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SPHINX31 suppresses splicing factor phosphorylation and inhibits melanoma cell growth and aggressiveness

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Abstract

Melanoma is a tumor resulting from the malignant transformation of skin or ocular melanocytes, and a serious health problem in countries with high UV exposure. The late detection, high invasive and metastatic potential of melanoma cells, and lack of effective treatments have led to poor prognosis and a high mortality rate among melanoma patients. The aberrant mRNA transcripts derived from alternative splicing have contributed to the progression of various types of cancer. Serine/Arginine-rich Splicing Factors (SRSFs) are responsible for mRNA splicing under the specific regulation of Serine-Arginine Protein Kinases (SRPKs). This study investigates the effects of the SRPK1-specific inhibitor SPHINX31. Cell viability was determined in A375 (cutaneous melanoma cell) in comparison to 92-1 (ocular melanoma cell) by MTT viability assays. The inhibitory effect of SPHINX31 on melanoma cell viability is presented in a dose- and time-dependent manner, with western blot analysis then performed to observe the suppression of kinase activity by SPHINX31. A decrease in phosphorylated SRSFs (pSRSFs) was demonstrated by both cells. The growth inhibition of SPHINX31 was examined by clonogenic assay, with the size and number of both A375 and 92-1 cell colonies decreasing. Remarkably, the results of SPHINX31 in other cancer phenotypes studies on A375 cells showed a significant effect on growth inhibition. The findings of this study reveal that SPHINX31 reduces the dead-evasion and migration abilities of the A375 cell. The collected data should serve as a strong foundation for developing new alternative therapeutic strategies for melanoma treatment by targeting SRPK1 activation.

Keywords**:** *alternative splicing; cancer phenotype; melanoma; phosphorylation; splicing factor; SRPK1*

1. Introduction

Melanoma is a tumor resulting from the malignant transformation of melanocytes in various anatomic locations. Classification of melanoma is based on the tissue origins and two common types are cutaneous melanoma and ocular melanoma. The incidence of melanoma is high in Australasian, North American, and European populations (Laikova et al., 2019). However, the risk factors of melanoma are exposure to ultraviolet (UV) radiation and genetic defects, leading to the promotion of melanoma development (Nasti & Timares, 2015; Laikova et al., 2019). In high UV exposure counties, including Thailand, melanoma is the most common cause of mortality (Suwanrungraung & Kamsa-ard, 2007). Although there are several therapeutic strategies for melanoma treatment, they remain ineffective on highly toxic or resistant-presenting phenomena (Menaa, 2013; White, Knight, Butler, & Burnstock, 2009; Megahed, Schön, Selimovic, & Schön, 2002). Moreover, metastatic melanoma is one of the most highly mutated, heterogenous, and lethal types of

this cancer (Kozar, Margue, Rothengatter, Haan, & Kreis, 2019).

Many recent studies represent the translational impact of aberrant alternative splicing, including the upregulation and dysregulation of various cancer models. Alternative splicing is the post-transcriptional modifications process of RNA synthesis, resulting in a single gene (multi-exon gene) being coded for multiple mRNA transcripts, for potential translation into various protein isoforms (Bonomi et al., 2013). Aberrant alternative splicing contributes to the development and progression of various types of human cancer including lung, liver, and breast cancer (Pio & Montuenga, 2009; Liu et al, 2014; Xiping, Qingshan, Shuai, Hongjian, & Xiaowen, 2017), and recently, a high incidence of cholangiocarcinoma and mortality have been reported among the Thai population (Yosudjai, Wongkham, Jirawatnotai, & Kaewkong, 2019). Therefore, aberrant alternative splicing is already recognized as another hallmark of cancer (Ladomery, 2013).

The mechanism of alternative splicing is regulated by the nucleic acid binding proteins, Serine/Arginine-rich (SR) family. Serine/Arginine-rich Splicing Factor or SRSFs play an essential role in the spliceosome function for processing pre-mRNA into mature mRNA. There are 12 SRSFs (SRSF1-SRSF12), containing different numbers of RNA recognition motifs (RRMs) and arginine-serine rich (RS) domains. Each SRSF presents specific 6–10 nucleotide sequences for the RRM target region (Änkö et al., 2014), binding to their mRNA targets. Dysregulation or overexpression of SRSFs can facilitate abnormal mRNA splicing which might be generated by the aberrant transcript variants. The derived cancer related-protein isoforms then present their oncogenic properties and potentially contribute to cancer aggression (da Silva et al., 2015).

Serine-Arginine Protein Kinase (SRPK) phosphorylates protein into the RS domain of SRSF. Several studies have demonstrated the oncogenic functions of SRPK in regulating the alternative splicing of various tumor suppressor genes to present the opposite function, or oncogenes to enhance the aggressiveness of cancer phenotypes (Gammons et al., 2014; Siqueira et al., 2015; Moreira et al., 2018). Interestingly, SRPK can serve as a target protein for cancer treatment, leading to the development of specific chemical substances to act as SRPK inhibitors. Recently, an SRPK1-specific inhibitor, namely SPHINX31, has been developed by structurally modifying SRPIN340, a pan-SRPK inhibitor (Batson et al., 2017). Although a previous report showed the upregulation of SRSF1 and SRPK1 in melanoma cell lines, the researchers used SRPIN340, which is a pan-inhibitor targeting both SRPK1 and SRPK2, thus identifying a research gap and the consequent availability of the SRPK1-specific inhibitor (SPHINX31). Therefore, this study aims to investigate the effect of SPHINX31 on cell viability in cutaneous and ocular melanoma cells, subsequently examining its inhibitory effects on SRSF phosphorylation, growth, death evasion, and the migration abilities of melanoma cells.

2. Objectives

To determine the effect of SPHINX31 on splicing factor phosphorylation and the aggressiveness of phenotypes in cutaneous and ocular melanoma cells including *in vitro* growth, death evasion, and migration abilities.

3. Materials and methods

3.1 Cell culture

The A375 (cutaneous melanoma) cells were provided for this study by the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand, while 92-1 (ocular melanoma cells) were purchased from Merck Millipore, MA, USA. Dulbecco's Modified Eagle Medium (DMEM) and RPMI 1640 were used to sub-passage and culture A375 and 92-1, respectively. The culture media were supplemented with 10% (v/v) fetal bovine serum (FBS) with 100 units/ml of penicillin and 100 μg/ml of streptomycin (Thermo Fisher Scientific, MA, USA), maintained at 37 °C and 5% CO_2. The cells were sub-cultured twice a week using 0.025% w/v trypsin/EDTA.

3.2 SRPK inhibitor

The SPHINX31 was purchased from the Cayman Chemical Company, MI, USA. Cells at ~70% confluence were serum starved for at least 12 h and treated with different concentrations of SPHINX31 (0-80 μM, depending on the experimental design), with 0.5% (v/v) DMSO added for vehicle control.

3.3 Cell proliferation assay

The effect of SPHINX31 on cell proliferation was determined by measuring cell

viability using an assay of 3-[4,5-dimethylthiazole]-2,5-diphenyltetrazolium bromide (MTT). The A375 and 92-1 cells were seeded in 96-well plates at 2×10^3 cells/well and then treated with different concentrations of SPHINX31 (0, 20, 40, 60, and 80 µM), incubated for 24, 48, and 72 h. Subsequently, 100 µl of MTT was added to a final concentration of 0.5 µg/ml and incubated at 37 °C for 4 h. The medium containing MTT was then removed, and the formazan crystals dissolved by DMSO (Sigma-Aldrich, MO, USA). Absorbances at 540 nm were observed by a microplate spectrophotometer, with the OD used for calculating.

3.4 Protein extraction and concentration measurement

Whole cell proteins from each treatment (different concentrations of SPHINX31) in A375 and 92-1 cells were isolated using a radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 2 mM EDTA, 0.1% sodium dodecyl sulphate, 50 mM Sodium fluoride) with a phosphatase inhibitor (Merck Millipore, MA, USA). The protein concentrations were determined by the Bradford assay. Briefly, 0.5 mg/ml of BSA was diluted into 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/µl in 96-well plates as standard proteins, and 1 µl of extracted proteins (10-folds dilution) then used as the protein sample. They were mixed with 200 µl of Bradford solution. After a 4 h incubation period, the absorbance was measured at 595 nm to calculate the protein concentration.

3.5 Western blot analysis

Equal amounts of proteins from each treatment (different concentrations of SPHINX31) in A375 and 92-1 cells were loaded and separated using SDS-PAGE gel (5% stacking gel and 15% separating gel), and then transferred to PVDF membranes (Bio-Rad Laboratories, WA, USA). The membranes were blocked using a 5% chemiluminescent blocker (Merck Millipore, MA, USA), and subsequently probed with primary antibodies: 1:1,000 of mouse anti-human phospho-epitope SRSFs (Merck Millipore, MA, USA) at 4 °C and left overnight and probed with secondary antibodies: 1:5,000 of HRP-conjugated goat-anti-mouse IgG (Thermo Fisher Scientific, Bremen, Germany) for 1 h. The protein bands were detected with Immobilon® ECL Ultra Western HRP Substrate (Merck Millipore, MA, USA), imaged by ImageQuant™ LAS 500 (GE Healthcare Life Sciences, Buckinghamshire, UK). The signal intensity of each band was quantitated using ImageJ software as a semi-quantitative expression, normalized by the intensity of the GAPDH protein.

3.6 Clonogenic assay

The A375 and 92-1 cells were plated at 500 and 2,000 cells/well, respectively, in a 6-well plate. After 24 h, the cells were treated with SPHINX31 at 20 and 40 µM for 72 h. The medium was changed every three days over a period of 10 days for the A375 cells, and 20 days for 92-1 cell. Cancer cell colonies were fixed in 4% (v/v) paraformaldehyde, stained by crystal violet, and then imaged and counted under the microscope. The colonies were counted using ImageJ software and the results expressed as a percentage of the untreated control cultures.

3.7 Live and dead staining assay

The A375 cells were plated at 16,000 cells/well in a 96-well black plate (SPL Life Sciences, Seoul, South Korea) for 24 h. The cells were treated with SPHINX31 at 0, 10, and 20 µM for 24 h. The cells were collected by centrifugation at 1,500 rpm for 5 min and the supernatant discarded. The cells were subsequently incubated using a staining solution (mix 1 µM Cyto-Dye, 1 mg/ml PI and 1,000 µl staining buffer) and live/dead cell dual staining kit (Merck Millipore, MA, USA) for 15 min before imaging and analyzing under a fluorescence microscope. Live cells were stained only with the cell-permeable Cyto-Dye to represent green fluorescence, whereas dead cells were stained with both the cell-permeable Cyto-Dye and non-permeable propidium iodide to represent orange-to-red fluorescence. The dead (orange-to-red) cells were counted and interpreted as the relative count of dead cells compared to control (0 µM of SPHINX31).

3.8 Migration assay

The A375 cells were plated at 400,000 cells/well into 12-well plates and cultured in 0, 10, and 20 µM of SPHINX31 for 24 h before creating a scratch wound on the monolayer of the cells using a 200 µl-pipette tip. The cells were then incubated in 1% FBS-media at 37 °C with 5% CO_2. Phase-contrast images were captured at 0, 24, and 48 h. The wound distance was observed at 24 and 48 h and compared with the wound distance at 0 h,

interpreted as the relative wound distance compared to control (0 µM of SPHINX31).

3.9 Statistical analysis

All data was expressed as the mean ± standard deviation (SD) from biological triplicate experiments. The different statistical significances between at least three different groups were determined with ANOVA. $P<0.05$ was considered to indicate a statistically significant difference, with the significant indicators being * $P<0.05$, ** $P<0.01$, and ***$P<0.001$.

4. Results and discussion

4.1 Effect of SPHINX31 on cell viability in A375 and 92-1 cells.

The cytotoxic potential of SPHINX31 was evaluated in A375 and 92-1 cells. Cell viability was determined, with the 92-1 cell demonstrating higher sensitivity to SPHINX31, even in a low concentration of 20 µM and showing just a slight decrease in viability until 80 µM-treatment. It should be noted that even though the A375 cell did not respond to SPHINX31 in low concentrations (20 and 40 µM) it was significantly sensitive to SPHINX31 in 60 and 80 µM in a dose- and time-dependent manner. Interestingly, the viability of A375 decreased to less than 50% with the 80 µM-treatment at 48 and 72 h (Figure 1). Therefore, the effect of SPHINX31 on cell viability can be summarized as being dose- and time-dependent, particularly in the A375 cells.

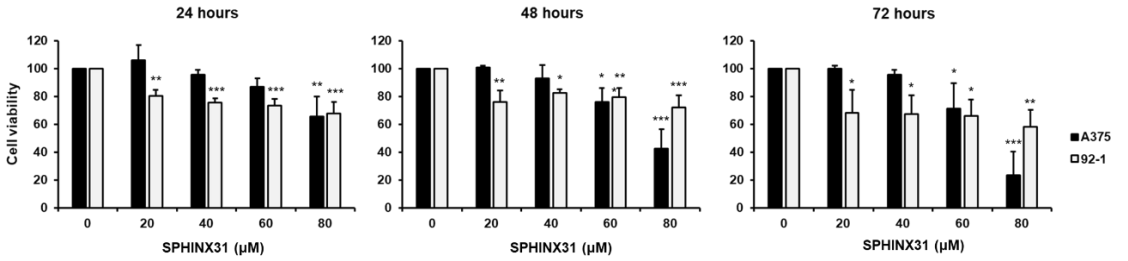

Figure 1 The effect of SPHINX31 treatments (in varying concentrations) on the viability of A375 and 92-1 cells in 24-hour intervals for three days. * $P<0.05$, ** $P<0.01$, and ***$P<0.001$.

4.2 Suppression of SRSF phosphorylation using SPHINX31

Regarding cell viability, 20 and 40 µM of SPHINX31 were selected to study the molecular function of SPHINX31 in melanoma cells. Following SPHINX31 treatment in A375 and 92-1 cells and the collection of whole cell proteins, western blot analysis was performed to evaluate the kinase activity of SPHINX31. The phosphorylation of SRSFs was evaluated using anti-phospho-epitope SR protein and represented in various molecular weights (MW) of phosphorylated SRSFs (pSRSFs). The band intensities of predominant pSRSFs are shown in Figures 2a and 2b. After semi-quantification of the band intensities normalized by the GAPDH protein band from experiments performed in triplicate, the band intensities of pSRSF1, pSRSF2, and pSRSF9 decreased in SPHINX31 treated cells, especially in 40 µM of SPHINX31 treatment (Figures 2c and 2d).

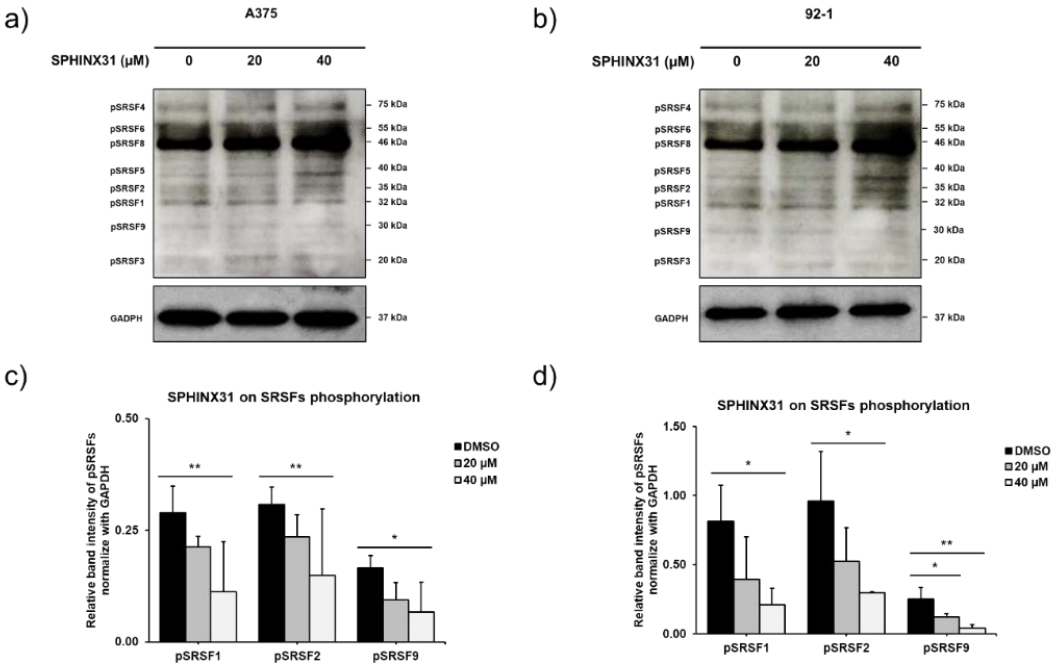

Figure 2 The effect of SPHINX31 treatment on SRSF phosphorylation (pSRSFs) in A375 cells (a) and 92-1 cells (b), with the semi-quantitative analysis of pSRSF1, pSRSF2, and pSRSF9 band intensities from triplicate experiments on A375 (c) and 92-1 (d). * P<0.05 and ** P<0.01.

4.3 The effect of SPHINX31 on the ability of cancer cell colony formation in melanoma cells

After the cells had been treated with SPHINX31, colony formation was determined by clonogenic assay and monitored over 10 days for A375, and 20 days for 92-1. The formation of cancer cell colonies was measured by ImageJ software, with the results revealing that SPHINX31 significantly decreased the size and number of A375 cell colonies in comparison to the 92-1 cell colonies (Figure 3a). This decreasing trend demonstrates dose-dependency, especially in the A375 cell treated in 40 μM SPHINX31 (Figure 3b). Therefore, the suppression of SRSF phosphorylation by SPHINX31 decreases the growth ability of A375 cells. According to the results for growth inhibition between the two cells, A375 was selected for subsequent experiments to evaluate its death evasion and migration abilities.

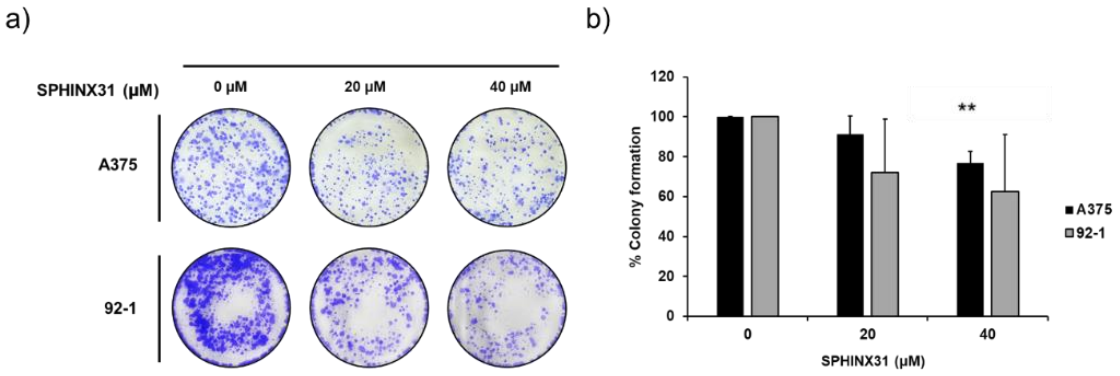

Figure 3 Suppression of SRSF phosphorylation by SPHINX31 and its effect on the cancer cell colony formation ability of A375 and 92-1 cells (a), trending in a dose-dependent manner after quantitative analysis of the percentage colony formation (b). **P<0.01.

4.4 The effect of SPHINX31 on death evasion and the migration abilities of the A375 cell

After the A375 cells had been treated using SPHINX31 at 10 and 20 µM, the live and dead cells were determined by live and dead staining and monitored for 24 h in triplicate experiments. The results revealed that SPHINX31 induced A375 cell death by decreasing the confluency of living cells (green fluorescent cells), while significantly increasing the number of dead cells (orange-to-red fluorescent cells) in a dose-dependent manner (Figures 4a and 4b). In addition, to evaluate the aggressiveness of other cancer phenotypes, their metastasis-associated migration ability was investigated using scratch wound assay with the closure gap monitored for 24 and 48 h using triplicate experiments. After observing the migration rate, the SPHINX31 treated cells presented a significantly slower rate of migration with the wound remaining open until 48 h of monitoring, while the wound of the control cells was entirely closed (Figures 4c and 4d). Therefore, the suppression of SRSF phosphorylation can decrease the survivability and migration activity of A375 cells.

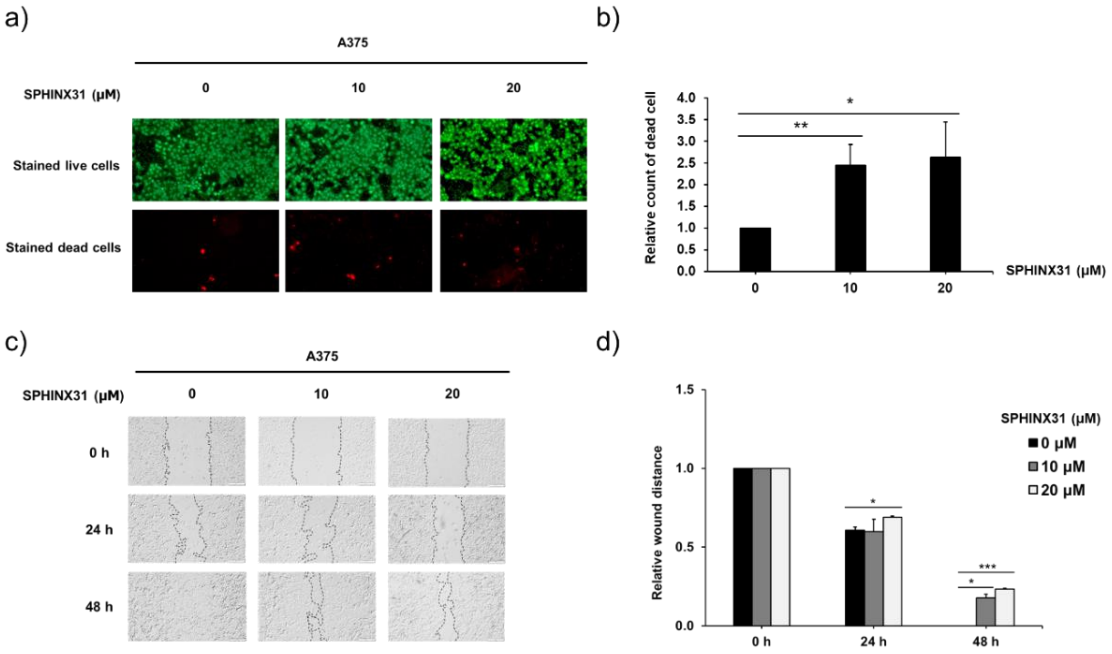

Figure 4 Suppression of SRSF phosphorylation by SPHINX31 and its effect on death evasion and the migration abilities of the A375 cell. Live and dead staining of SPHINX31 treated A375 cells (a) with the relative count of dead cells (orange-to-red fluorescence) (b). Migration ability of the SPHINX31 treated A375 cell was accessed using the scratch wound method for 48 h (c) with the relative wound distance compared to the distance at 0 h (d). * P<0.05, ** P<0.01, and ***P<0.001.

5. Discussion

Melanoma is one of the most aggressive cancers and a serious health problem in countries with high UV exposure, including Thailand. Although it is a rare disease, mortality is often caused by the cancer cells metastasizing to secondary sites, including the lungs, liver, brain, bones, and lymph nodes (Braeuer et al., 2014), resulting in high severity with poor prognosis and ineffective treatment. The results of this study reveal the contribution of aberrant gene splicing to the development and progression of cancer. It should be noted that the expression and activation of SRPKs may lead to aberrant alternative splicing, potentially contributing to various diseases, and the development of cancer (da Silva et al., 2015), and crucially, to cancer cell aggressiveness (David & Manley, 2010; Kim & Kim, 2012). This study attempts to demonstrate an association between the specific SRPK1 function with the downstream

activation of phosphorylate splicing factors (pSRSFs), and the effect on the basic aggressive behavior of cancer cells, such as the promotion of melanoma cell growth, a reduction in death evasion ability, and migration ability.

In a previous study in 2018, the researcher presented the effect of the SRPK1-specific inhibitor, SPHINX31, on the viability of acute myeloid leukemia cells, finding that it decreased leukemia cell viability through direct association with the reduced SRPK1 function (Tzelepis et al., 2018). The results of this current study show that SPHINX31 can decrease melanoma cell viability in both cutaneous melanoma (A375) and ocular melanoma (92-1) cells, particularly regarding the dose- and time-dependency of the A375 cell (Figure 1). Remarkably, even the A375 cell did not respond to SPHINX31 in low concentrations but was significantly sensitive to SPHINX31 in high concentrations when it exhibited dose- and time-dependency. To obtain molecular insight, a previous study reported a decrease in the phosphorylation of SRSFs when applying the SRPK1/2-specific inhibitor (SRPIN340) to the treatment of leukemia cells (Siqueira et al., 2015). Furthermore, a report on prostate cancer also verified the dose response of SPHINX31 in PC3 cells which specifically inhibits the phosphorylation of SRSF1 (Batson et al., 2017). Therefore, in this current study an experiment was performed to validate phosphorylation inhibition efficiency; the results of which indicate that SPHINX31 can decrease the kinase activity of SRPK1 by reducing the phosphorylation form of SRSFs in both A375 and 92-1 cells (Figure 2). Therefore, SPHINX31 might reduce the proliferation ability of cancer cells through the inhibition of SRSF phosphorylation.

Moreover, a previous study undertaken in 2014 revealed the functional involvement of SRPK1 in the tumor angiogenesis of metastatic melanoma. The findings revealed that tumor growth was attenuated after SRPIN340 treatment (Gammons et al., 2014). In another study, the effect of SRPK inhibitors on colony formation in B16F10 murine melanoma cells were examined, with the results revealing that SRPK inhibitors can reduce colony formation in murine melanoma cells in a dose-dependent manner (Moreira et al., 2018). In this current study, the effect of SPHINX31 in A375 and 92-1 melanoma cells has been confirmed and a reduction in the size and number of melanoma cell colonies demonstrated after SPHINX31 treatment. Specifically, a significant decrease was obtained when A375 cells were treated in high concentrations of SPHINX31 (Figure 3). In the subsequent experiment, we selected A375 cells to explore the activity of SPHINX31 on death evasion and migration ability. The aggressive behaviors of cancer cells were tracked by two assays, representing other cancer hallmarks, as well as death-evading and metastasis-associated phenotypes. The death evasion ability of SPHINX31-treated A375 cells was found to decrease in comparison to the control group (Figures 4a and 4b), while the migration was also significantly slower, especially in the 48 h SPHINX31 treatment (Figures 4c and 4d). Furthermore, the effect of SPHINX31 can decrease the death evasion and migration ability of A375 cells.

The role of SRPKs in tumor metastasis has been accredited to their expression of pro-metastatic spliced isoform benefits, such as Rac1b (Gonçalves et al., 2014), RONΔ165 (Moon et al., 2014), and the pro-angiogenic VEGF165, which have been related to metastatic melanoma (Gammons et al., 2014). Therefore, this data collectively suggests that SRPK is a potential target for the development of antimetastatic drugs. Therefore, this study attempts to demonstrate the effect of the SRPK1-specific inhibitor SPHINX31 on the growth, death evasion, and migration ability of melanoma cells through the inhibiting phosphorylation of SRSFs. The results of this study will serve as a basis for targeting SRPKs as an alternative therapeutic strategy in melanoma treatment. It should be noted that the molecular insight of SRPK targeting in melanoma cells could be further designed to reveal the specific activation of a signaling pathway, derived from an SRPK inhibitor, such as SPHINX31 or combined with chemotherapeutic cancer drugs to gain synergistic treatment outcomes.

6. Conclusion

Serine-Arginine Protein Kinase 1 or SRPK1 regulates alternative splicing through the phosphorylation of SRSFs. Several studies present the overexpression of SRPK1 associated with the aberrant alternative splicing of many oncogenic genes and proteins. To study the specific SRPK1 function in melanoma, the SRPK1-specific inhibitor, SPHINX31, is demonstrated in this study to decrease the viability of cutaneous and ocular melanoma cells through the inhibition of SRSF

phosphorylation. The suppression of SRSF phosphorylation is shown to affect the cancer cell colony formation of melanoma as demonstrated by its smaller size and lower number of A375 and 92-1 melanoma cell colonies following treatment with SPHINX31, especially the A375 cutaneous melanoma cell. Furthermore, other aggressive behaviors exhibited by cancer cells were explored in the A375 cell. Suppression of SRSF phosphorylation by SPHINX31 was found to promote cell death and suppress the migration ability of A375 cells. This study will serve as the basis for targeting SRPKs as an alternative therapeutic strategy for melanoma treatment.

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