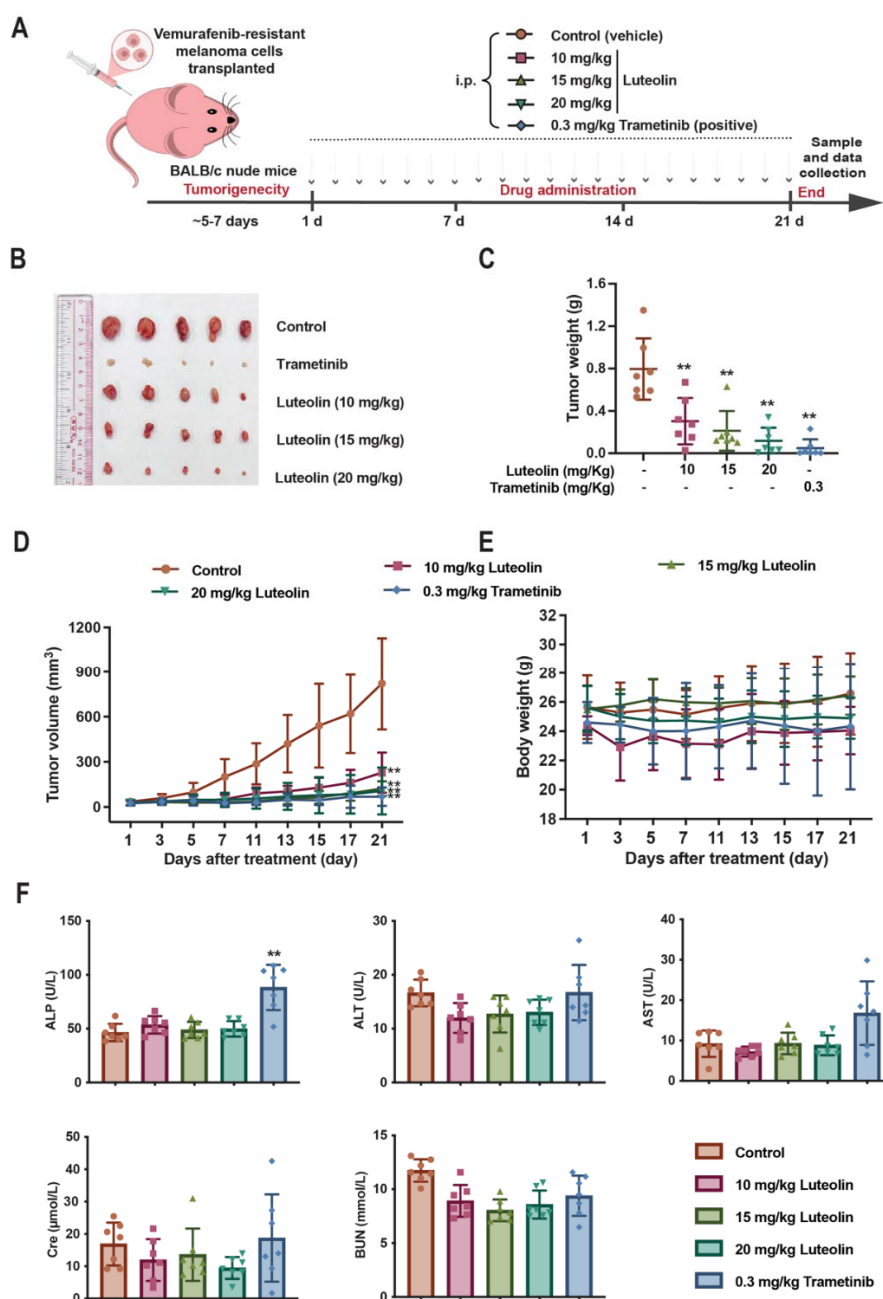


Fig. 4. Luteolin suppresses tumor growth and exhibits no overt toxicity in A375-VR bearing mice. (A) Schematic diagram of the animal experimental procedure. (B) Representative images of A375-VR tumors from each group. (C) Tumor weight at endpoint. (D) Tumor volume during treatment. (E) Mouse body weight during treatment. (F) Serum biochemical parameters. Data are presented as mean $\pm$ SD ($n = 7$ per group). * $P < 0.05$, ** $P < 0.01$, vs. control group.

3.4. Network pharmacology analysis implicates the PI3K/AKT and MAPK signaling cascades in mediating luteolin's effects on BRAFi-resistant cutaneous melanoma

To elucidate the molecular mechanisms underlying luteolin's activity against BRAFi-resistant cutaneous melanoma, we performed a network pharmacology analysis. The GSE50509 dataset from the GEO database—featuring paired pre- and post-progression melanoma samples from BRAFi-treated patients—was analyzed to capture transcriptomic changes associated with acquired resistance. In total, 669 DEGs were identified, including 573 upregulated and 96 downregulated genes (Fig. 5A). Additional melanoma genes associated with

BRAFi resistance were obtained from GeneCards (296 genes) and OMIM (54 genes), and after removal of duplicates, a combined set of 935 unique genes was compiled.

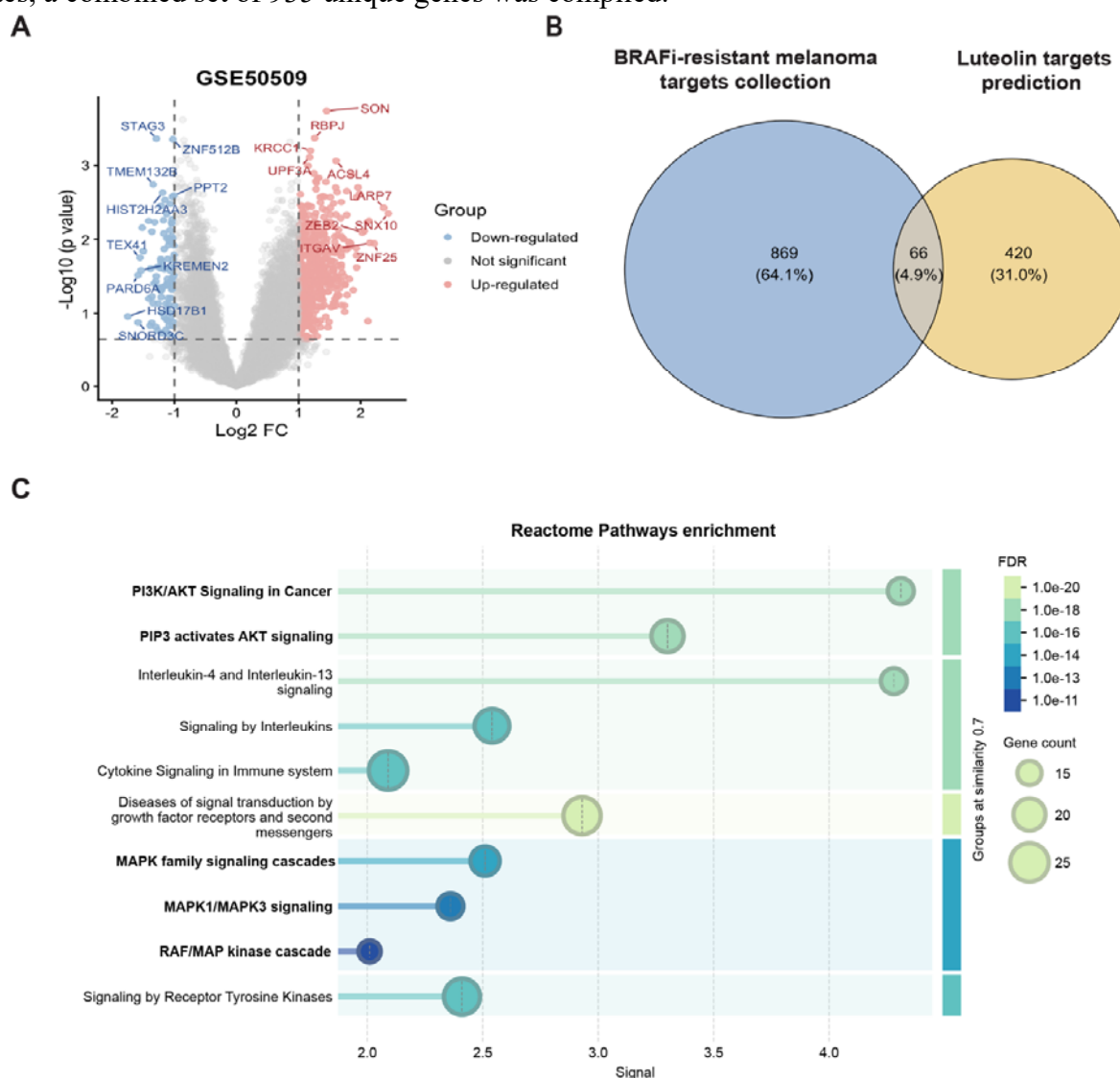


Fig. 5. PI3K/AKT and MAPK signaling pathways are involved in the effects of luteolin. (A) Volcano plot of differentially expressed genes (DEGs) between pre-treatment and post-progression melanoma samples from BRAFi-treated patients. Red and blue dots represent up- and downregulated genes, respectively; gray dots represent non-significant genes. (B) Venn diagram of the targets related to luteolin and BRAFi-resistant melanoma. (C) Reactome pathway enrichment analysis illustrating potential signaling pathways associated with BRAFi resistance and targeted by luteolin in melanoma.

Potential molecular targets of luteolin were predicted using TCMSP (55 targets), SwissTargetPrediction (100 targets), and PharmMapper (365 targets), yielding 486 unique targets after removal of duplicates. By intersecting the predicted luteolin targets with the BRAFi-resistant melanoma gene set, 66 overlapping targets were identified (Fig. 5B, Table S2). Within the PPI network, node size and color intensity reflected interaction strength, with larger and deeper purple nodes indicating greater connectivity (Fig. S3A). The top 20 hub genes were ranked by degree centrality (Fig. S3B), with TP53, EGFR, CASP3, HSP90AA1, and AKT1 exhibiting the highest degree values (57, 56, 56, 56, and 54, respectively), underscoring their central roles in BRAFi-resistant melanoma and potential involvement in luteolin's pharmacological effects.

Reactome pathway enrichment analysis of the 66 common targets identified 22 significantly enriched pathways. The top 10 pathways (adjusted $P < 0.05$, gene count > 15 , group similarity set to 0.7) are shown in

Fig. 5C. These pathways primarily involved receptor tyrosine kinase activity, cytokine-mediated signaling, and nuclear receptor regulation. Importantly, multiple PI3K/AKT and MAPK signaling terms were highly enriched, including PI3K/AKT Signaling in Cancer (ranked 1st) and PIP3 activates AKT signaling (ranked 2nd), and MAPK family signaling cascades (ranked 7th), MAPK1/MAPK3 signaling (ranked 8th), and RAF/MAP kinase cascade (ranked 9th). The enrichment of these pathways highlights their pivotal roles in BRAFi resistance, in line with previous melanoma studies^[3,6,38]. Accordingly, we prioritized PI3K/AKT and MAPK for subsequent mechanistic validation.

3.5. Luteolin inhibits PI3K/AKT signaling in BRAFi-resistant cutaneous melanoma

Given the prominent enrichment of the PI3K/AKT pathway in our network pharmacology analysis, we next investigated whether luteolin suppresses this signaling axis in BRAFi-resistant melanoma. Western blot results showed that luteolin markedly decreased AKT1 phosphorylation at Ser473 in both A375-VR xenograft tumors (Fig. 6A) and in BRAFi-resistant cell lines (A375-VR and A375-DR; Fig. 6B). A concomitant reduction in PI3K-p110 α subunit levels was observed (Fig. 6B).

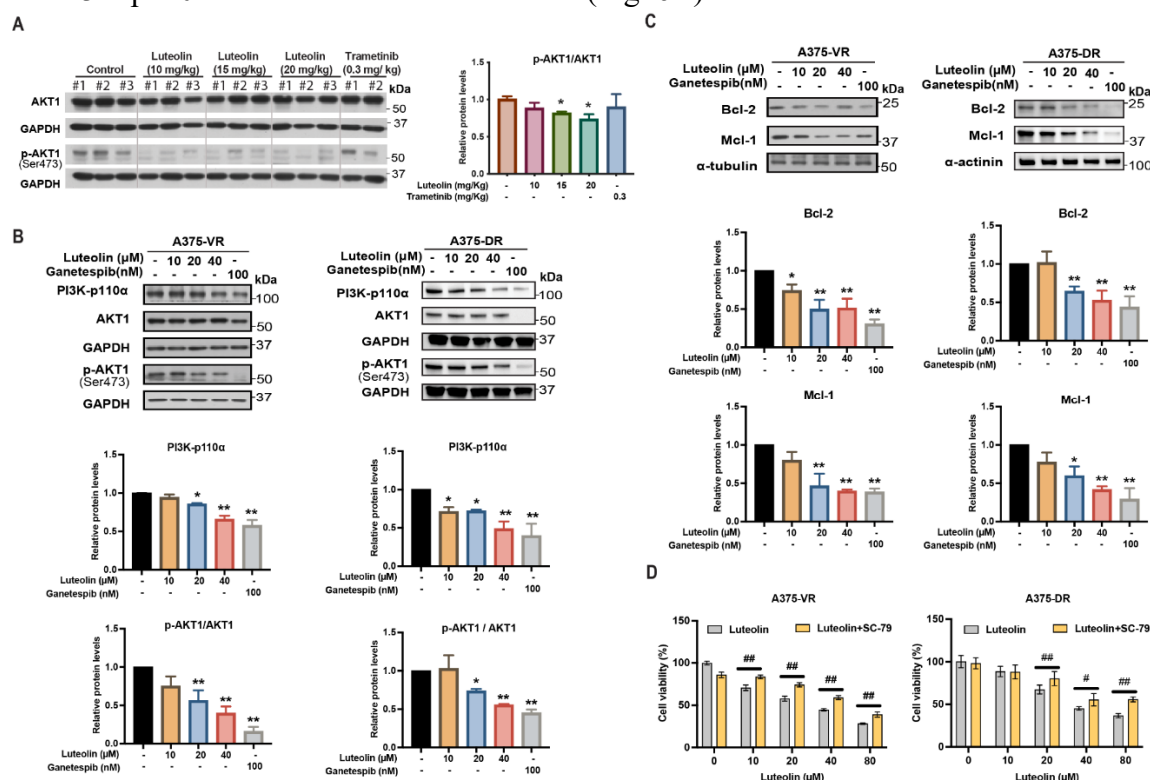


Fig. 6. Luteolin suppresses the PI3K/AKT signaling pathway in BRAFi-resistant cutaneous melanoma cells and tumor xenografts. (A) Immunoblotting analysis of AKT1 and phosphorylated AKT1 (Ser473) in tumor tissues.

Representative blots from each group are shown on the left, and relative band intensities are quantified on the right.

Phospho-AKT1 (Ser473) expression was normalized to total AKT1. GAPDH served as a loading control. Data are presented as mean $\pm$ SD from three individual samples in the control and luteolin-treated groups, and from two independent samples in the trametinib-treated group. (B-C) Immunoblotting analysis of PI3K-p110 α , AKT1 and phospho-AKT1 (Ser473), and downstream molecules Bcl-2 and Mcl-1 in A375-VR and A375-DR cells. Representative bands from each group are presented in the upper panels, and relative band intensities are shown in the lower panels. Phospho-AKT1 (Ser473) expression was normalized to total AKT1. GAPDH, α -tubulin, or α -actinin served as a loading control. Data are presented as mean $\pm$ SD from three independent experiments. (D) AKT overactivation diminished luteolin's suppression of cell viability. A375-VR and A375-DR cells were pretreated with the AKT activator SC-79 (10 μ mol/L) for 1 hour, followed by treatment with 0, 10, 20, 40, or 80 μ mol/L luteolin for 48 hours. Cell viability was measured using MTT assays. Data are presented as mean $\pm$ SD from three independent experiments. * P < 0.05, ** P < 0.01, vs. control group. ## P < 0.01, vs. the corresponding luteolin group without SC-79 pretreatment.

Since the PI3K/AKT pathway regulates key anti-apoptotic proteins, including Mcl-1 and Bcl-2^[39], we further examined their expression after luteolin treatment. Luteolin significantly downregulated Mcl-1 and Bcl-2 protein levels in both resistant cell lines (Fig. 6C), in line with its pro-apoptotic activity (Fig. 3A). Of note, Bcl-2 has been reported to interact strongly with luteolin (binding energy: -31.80 kJ/mol)^[40], suggesting that Bcl-2 may be a direct target of luteolin.

To confirm that PI3K/AKT inhibition contributes to the action of luteolin in BRAFi-resistant melanoma, rescue experiments were conducted using the AKT activator SC-79. BRAFi-resistant cells were pretreated with SC-79 (10 μ mol/L, 1 hour)^[41] prior to luteolin or vehicle exposure for 48 hours. As shown in Fig. 6D, AKT activation partially reversed the growth-suppressive effects of luteolin in both A375-VR and A375-DR cells. In A375-VR cells, SC-79 pretreatment increased viability from 57.81%, 44.71%, and 28.32% to 74.20%, 59.01%, and 39.04% at 20, 40, and 80 μ mol/L luteolin, respectively. Similarly, in A375-DR cells, viability rose from 67.24%, 45.07%, and 36.64% to 80.23%, 55.44%, and 55.84% under the same treatments.

In summary, these data demonstrate that luteolin suppresses PI3K/AKT signaling and downregulates Mcl-1 and Bcl-2, thereby facilitating apoptosis and inhibiting tumor growth in BRAFi-resistant cutaneous melanoma cells and xenograft models.

3.6. Luteolin inhibits MAPK signaling in BRAFi-resistant cutaneous melanoma

Resistance to BRAF inhibition in melanoma is largely due to reactivation of the MAPK (RAF/MEK/ERK) signaling cascade^[3]. In addition to the PI3K/AKT axis, our network pharmacology analysis identified the MAPK pathway as another key signaling route potentially involved in luteolin's anticancer effects in BRAFi-resistant cutaneous melanoma (Fig. 5C).

Western blot analysis of xenograft tumor lysates showed that luteolin markedly reduced phosphorylated ERK1/2 (Thr202/Tyr204) and slightly decreased total ERK1/2 levels, confirming suppression of MAPK signaling *in vivo* (Fig. 7A). In BRAFi-resistant melanoma cells, luteolin significantly inhibited MAPK pathway activation, as evidenced by reduced expression of C-Raf and B-Raf (V600E), along with decreased phosphorylation of MEK1/2 (Ser217/221) and ERK1/2 (Thr202/Tyr204) (Fig. 7B).

To further clarify the involvement of ERK1/2 in luteolin's mechanism, we performed rescue assays with pretreatment using the ERK1/2 activator Ro 67-7476 (1 μ mol/L, 5 minutes)^[42], before exposure to luteolin (10–80 μ mol/L, 48 hours) or vehicle in A375-VR and A375-DR cells. As shown in Fig. 7C, Ro 67-7476 pretreatment attenuated luteolin's inhibitory effects on cell viability in both BRAFi-resistant melanoma models. Specifically, in A375-VR cells, Ro 67-7476 increased viability from 60.92%, 49.17%, and 25.67% to 81.00%, 65.35%, and 40.32% at 20, 40, and 80 μ mol/L luteolin, respectively. Similarly, in A375-DR cells, viability increased from 70.49%, 46.67%, and 35.26% to 84.27%, 58.09%, and 46.76% under the same conditions.

Overall, these findings demonstrate that inhibition of the RAF/MEK/ERK signaling contributes to the anticancer effects of luteolin in BRAFi-resistant cutaneous melanoma models.

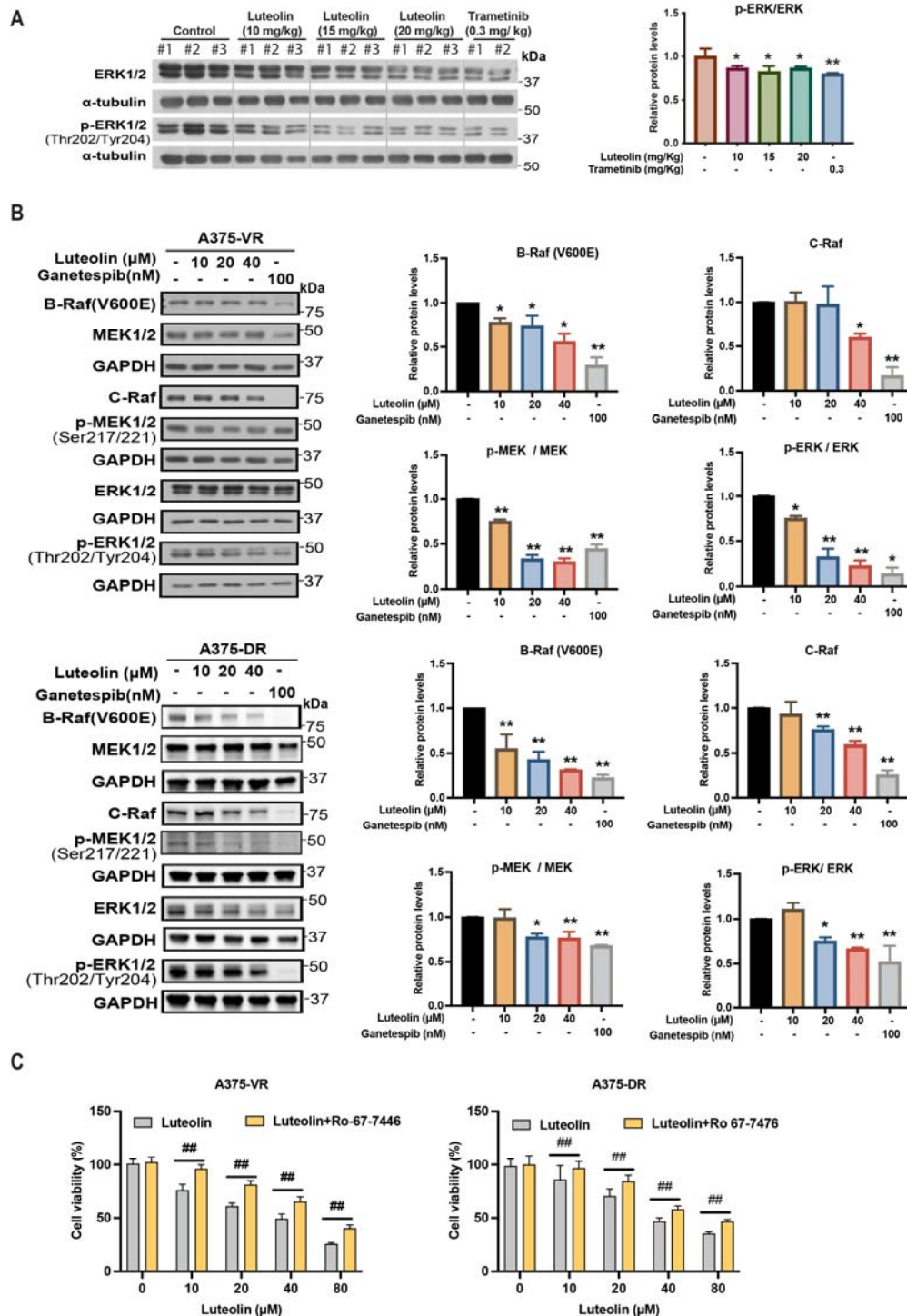


Fig. 7. Luteolin inhibits MAPK signaling in BRAFi-resistant cutaneous melanoma cells and tumor xenografts. (A) Immunoblotting analysis of ERK1/2 and phosphorylated ERK1/2 (Thr202/Tyr204) in tumor tissues. Representative blots from each group are shown on the left, and relative band intensities are quantified on the right. Phospho-ERK1/2 (Thr202/Tyr204) expression was normalized to total ERK1/2. α -tubulin served as a loading control. Data are presented as mean $\pm$ SD from three individual samples in the control and luteolin-treated groups, and from two independent samples in the trametinib-treated group. (B) Immunoblotting analysis of B-Raf (V600E), C-Raf, phospho-MEK1/2 (Ser217/221) and phospho-ERK1/2 (Thr202/Tyr204) protein levels in A375-VR and A375-DR cells. Phosphorylated protein levels were normalized to respective total protein. GAPDH was used as a loading control. Representative bands from each group are presented in the left panels, and relative band intensities are shown in the right panels. Data are presented as mean $\pm$ SD from three independent experiments. (C) ERK overactivation diminished luteolin's suppression of cell viability. A375-VR and A375-DR cells were pretreated with the ERK activator Ro 67-7446 (1 μ mol/L) for 5 minutes, followed by treatment with 0, 10, 20, 40, or 80 μ mol/L luteolin for 48 hours. Cell viability was determined using MTT assays. Data are presented as mean $\pm$ SD from three independent experiments. * P < 0.05, ** P < 0.01, vs. control group. # P < 0.05, ### P < 0.01, vs. the corresponding luteolin group without Ro 67-7446 pretreatment.

4. Discussion

Resistance to BRAF inhibitors remains a major clinical challenge in cutaneous melanoma. Here, we report, to our knowledge, the first evidence that the dietary flavonoid luteolin exerts anticancer activity in cellular and animal models of acquired BRAFi-resistant cutaneous melanoma without inducing overt toxicity. Using an integrated approach of network pharmacology and experimental validation, we show that luteolin concurrently suppresses the RAF/MEK/ERK and PI3K/AKT signaling pathways (Fig. 8), two critical axes implicated in BRAFi resistance. These findings support luteolin as a natural, multitarget candidate for the adjunct management of BRAFi-resistant cutaneous melanoma.

Luteolin is a widely available supplement (e.g., Lutimax®, SuperSmart®) that is generally well tolerated, with reported LD₅₀ values of 460 mg/kg in mice (intraperitoneal)^[43] and > 5,000 mg/kg in rats (oral)^[44]. In our xenograft model, daily intraperitoneal administration of luteolin (20 mg/kg for 21 days) significantly suppressed tumor growth without affecting body weight, organ indices, or serum biomarkers, consistent with a favorable safety profile. In contrast, intraperitoneal trametinib (0.3 mg/kg) caused diarrhea, an increased kidney index (Fig. S2E), and elevated serum ALP (Fig. 4F), indicating renal/hepatic toxicity under our experimental conditions. Although these findings suggest better tolerability for luteolin than trametinib in this preclinical setting, therapeutic success also depends on bioavailability^[45]. Luteolin's oral bioavailability is limited (~26% in rats)^[31], owing to poor lipophilicity, low solubility, and rapid systemic clearance^[46]. Following oral administration in rats, luteolin shows rapid absorption ($T_{max} \approx 1$ hour) and moderate elimination ($t_{1/2} \approx 5$ hours)^[47], motivating formulation optimization. Approaches such as nanoparticle encapsulation (yielding ~5-fold higher plasma concentrations^[48]) and phospholipid complexes^[49] have improved luteolin exposure and should be prioritized to enable oral dosing. Future studies should directly compare optimized oral luteolin formulations with orally administered trametinib to assess pharmacokinetics (PK), efficacy, and tolerability, better aligning preclinical evaluation with clinical use. Because luteolin penetrates the blood-brain barrier^[50] and human skin^[51], alternative routes (e.g., topical for cutaneous lesions, intravenous for systemic disease) merit investigation, particularly for melanoma with brain metastases.

Importantly, luteolin displayed minimal effects on normal melanocytes (PIG1) compared with BRAFi-resistant melanoma cells (Figs. 1C–F), suggesting tumor-selective activity. We hypothesize that this differential sensitivity reflects the lesser reliance of normal melanocytes on the oncogenic survival and stress-adaptation pathways (PI3K/AKT and MAPK) that are hyperactivated in BRAFi-resistant melanoma and inhibited by luteolin. Furthermore, melanocytes maintain robust antioxidant defenses (e.g., glutathione and NRF2-driven responses)^[52] that may mitigate luteolin-induced oxidative stress, whereas melanoma cells often operate closer to a pro-oxidant threshold and are thus more vulnerable. This mechanistic interpretation requires further validation.

Although melanoma-directed clinical trials of luteolin are lacking, its human safety and biological activity are supported by ongoing and completed studies for neuronal indications (e.g., luteolin for schizophrenia/cognitive impairment, NCT05204407^[53]; PEA-LUT—a luteolin-containing nutraceutical—for

frontotemporal dementia, NCT04489017^[54]). Moreover, clinical benefits of luteolin in combination with quercetin have been reported in autism spectrum disorder^[55]. Our preclinical data, together with these records, provide a strong rationale for the clinical exploration of luteolin as an adjunct therapy in BRAFi-resistant cutaneous melanoma, including in scenarios involving brain metastases.

We observed model-dependent differences in susceptibility: luteolin elicited stronger anti-proliferative effects in cells with acquired resistance (A375-VR, A375-DR) than in intrinsically resistant RPMI-7951 cells (Figs. 1C–E). A similar differential response pattern was seen with the Hsp90 inhibitor ganetespib. Both A375 and RPMI-7951 cells harbor the BRAF(V600E) and TERT promoter mutations; however, RPMI-7951 carries homozygous PTEN and TP53 deletions and overexpresses MAP3K8 (COT). These alterations are known to drive constitutive PI3K-AKT activation, impair apoptosis, and reactivate ERK independently of RAF inhibition^[36,56], thereby underpinning the observed intrinsic resistance. Our Western blot data show that luteolin and ganetespib suppress PI3K/AKT and RAF/MEK/ERK signaling in the acquired-resistance models (Figs. 6–7)—mechanisms that are likely more effective when PTEN and RAF signaling remain functionally targetable. Additionally, differences in Hsp90 client dependence and induction of cytoprotective responses (e.g., Hsp70 upregulation upon Hsp90 blockade^[34]) may further influence responsiveness to ganetespib. Although these hypotheses align with prior reports, the precise mechanistic basis for these model-dependent responses was not systematically investigated and remains to be elucidated.

Beyond these differences, we observed distinct 2D versus 3D growth phenotypes for the two acquired-resistance sublines. A375-DR cells proliferated robustly in 2D yet formed loose spheroids, whereas A375-VR cells proliferated less in 2D but generated compact, rapidly expanding spheroids in 3D (Figs. 2A–B). Prior studies in A375 models have reported that vemurafenib resistance can enhance 3D proliferation and cohesion^[33], whereas dabrafenib resistance can promote invasive traits characterized by increased VEGF-A and MMP-9, extracellular matrix (ECM) remodeling, and weakened adhesion^[57], which may contribute to the loose spheroids observed in A375-DR. These observations suggest that long-term exposure to different BRAFi selects for heterogeneous adaptive states that rewire adhesion/ECM signaling—potentially via integrin β 1/FAK pathways^[58]—and drive context-dependent growth. Side-by-side comparisons with parental cells and systematic adhesion/ECM profiling will be needed to delineate how resistance reshapes these properties.

Mechanistically, dual blockade of the PI3K/AKT and RAF/MEK/ERK pathways is a rational strategy to counteract BRAFi resistance^[59,60], given the frequent compensatory activation of PI3K/AKT when MAPK signaling is suppressed^[38]. However, clinical trials combining PI3K/AKT and MEK inhibitors in melanoma have been constrained by poor tolerability^[61,62]. A prior biochemical study reported that luteolin competitively inhibits PI3K and noncompetitively inhibits RAF in cell-free assays^[63]. Here, our Western blot analyses confirm that luteolin concomitantly inhibits PI3K/AKT and RAF/MEK/ERK signaling in BRAFi-resistant cutaneous melanoma cells and mouse tumors. This dual-pathway mechanism warrants evaluation of luteolin both as a monotherapy and as an adjunct to BRAFi/MEKi or other targeted agents, with the potential

to mitigate compensatory signaling while maintaining tolerability. Rigorous dose-finding, PK modeling, and assessment of drug-drug interactions will be essential to translate such combinations safely.

In this study, we did not perform matched, head-to-head dose-response comparisons between parental and BRAFi-resistant lines under identical conditions; therefore, differences in drug sensitivity between BRAFi-sensitive and BRAFi-resistant models cannot be inferred. To address this, future work will include parallel dose-response analyses and time-course studies in parental cells and their resistant derivatives to rigorously evaluate potential differences in luteolin sensitivity. Additionally, our *in vivo* studies used male BALB/c nude mice, which lack mature T cell-mediated immunity^[64], thereby limiting assessment of immunomodulatory effects—particularly relevant given luteolin's anti-inflammatory properties^[65] and the enrichment of immune-related pathways in our analysis (Fig. 5C): Interleukin-4 and Interleukin-13 signaling (ranked 3rd), Signaling by Interleukins (ranked 4th), and Cytokine Signaling in Immune System (ranked 5th). Immunocompetent models, co-culture systems, and syngeneic or orthotopic studies will be important to evaluate luteolin's effects on antitumor immunity in BRAFi-resistant melanoma models.

5. Conclusions

In conclusion, our study demonstrates that luteolin, a naturally occurring dietary flavonoid, exerts significant anticancer effects in preclinical models of acquired BRAF inhibitor-resistant cutaneous melanoma, both *in vitro* and *in vivo*. Our data are consistent with a mechanism involving concurrent inhibition of the PI3K/AKT and RAF/MEK/ERK signaling pathways. Luteolin demonstrated favorable tolerability and relative tumor selectivity in our models, supporting its potential for further development. Beyond its established health benefits, these findings highlight a novel application for luteolin in cancer therapy and underscore its translational relevance as a functional food or nutraceutical. Further investigation into formulation optimization, bioavailability enhancement, and clinical validation is warranted to advance luteolin-based strategies for managing drug-resistant cutaneous melanoma.

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Declaration of competing interest

The authors declare no competing interests.

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