

20(S)-Ginsenoside Rh2 Promotes Apoptotic and Autophagic Cell Death in Melanoma Cells by Inhibiting Src/STAT3 Signaling Pathway

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ABSTRACT

20(S)-Ginsenoside Rh2 (GRh2) has been the subject of extensive research due to its wide range of health benefits. Nevertheless, its specific impact on melanoma is still not fully elucidated. The current work examined the anti-melanoma properties of GRh2 along with the underlying mechanisms involved. MTT assays, EdU staining, flow cytometry, cellular thermal shift assay (CETSA), confocal microscopy, molecular docking, molecular dynamics (MD) simulations, immunoblotting, and a B16F10 cell-bearing mouse model were utilized to evaluate the anti-melanoma activity and mechanism of GRh2. GRh2 inhibited cell proliferation, caused G0/G1 phase cell cycle arrest, and promoted apoptosis in melanoma cells. The compound also induced autophagy and reduced the activity of mTOR, a major negative regulator of autophagy, in these cells. Inhibition of autophagy further strengthened the anti-melanoma activity of GRh2. Molecular docking, MD simulations, and CETSA experiments indicated that GRh2 binds stably to the Src protein, an upstream kinase of STAT3. GRh2 suppressed the activation of both Src and STAT3, thereby blocking STAT3 nuclear translocation in melanoma cells. Over-activation of STAT3 diminished the cytotoxic, pro-apoptotic, and autophagy-inducing effects of GRh2. In addition, GRh2 inhibited the growth of B16F10 tumors in mice without causing noticeable toxicity. It decreased the protein levels of phospho-Src, phospho-STAT3, phospho-mTOR, and Mcl-1, while increasing cleaved-PARP and LC3B-II levels within the B16F10 tumors. GRh2 displays anti-melanoma effects mainly by suppressing Src/STAT3 signaling. This research enhances understanding of the anti-melanoma mechanism of GRh2 and suggests that consumption of GRh2 may help slow melanoma progression.

Keywords: 20(S)-Ginsenoside Rh2, Melanoma, Apoptosis, Autophagy, Src/STAT3 signaling

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Introduction

Melanoma is a highly aggressive cancer [1] that presents a serious challenge to public health. Available treatment options include surgery, radiotherapy, immunotherapy, targeted therapies, and chemotherapy. Although these interventions can restrain melanoma progression, their clinical effectiveness is often limited. Due to drawbacks such as severe side effects and the emergence of drug resistance, patient prognosis remains unsatisfactory [2]. This situation emphasizes the critical need for alternative therapeutic approaches.

Ginseng (*Panax ginseng*) is widely employed as a functional food and medicinal herb in Asia. Saponins isolated from ginseng roots are frequently used in herbal medicines and dietary supplements [3]. 20(S)-Ginsenoside Rh2 (GRh2), a key triterpenoid saponin component of ginseng, has shown potential cytotoxic activity against several cancer cell types, including those from breast, colorectal, cervical, and pancreatic cancers [4]. However, the anti-melanoma effects of GRh2 have not been clearly established.

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that is abnormally activated in melanoma tissues and plays an essential role in melanoma development [5]. STAT3 becomes phosphorylated at Tyr705 via upstream non-receptor tyrosine kinases such as Src. This phosphorylation promotes its nuclear translocation and the transcription of target genes [6]. STAT3 also regulates autophagy in different cancers [7]. Autophagy serves initially as a protective mechanism that supports cell survival under stress by removing damaged cellular components. Yet, its function in cancers including melanoma is complex and may depend on disease stage [8]. Recent findings highlight the dual outcomes of autophagy modulation in melanoma treatment [8], underscoring the importance of clarifying its role when developing new anti-melanoma agents. In summary, inhibition of STAT3 activation and appropriate manipulation of autophagy are considered promising strategies for melanoma therapy.

In this study, we observed that GRh2 exerts anti-melanoma effects, with suppression of the Src/STAT3 pathway being partly responsible for this activity.

Materials and Methods

Reagents and chemicals

Dacarbazine (DTIC) was purchased from MedChemExpress (NJ, USA). Dimethyl sulfoxide (DMSO), rapamycin (Rapa), bafilomycin A1 (Baf-A1), and chloroquine (CQ) were obtained from Sigma-Aldrich (MO, USA). The Cell-Light™ EdU Apollo®567 kit was acquired from Ribobio (Guangzhou, China). GRh2 (purity $\geq 98\%$ as determined by HPLC analysis), (**Figure 5a**) was provided by Chengdu Alfa Bio-Technology Co. Ltd (Chengdu, China).

Cell culture

A375, B16F10, and L929 cells obtained from ATCC were maintained in DMEM with 10% FBS and 1% penicillin/streptomycin at 37°C under 5% CO₂.

Cell viability assays

Owing to melanin production in B16F10 cells that interferes with absorbance measurements, crystal violet staining was used to assess GRh2 cytotoxicity in these cells. MTT assays were employed for A375 cells. A375 cells (5×10^3) seeded in 96-well plates and B16F10 cells (2×10^5 cells/well) seeded in 6-well plates were exposed to GRh2 (0–60 μ M) for 24 or 48 h [9]. Standard MTT and crystal violet procedures were followed.

Colony formation and 5-ethynyl-2'-deoxyuridine (EdU) staining assays

For EdU staining, 4×10^4 melanoma cells seeded in 4-well plates were treated with GRh2 for 24 h. Proliferation was evaluated using the EdU assay kit per manufacturer instructions. For colony formation, 400 melanoma cells per well in 6-well plates were treated with GRh2 for 24 h, after which the medium was replaced and cells were cultured for another 7 days [10].

Flow cytometric analysis

Melanoma cells were treated with GRh2 (0–40 μ M) for 24 h. For cell cycle analysis, cells were fixed in cold 70% ethanol and stained with PI/RNase A buffer. Apoptosis was measured using an Annexin V/PI detection kit. Data were collected on a flow cytometer.

Subcellular fractionation

Cells in 90-mm dishes were treated with GRh2 for 24 h. Nuclear and cytoplasmic fractions were isolated using the NE-PER kit following manufacturer instructions.

Molecular docking and molecular dynamics (MD)

Binding interactions between Src (PDB ID: 1YOL) and GRh2 (PubChem CID: 119307) were investigated by molecular docking with AutoDockTools 1.5.6. MD simulations were conducted using YASARA as described previously [11].

Cellular thermal shift assay (CETSA)

Cell lysates were prepared with RIPA buffer containing inhibitors, incubated with GRh2 (80 μ M) or DMSO, and subjected to a temperature gradient (37–57°C) before immunoblotting [11].

Immunoblotting analysis

Proteins extracted from cells and tumors using RIPA buffer were analyzed by Western blotting as reported previously [12]. Antibodies targeted LC3B (#2775), CDK6 (#13331), Mcl-1 (#4572), Bcl-xL (#2764), Cyclin D1 (#2978), Src (#2108), phospho-Src (Tyr416) (#6943), STAT3 (#9139), phospho-STAT3 (Tyr705) (#9145), Cyclin A2 (#91500), CDK4 (#12790), PARP (#9542), CDK2 (#2546), phospho-mTOR (Ser2448) (#5536), mTOR (#2983) from CST, and β -actin from Santa Cruz.

LC3 puncta analysis

Cells transfected with Premo™ RFP-GFP-LC3B tandem sensor were treated with GRh2 and imaged by confocal microscopy [10].

Immunofluorescence analysis

Cells were treated with GRh2, fixed, permeabilized, blocked, and stained with anti-STAT3 antibody followed by fluorescent secondary antibody and DAPI. Images were acquired by fluorescence microscopy.

STAT3 overactivation in A375 cells

STAT3-overactivated A375 cells were established as previously described [9].

In vivo studies

B16F10 cells ($1 \times 10^6/100 \mu\text{l}$ PBS) were injected subcutaneously into C57BL/6 mice. Mice were randomized into groups receiving vehicle, DTIC (50 mg/kg), or GRh2 (20, 40, or 60 mg/kg) intraperitoneally daily for 15 days. Tumor volumes and body weights were monitored. All procedures were approved by the Department of Health, Hong Kong [No.: (22–147) in DH/HT&A/8/2/6 Pt.6].

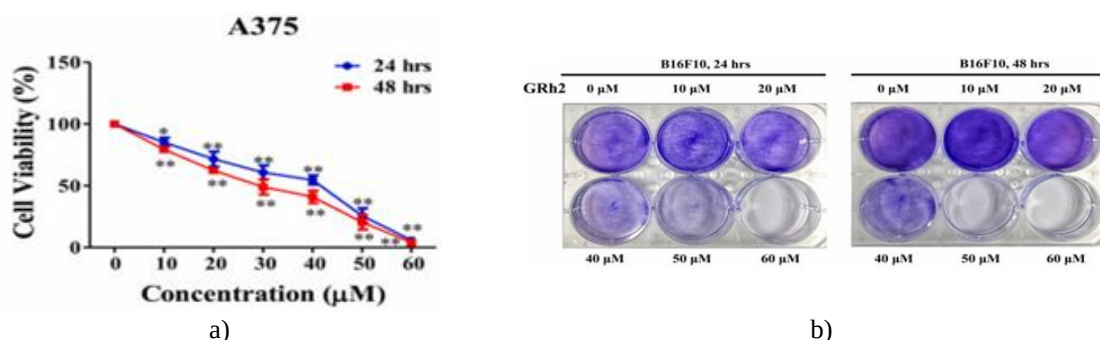
Statistical analysis

Results are expressed as means $\pm$ SD from triplicate experiments. Data were analyzed using GraphPad Prism 9 with Student's t-test or one-way ANOVA with Dunnett's test. $P < 0.05$ was considered statistically significant.

Results and Discussion

GRh2 inhibits melanoma cell proliferation

MTT and crystal violet assays in **Figures 1a and 1b** showed that GRh2 decreased viability of A375 and B16F10 cells, respectively. Cytotoxicity was lower in normal L929 fibroblasts than in melanoma cells. EdU staining and colony formation assays demonstrated that GRh2 significantly reduced EdU-positive cells (**Figure 1c**) and colony numbers in melanoma cells (**Figure 1d**).



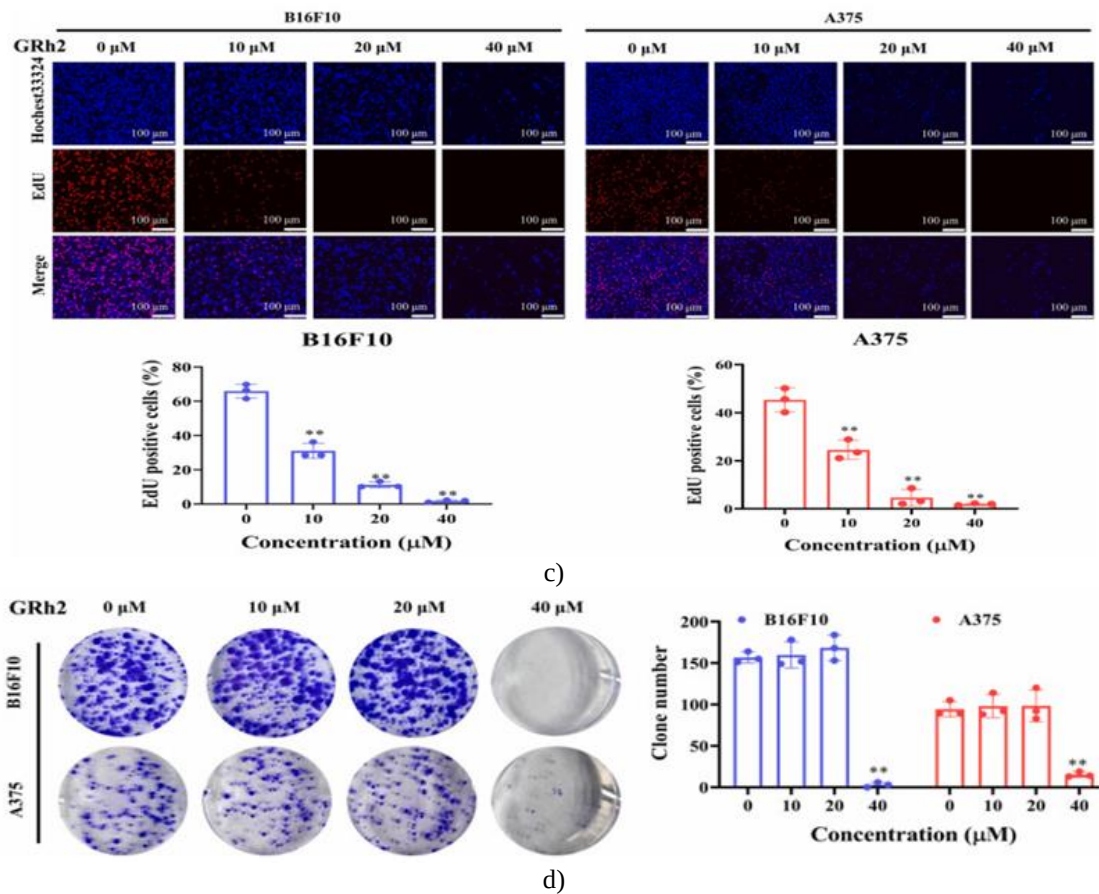


Figure 1. GRh2 suppresses proliferation of melanoma cells. Treatment with GRh2 led to reduced viability in both (a) A375 and (b) B16F10 cell lines. (c) The fraction of EdU-positive melanoma cells declined following GRh2 exposure. (d) Colony formation by melanoma cells was also significantly diminished. * $P < 0.05$, ** $P < 0.01$ versus the untreated control group.

GRh2 causes cell cycle arrest at the G0/G1 phase in melanoma cells

Relative to the untreated control, GRh2 exposure resulted in a substantial increase in the G0/G1 population in both B16F10 and A375 melanoma cells, accompanied by a clear reduction in the S-phase fraction (**Figure 2a**). Consistent with this observation, immunoblotting showed that GRh2 markedly lowered the protein expression of several critical G0/G1 regulators, such as CDK2, CDK4, CDK6, Cyclin A2, and Cyclin D1 (**Figure 2b**).

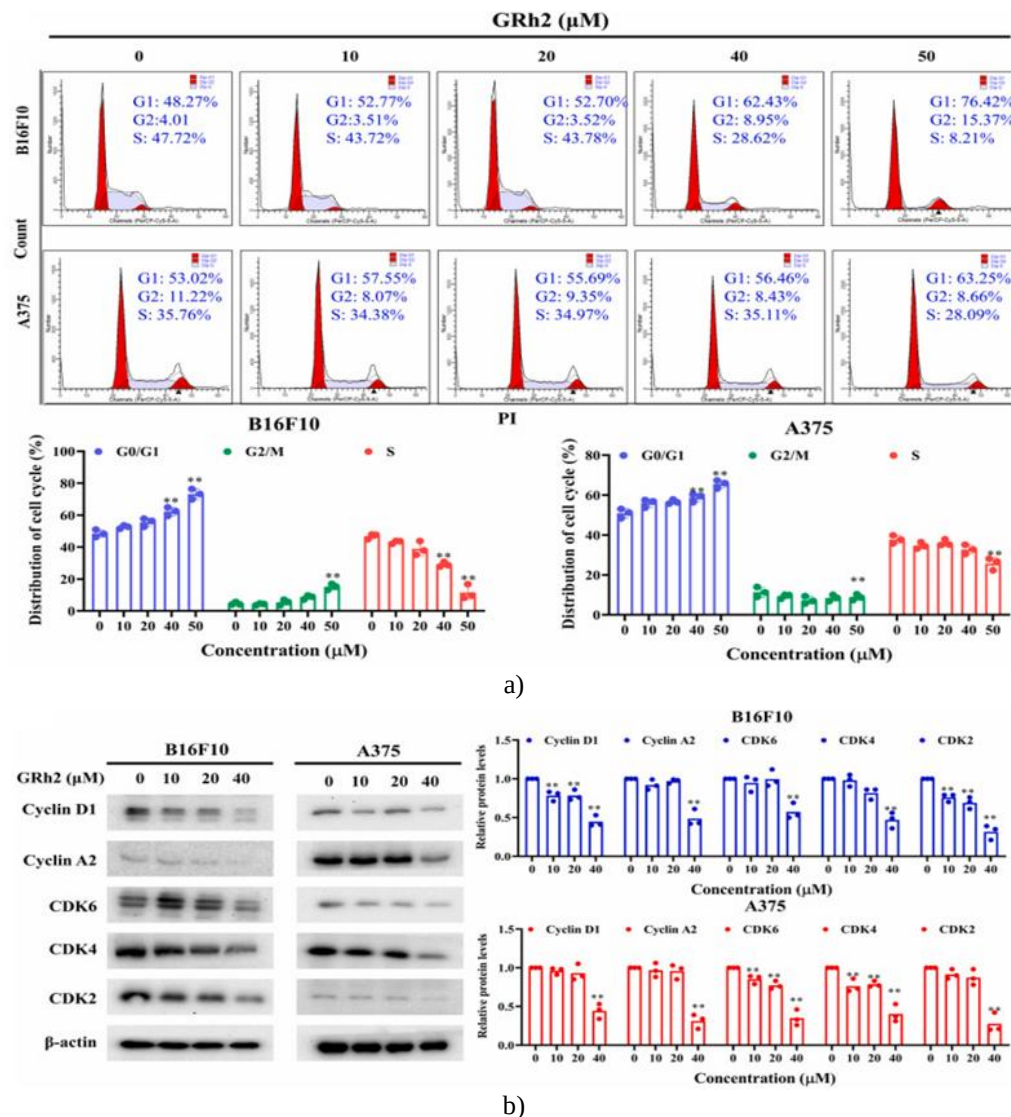


Figure 2. GRh2 induces G0/G1 phase cell cycle arrest in melanoma cells. (a) Impact of GRh2 treatment on cell cycle distribution in melanoma cells. (b) Western blot showing changes in the expression of proteins associated with G0/G1 phase regulation. **P < 0.01 versus control group.

GRh2 triggers apoptosis in melanoma cells

As illustrated in **Figure 3a**, GRh2 substantially increased the percentage of apoptotic cells in both B16F10 and A375 lines. Cleavage of PARP serves as a well-established indicator of apoptosis. In addition, pro-apoptotic members (such as Bax) and anti-apoptotic members (such as Bcl-XL and Mcl-1) are known to critically regulate the apoptotic process. The results demonstrated that GRh2 notably raised the levels of cleaved-PARP and Bax while reducing the expression of the anti-apoptotic proteins Bcl-XL and Mcl-1 in melanoma cells (**Figure 3b**). In contrast, GRh2 failed to cause PARP cleavage in L929 normal cells, indicating that it does not promote apoptosis in non-cancerous cells.

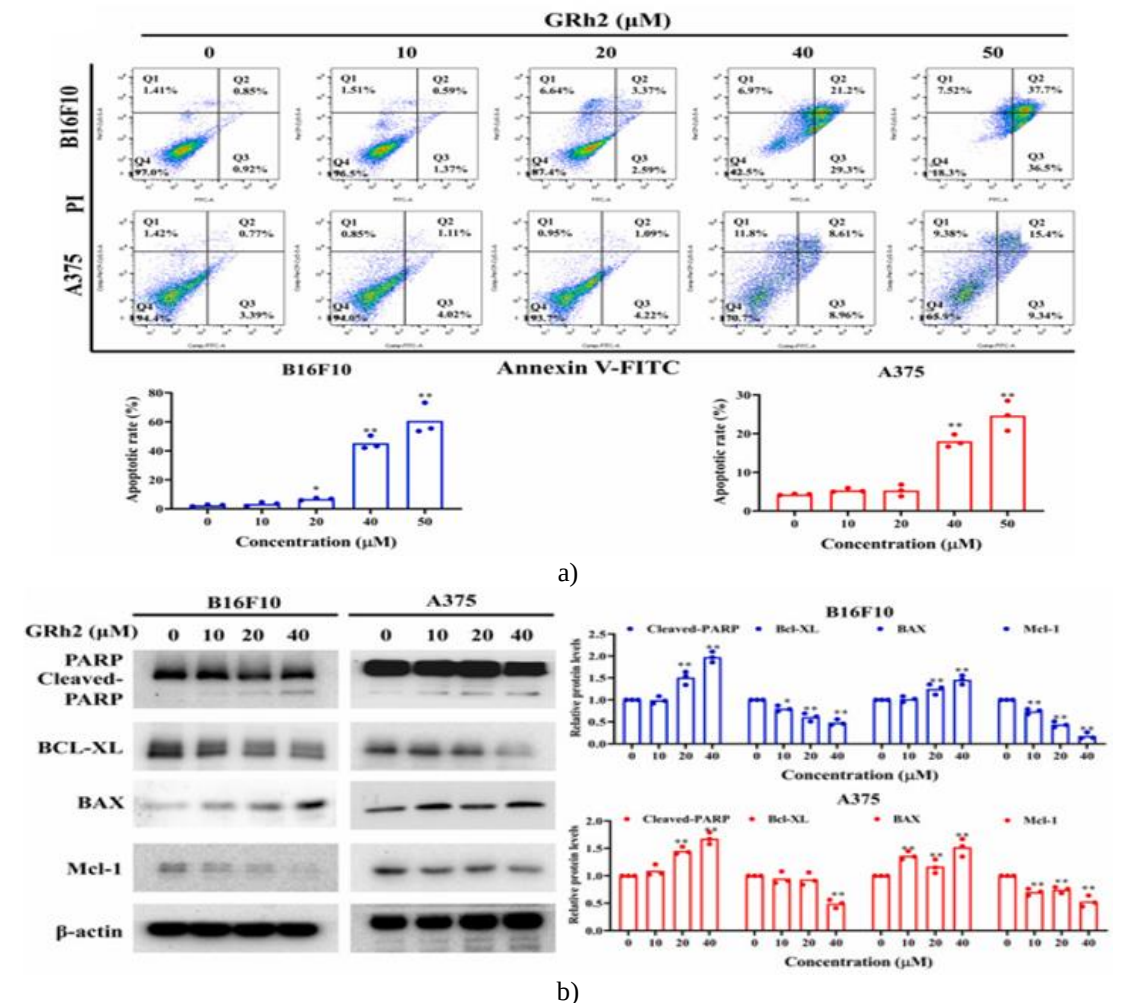
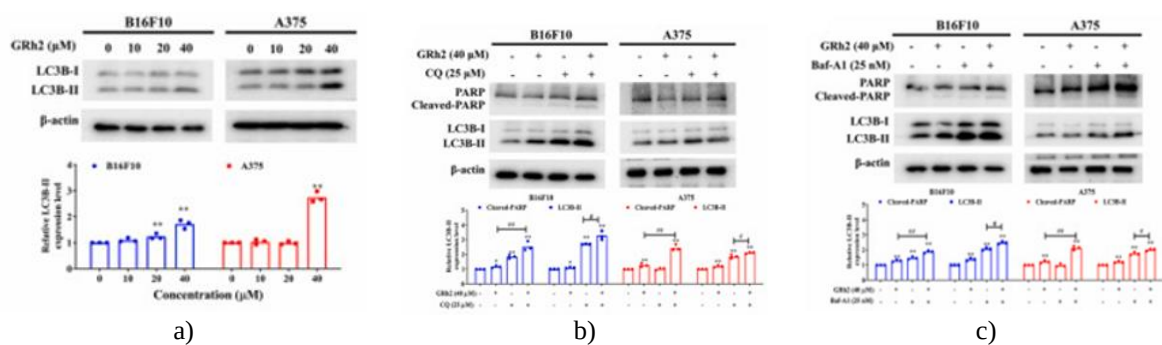


Figure 3. GRh2 promotes apoptosis in melanoma cells. (a) GRh2 raises the number of cells undergoing apoptosis. (b) GRh2 modifies the expression levels of PARP, Bcl-XL, BAX, and Mcl-1 proteins in melanoma cells. *P < 0.05, **P < 0.01 versus control group.

GRh2 stimulates autophagy in melanoma cells

To investigate the influence of GRh2 on autophagy processes in melanoma cells, researchers analyzed LC3B protein expression through Western blotting. When compared to DMSO-treated controls, GRh2 exposure caused a clear elevation in LC3B-II levels in both B16F10 and A375 cells. This observation points to GRh2 promoting the formation of autophagosomes in melanoma cells (**Figure 4a**). However, because accumulation of LC3B-II can also result from interrupted autophagic flux [13], additional tests were performed to distinguish between these possibilities. Pretreatment with the autophagy blockers CQ (**Figure 4b**) and Baf-A1 (**Figure 4c**) produced even higher LC3B-II accumulation than GRh2 alone in both cell lines. These results demonstrate that GRh2 actively enhances autophagic flux rather than blocking it in melanoma cells.



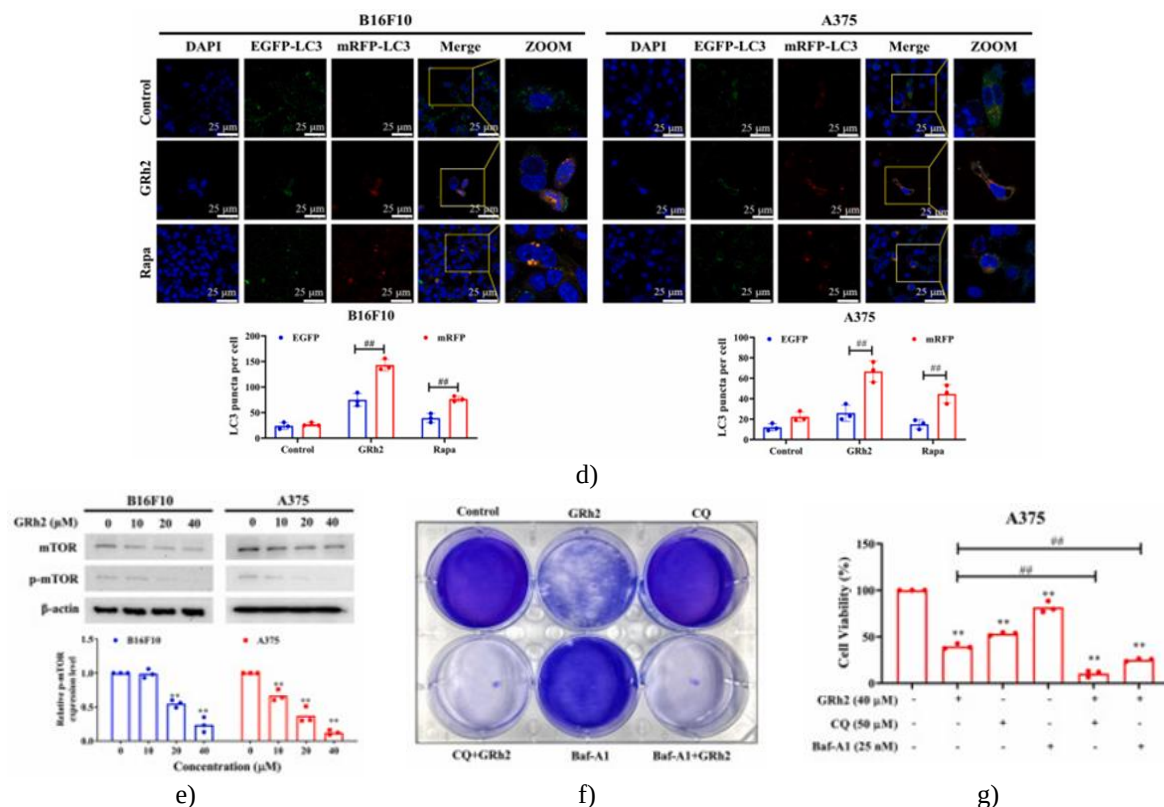
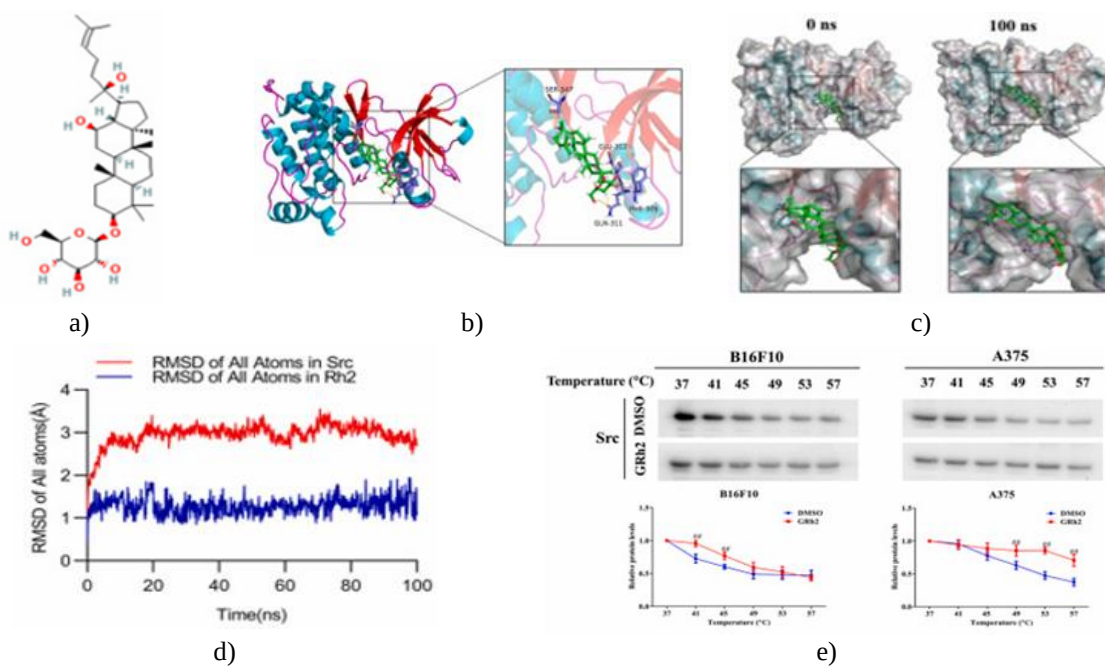


Figure 4. GRh2 induces protective autophagy in melanoma cells. (a) Changes in LC3B-II protein abundance in melanoma cells upon GRh2 treatment. (b and c) Western blotting results showing LC3B levels in melanoma cells treated with GRh2 alone or in combination with pretreatment using (b) CQ (25 μ M) and (c) Baf-A1 (25 nM). (d) GRh2 increases autophagic flux observed in B16F10 and A375 cells. Scale bar = 25 μ m. (e) GRh2 decreases the level of phosphorylated mTOR protein in melanoma cells. Effects on cell viability when GRh2 is combined with CQ or Baf-A1 in (f) B16F10 and (g) A375 cells. ** $P < 0.01$ versus control group. # $P < 0.05$, ## $P < 0.01$.



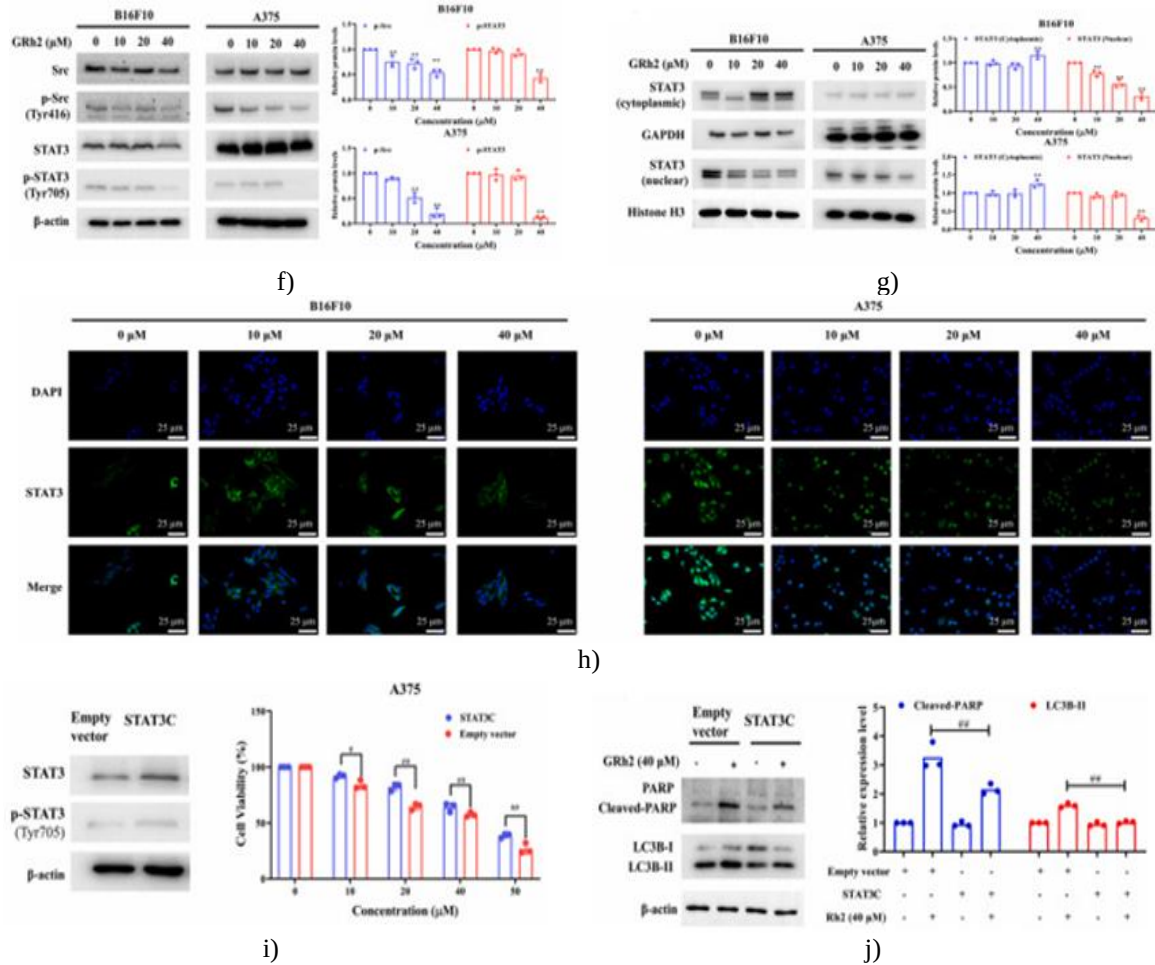


Figure 5. GRh2 displays anti-melanoma activity through inhibition of Src/STAT3 signaling. (a) Molecular structure of GRh2. (b) Three-dimensional docking pose showing GRh2 (green) bound within the Src pocket. (c) Surface views of the Src-GRh2 complex at 0 ns (left panels) and after 100 ns (right panels) of simulation. (d) RMSD trajectories of heavy atoms for free Src (blue line) and the Rh2-bound Src complex (red line). (e) CETSA results confirming GRh2 interaction with Src protein in melanoma cell lysates. ### $P < 0.01$ vs. DMSO treatment. (f) GRh2 blocks activation of the Src/STAT3 pathway in melanoma cells. (g) GRh2 decreases nuclear accumulation of STAT3 protein in melanoma cells. (h) Immunofluorescence examination of GRh2's effect on STAT3 nuclear translocation in B16F10 and A375 cells. After 24 h GRh2 incubation, STAT3 was stained green and nuclei with DAPI. Scale bar, 25 μ m. (i) Constitutive STAT3 activation reduced GRh2's ability to inhibit A375 cell viability. (j) Influence of GRh2 on LC3B and PARP protein expression in control vector versus STAT3C-overexpressing A375 cells. ** $P < 0.01$ versus control group. # $P < 0.05$, ### $P < 0.01$.

Melanoma cells were transiently transfected with a tandem RFP-GFP-LC3B reporter plasmid to validate autophagic flux induction by GRh2. Data in **Figure 4d** revealed a substantial increase in the mean number of red fluorescent puncta in GRh2-exposed B16F10 and A375 cells compared to DMSO controls, confirming autophagy stimulation. The autophagy inducer Rapa (positive control) likewise produced a strong elevation in red puncta counts (**Figure 4d**). As mTOR is a well-known suppressor of autophagy, its phosphorylated form was examined; GRh2 treatment reduced p-mTOR protein levels in melanoma cells (**Figure 4e**).

Further investigation addressed the connection between GRh2-driven autophagy and cell death. Results indicated that pharmacological inhibition of autophagy led to additional viability loss in GRh2-treated B16F10 and A375 cells (**Figure 4f**). Western blotting demonstrated markedly higher cleaved-PARP levels when GRh2 was combined with CQ (**Figure 4b**) or Baf-A1 (**Figure 4c**) compared to GRh2 monotherapy. These observations establish that GRh2 elicits cytoprotective autophagy in melanoma cells (**Figures 4b and 4c**). Therefore, autophagy blockade augments the therapeutic efficacy of GRh2 against melanoma.

Blockade of Src/STAT3 signaling participates in GRh2-mediated apoptosis and autophagy in melanoma cells

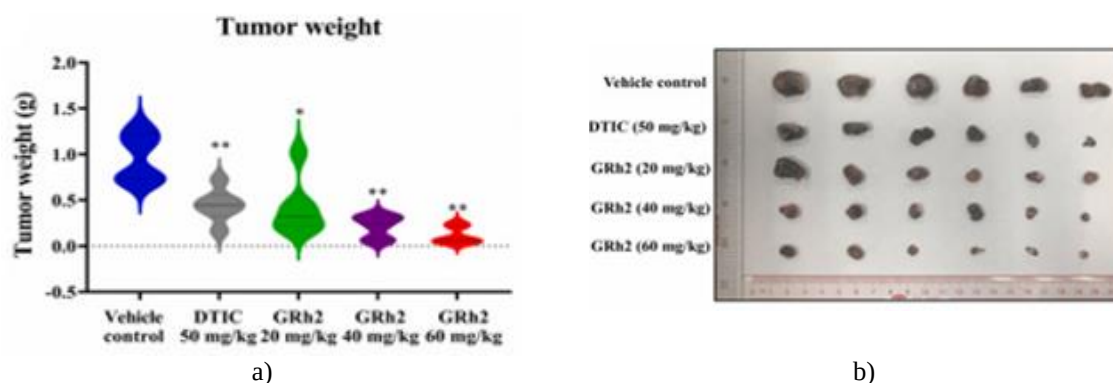
STAT3 functions as an important downstream component of Src-mediated survival signaling in melanoma cells [14]. Molecular docking studies were performed to assess whether Src represents a direct binding target for GRh2. GRh2 was found to occupy a hydrophobic groove lined by SER-347, GLU-312, PHE-309, and GLN-311 residues, consistent with interaction at the SH1 kinase domain of Src. The estimated binding affinity was -8.79 kcal/mol (**Figures 5a and 5b**). MD simulations were then run to corroborate the docking findings. Surface models illustrated that GRh2 stayed firmly anchored in the central binding site of Src throughout the entire 100 ns trajectory (**Figure 5c**). RMSD analysis showed that the GRh2-Src complex fluctuated mildly between 2.0–3.0 Å, whereas apo-Src remained more stable near 1.1 Å during the simulation period (**Figure 5d**).

CETSA experiments provided further evidence of direct binding in cellular lysates. GRh2 treatment increased the thermal stability of Src, shifting its melting temperature from 41°C to 49°C in B16F10 cells and from 49°C to 57°C in A375 cells relative to DMSO controls (**Figure 5e**). Collectively, these experiments demonstrate stable and direct interaction between GRh2 and Src in melanoma cells.

Phosphorylation of Src at Tyr416 within the SH1 domain is essential for its kinase activity [15]. Western blot experiments confirmed that GRh2 suppressed Src kinase function by reducing phospho-Src (Tyr416) and downstream phospho-STAT3 (Tyr705) levels (**Figure 5f**). GRh2 also decreased nuclear STAT3 while increasing its cytoplasmic retention (**Figure 5g**). These findings were supported by immunofluorescence imaging showing blocked nuclear translocation of STAT3 in both cell lines after GRh2 treatment (**Figure 5h**). To examine the functional importance of STAT3 inhibition, A375 cells engineered to overexpress activated STAT3 were utilized. MTT assays indicated that STAT3 hyperactivation significantly rescued cell viability from GRh2 cytotoxicity (**Figure 5i**). In these cells, GRh2-induced increases in LC3B-II and cleaved-PARP were notably attenuated compared to empty vector controls (**Figure 5j**), indicating that downregulation of STAT3 is required for GRh2's effects on apoptosis and autophagy.

GRh2 suppresses tumor progression in mice bearing B16F10 xenografts

After 15 days of daily administration, GRh2 produced clear, dose-dependent inhibition of B16F10 tumor development compared with vehicle-treated animals. The highest dose (60 mg/kg) achieved the greatest antitumor effect among the tested doses. Relative to the control, tumor weights were lowered by 56.5%, 78.1%, and 88.3% in the 20, 40, and 60 mg/kg GRh2 groups, respectively (**Figure 6b**). Tumor volumes were also significantly smaller in all GRh2 groups starting after 9 days of treatment (**Figure 6c**). By the endpoint, volumes reached only 49.1%, 21.6%, and 19.8% of control values in the 20, 40, and 60 mg/kg cohorts. The reference compound DTIC (50 mg/kg) similarly reduced tumor burden. No overt toxicity was detected through clinical observation or major organ assessment, and body weight changes did not differ meaningfully across treatment groups (**Figure 6d**). Analysis of excised tumors by Western blotting showed GRh2-mediated reductions in phospho-Src, phospho-mTOR, and phospho-STAT3, together with elevations in cleaved-PARP and LC3B-II (**Figure 6e**). These *in vivo* data indicate that GRh2 impedes melanoma growth and suppresses Src/STAT3 signaling within tumor tissues.



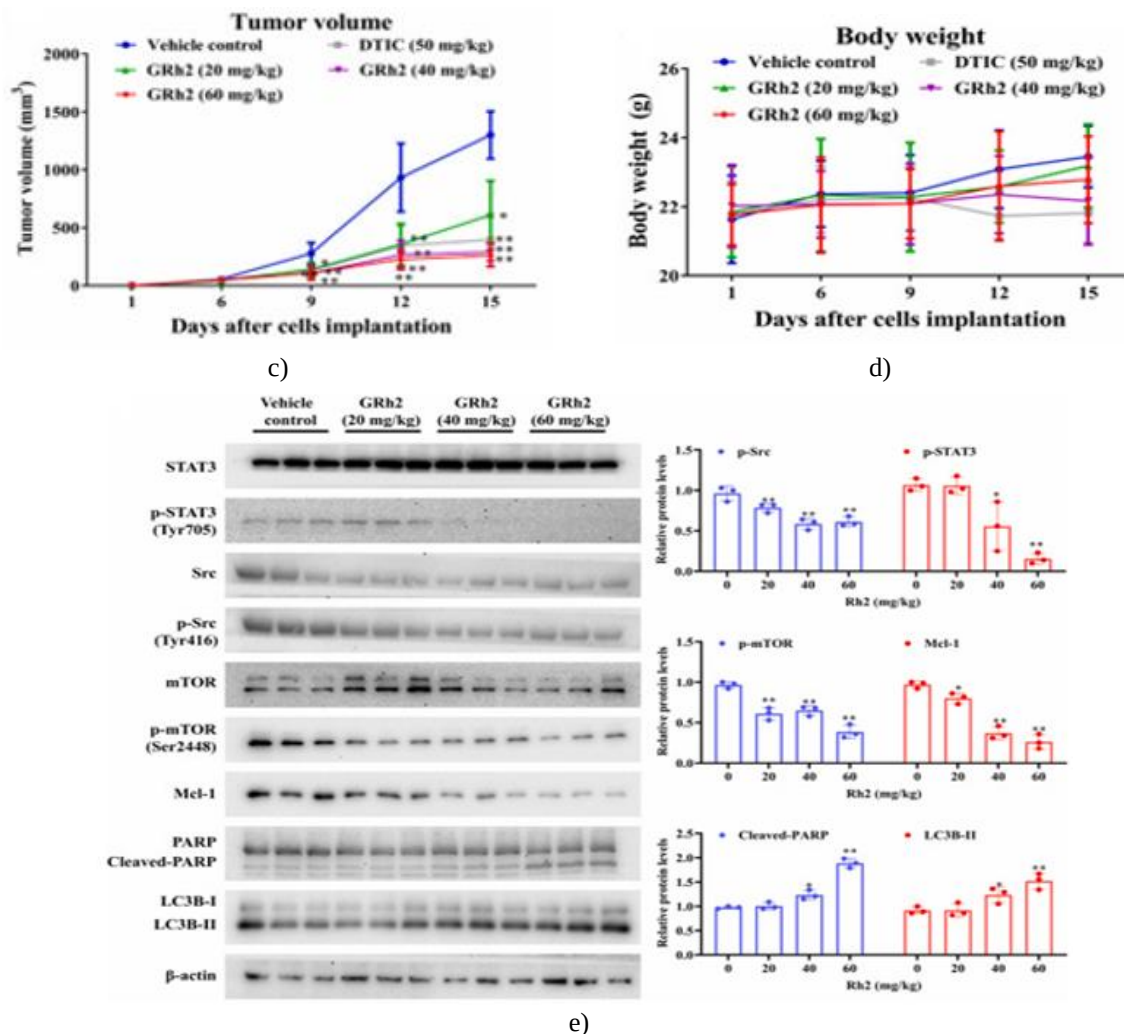


Figure 6. GRh2 hinders tumor growth in mice. (a) GRh2 lowers average tumor weights. (b) Photographs of excised tumors. (c) GRh2 decreases tumor volumes over time in mice. (d) No significant changes in body weights of mice treated with GRh2. $n = 6$ for panels (a) to (d). (e) Western blotting results showing the impact of GRh2 on protein levels of Src, phospho-Src, STAT3, phospho-STAT3, PARP, and LC3B in B16F10 allograft tumors. * $P < 0.05$, ** $P < 0.01$ versus the vehicle control group.

Ginseng is recognized as one of the top-selling natural products globally because of its notable tonic and health-supporting properties [16]. Ginsenosides possess substantial potential for development as anticancer agents, usable either independently or as adjunct therapies [17]. Among them, GRh2 has exhibited strong anticancer activity in various cancer models [18]. Nevertheless, investigations into the anti-melanoma activity of GRh2 are scarce. In this study, GRh2 was demonstrated to inhibit cell proliferation, induce G0/G1 phase cell cycle arrest, trigger apoptosis, and activate autophagy in melanoma cells. In addition, GRh2 markedly suppressed B16F10 tumor growth in vivo. Consistent with prior research, GRh2 treatment did not produce obvious toxicities [19], suggesting it is a relatively safe and effective candidate for melanoma management.

Autophagy plays a complex and dual role in cancer development and therapy, functioning as either cytoprotective or cytotoxic depending on the circumstances [20]. As such, its specific contribution must be thoroughly assessed during the development of new cancer treatments [21]. Here, GRh2 was found to promote autophagic flux in melanoma cells. Given that mTOR serves as a key negative regulator of autophagy [22], the observed reduction in phospho-mTOR protein levels by GRh2 supports its autophagy-inducing effect in melanoma cells. Further experiments showed that pharmacological inhibition of autophagy using specific inhibitors led to greater reductions in cell viability and enhanced apoptosis in GRh2-treated melanoma cells. These observations indicate that the autophagy triggered by GRh2 is cytoprotective in nature. Therefore, the strategy of combining GRh2 with autophagy inhibitors may offer an improved approach for melanoma treatment.

Activation of STAT3, which occurs through phosphorylation at the Tyr705 residue, is closely associated with enhanced cell survival, resistance to apoptosis, invasion, and metastasis [23]. Persistent STAT3 activation is frequently observed in melanoma [24], positioning STAT3 as a valuable therapeutic target for cancer drug development [25]. Although STAT3 inhibitors have demonstrated therapeutic benefits, their clinical translation is often limited by serious side effects [26]. Thus, the identification of safer and more effective STAT3 inhibitors holds considerable importance. Previous studies have shown that GRh2 can suppress STAT3 activation in non-small-cell lung cancer [27] and liver cancer cells [28]. The present results extend these findings by showing that GRh2 effectively represses STAT3 activity in melanoma cells as well as in allograft tumors. GRh2 also reduced nuclear STAT3 protein levels and downregulated STAT3 target genes such as Bcl-xL and Mcl-1 (which are associated with cell survival [12]) and Cyclin D1 (a regulator of G0/G1 phase progression) in melanoma cells. Overexpression of STAT3 weakened the anti-melanoma effects of GRh2, confirming that STAT3 suppression contributes significantly to GRh2's activity. Moreover, this study revealed that GRh2 directly binds to Src, an important upstream kinase responsible for STAT3 activation, with strong affinity, thereby inhibiting its activity and subsequently suppressing STAT3 signaling.

Earlier reports indicated that STAT3 inhibitors can promote autophagy in cancer cells by disrupting the STAT3-PKR interaction [29]. Other research showed that STAT3 negatively regulates autophagy through the Bcl2-Becclin 1 axis in cervical cancer cells [30]. Nuclear translocation of STAT3 further enables transcriptional control of multiple autophagy-related genes, including PIK3C3, CTSB, Bcl-2, BECN1, and CTSL [31]. Consistent with these mechanisms, STAT3 overactivation in our experiments resulted in decreased LC3B-II protein expression in A375 cells.

However, the current study has certain limitations. Melanoma commonly features somatic mutations in genes such as BRAF, PTEN, NRAS, and p53, with BRAFV600E representing the most common alteration [32]. Although A375 cells harbor the BRAFV600E mutation, future investigations will incorporate additional melanoma cell lines with varied genetic backgrounds, including A2058 (BRAFV600E, p53V274F, PTENnull), Hs294T (BRAFwt, p53 wt), WM-115 (BRAFV600D), IGR1 (BRAFV600K), and SK-MEL-2 (NRASQ61R, p53S245G), to more comprehensively evaluate GRh2's anti-melanoma effects. Furthermore, GRh2 was observed to substantially increase LC3B-II protein levels in L929 normal cells. Whether this leads to actual autophagy induction in L929 cells will be examined in subsequent studies. Finally, the detailed molecular mechanisms by which GRh2 inhibits STAT3 and induces autophagy require further clarification in future research.

Conclusion

In summary, GRh2 exerts anti-melanoma effects at least partially through the inhibition of Src/STAT3 signaling. Concurrent blockade of autophagy enhances the anti-melanoma efficacy of GRh2. The findings from this study provide deeper insights into the anti-melanoma mechanism of GRh2 and suggest that intake of GRh2 may help retard melanoma progression.

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Conflict of Interest: None

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Ethics Statement: None

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