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Scoparone attenuates PD-L1 expression in human breast cancer cells by MKP-3 upregulation

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ABSTRACT

Breast cancer is a frequently occurring malignant tumor that is one of the leading causes of cancer-related deaths in women worldwide. Monoclonal antibodies that block programed cell death 1 (PD-1)/programed cell death ligand 1 (PD-L1) – a typical immune checkpoint – are currently the recommended standard therapies for many advanced and metastatic tumors such as triple-negative breast cancer. However, some patients develop drug resistance, leading to unfavorable treatment outcomes. Therefore, other approaches are required for anticancer treatments, such as downregulation of PD-L1 expression and promotion of degradation of PD-L1. Scoparone (SCO) is a bioactive compound isolated from *Artemisia capillaris* that exhibits antitumor activity. However, the effect of SCO on PD-L1 expression in cancer has not been confirmed yet. This study aimed to evaluate the role of SCO in PD-L1 expression in breast cancer cells in vitro. Our results show that SCO downregulated PD-L1 expression in a dose-dependent manner, via AKT inhibition. Interestingly, SCO treatment did not alter PTEN expression, but increased the expression of mitogen-activated protein kinase phosphatase-3 (MKP-3). In addition, the SCO-induced decrease in PD-L1 expression was reversed by siRNA-mediated MKP-3 knockdown. Collectively, these findings suggest that SCO inhibited the expression of PD-L1 in breast cancer cells by upregulating MKP-3 expression. Therefore, SCO may serve as an innovative combinatorial agent for cancer immunotherapy.

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Introduction

Breast cancer is the most frequently reported malignancy and leading cause of cancer-related deaths in women worldwide (Harbeck et al. 2019; Giaquinto et al. 2022). Immunotherapy has recently become a crucial treatment approach for cancer, in addition to surgery, chemotherapy, radiotherapy, and hormone therapy (Schneble et al. 2015; Waks and Winer 2019; Debien et al. 2023). In particular, immune checkpoint inhibitors – which block key immunosuppressive receptors, such as programed cell death 1 (PD-1), programed death ligand 1 (PD-L1), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), – have revolutionized the treatment of solid tumors (Ribas and Wolchok 2018; Jacob et al. 2023).

PD-L1 is one of the most important molecular pathways used by tumor cells to evade the immune checkpoints of T cells (Ricklefs et al. 2018; Kumagai et al. 2020). PD-L1 expressed on the surface of tumor cells bind to PD-1 expressed by activated T cells, resulting in the direct inhibition of T cell effector functions against tumor cells (Iwai et al. 2017; Han et al. 2020). PD-L1 is frequently overexpressed in most breast cancer tissues, particularly in triple-negative breast cancer (TNBC) (Gatalica et al. 2014; Mittendorf et al. 2014; Twyman-Saint Victor et al. 2015). It is associated with several clinicopathological parameters that indicate poor outcomes and may increase the risk of mortality (Ghebeh et al. 2006; Wang C et al. 2017).

Scoparone (SCO) is a biologically active compound isolated from *Artemisia capillaris* that possesses various biological properties, including anti-allergic (Choi YH and Yan 2009), antitumor (Kim JK et al. 2013; Wu X et al. 2023), antioxidant (Atmaca et al. 2011) and anti-inflammatory activities (Cho et al. 2016; Lu et al. 2018). However, the role of SCO in regulating PD-L1 expression

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or in cancer immunotherapy has not yet been elucidated.

Mitogen-activated protein kinase phosphatase-3 (MKP-3) is a cytoplasmic dual-specificity phosphatase that preferentially dephosphorylates ERK (Muda et al. 1996), which is a major trigger for the development of most types of cancers (Garcia-Gomez et al. 2018; Maik-Rachline et al. 2019). MKP-3 exerts a tumor-suppressive effect via the negative regulation of ERK in different types of cancers, including pancreatic (Furukawa et al. 2003), lung (Okudela et al. 2009), ovarian (Chan et al. 2008), colon (Kim HS et al. 2021), breast cancer (Luo et al. 2015), and melanoma (Warmka et al. 2004).

Therefore, in this study, we investigated the effects of SCO on PD-L1 expression in breast cancer cells and found that SCO reduced the expression of PD-L1, which was mediated by AKT inhibition through the induction of MKP-3. Furthermore, MKP-3 knockdown resulted in increased AKT phosphorylation and PD-L1 expression. Collectively, these data provide evidence for SCO-induced MKP-3 expression as a mechanism that regulates PD-L1 expression in breast cancer cells. This suggests the potential application of SCO in cancer immunotherapy.

Materials and methods

Materials

SCO was obtained from MedChem Express (Monmouth Junction, NJ, USA). Anti-PD-L1, anti-phospho-NF-κB (Thr172), anti-phospho-AKT (Ser473), anti-phospho-ERK1/2, anti-phospho-STAT1 (Tyr701), anti-phospho-STAT3 (Tyr705), and anti-PTEN antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA); anti-MKP-3 antibody was purchased from Abcam (Waltham, MA, USA). The anti-β-actin antibody was obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell culture

MCF7 and MDA-MB-231 cells were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea) and cultured in RPMI 1640 medium (WELGENE, Gyeongsan, Republic of Korea) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and 1% antibiotics (WELGENE). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Cell viability assay

The MDA-MB-231 cells were seeded at a density of 1×10^4 cells/well in 96-well plates. The cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Rockville, MD, USA) was used for cell viability assessment 24 h after incubation with SCO at various concentrations (0–500 μM), following the manufacturer's instructions. The absorbance of the plates was measured at 450 nm using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific) (Kim DB et al. 2022).

Western blot analysis

The cells were washed with ice-cold phosphate buffer saline and lysed on ice in a lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, and 0.5% sodium deoxycholate) supplemented with protease and phosphatase inhibitors (Son et al. 2022). Aliquots containing equal amounts of protein were loaded and separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) and probed with the indicated antibodies. Protein bands were detected by chemiluminescence using a ChemiDoc gel imaging system (Bio-Rad).

RNA extraction and real-time quantitative polymerase chain reaction

Total RNA was isolated from cells using the AccuPrep Universal RNA Extraction Kit (Bioneer, Daejeon, Republic of Korea), according to the manufacturer's instructions. Reverse transcription was performed using the AccuPower CycleScript RT PreMix (Bioneer) after RNA isolation and quantitation. Real-time quantitative polymerase chain reaction (RT-qPCR) experiments were performed using AccuPower 2X GreenStar qPCR Master Mix (Bioneer) and analyzed using a CFX Connect Real-Time PCR detection system (Bio-Rad). The normalization of gene expression levels was performed relative to the 18S ribosomal RNA (rRNA) as a reference housekeeping gene. The primer sequences used for RT-qPCR are listed in Supplementary Data 1.

Small-interfering RNA (siRNA) transfection

Pre-designed siRNAs targeting human MKP-3 (#1848-1) and control siRNA (#SN-1001) were purchased from Bioneer. MDA-MB-231 cells were seeded in 60-mm dishes, grown to approximately 80% confluence, and transfected with siRNA duplexes using Lipofectamine RNAiMAX (Thermo Fisher Scientific), according to the manufacturer's recommendations. To determine the extent of siRNA inhibition, the expression of MKP-3 in transfected cells was assessed using western blotting.

Statistical analysis

Data were evaluated using analysis of variance (Sigma Stat 12.0, Systat Software, San Jose, CA, USA) with parametric or nonparametric *post hoc* analysis, and multiple comparisons were made using the least significant difference method. All data are presented as the mean ± standard deviation of at least three independent experiments. Statistical comparisons of the results were made using one-way analysis of variance, and $p < 0.05$ was considered statistically significant.

Results

SCO decreases the expression of PD-L1 in human breast cancer cells

The chemical structure of SCO is shown in Figure 1(A). First, we performed a CCK-8 assay to examine the cytotoxic effect of SCO on MDA-MB-231 cells. As shown in Figure 1(B), SCO treatment with up to 500 μM for 24 h exhibited no cytotoxic effects on MDA-MB-231 cells. Wu et al. recently reported that high concentration (up to 1000 μM) and prolonged (up to 72 h) SCO treatment decreased viability and induced apoptosis in breast cancer cells. However, they did not observe any cytotoxicity below 500 μM for 24 h (Wu X et al. 2023), which is consistent with our results. Therefore, we used a relatively safe dose of 100 μM in subsequent experiments to explore the molecular mechanism underlying the inhibition of PD-L1 expression by SCO.

To examine the inhibitory effects of SCO, we used two breast cancer cell lines with differential PD-L1 expression under standard culture conditions. Consistent with the findings of previous studies (Rom-Jurek et al. 2018; Azadi et al. 2019), a high level of PD-L1 protein expression was observed in the MDA-MB-231 cells, whereas a low PD-L1 protein expression was observed in MCF-7 cells (Figure 2(A)). The effects of SCO on PD-L1 protein and mRNA expression levels in human breast cancer cells were examined. As shown in Figure 2(A and B), SCO treatment significantly downregulated PD-L1 expression by inhibiting transcription. SCO decreased PD-L1 expression in a dose-dependent manner (Figure 2(C)).

SCO inhibits activation of AKT in human breast cancer cells

The expression of PD-L1 in cancer cells is regulated by various oncogenic signaling pathways. MAPK (Wagner and Nebreda 2009; Guo et al. 2020) and PI3 K/AKT (Fresno Vara et al. 2004; Haddadi et al. 2018) are well-studied oncogenic pathways that play important roles in the pathogenesis of cancer by activating many downstream targets that regulate cell survival, proliferation, and motility. They are also vital for PD-L1 induction in cancer cells (Kim YB et al. 2019; Mansour et al. 2020; Zhang S et al. 2022). In addition, JAK-STAT pathway (Sasidharan Nair et al. 2018; Song et al. 2018) and NF-κB (Antonangeli et al. 2020) are related to the high

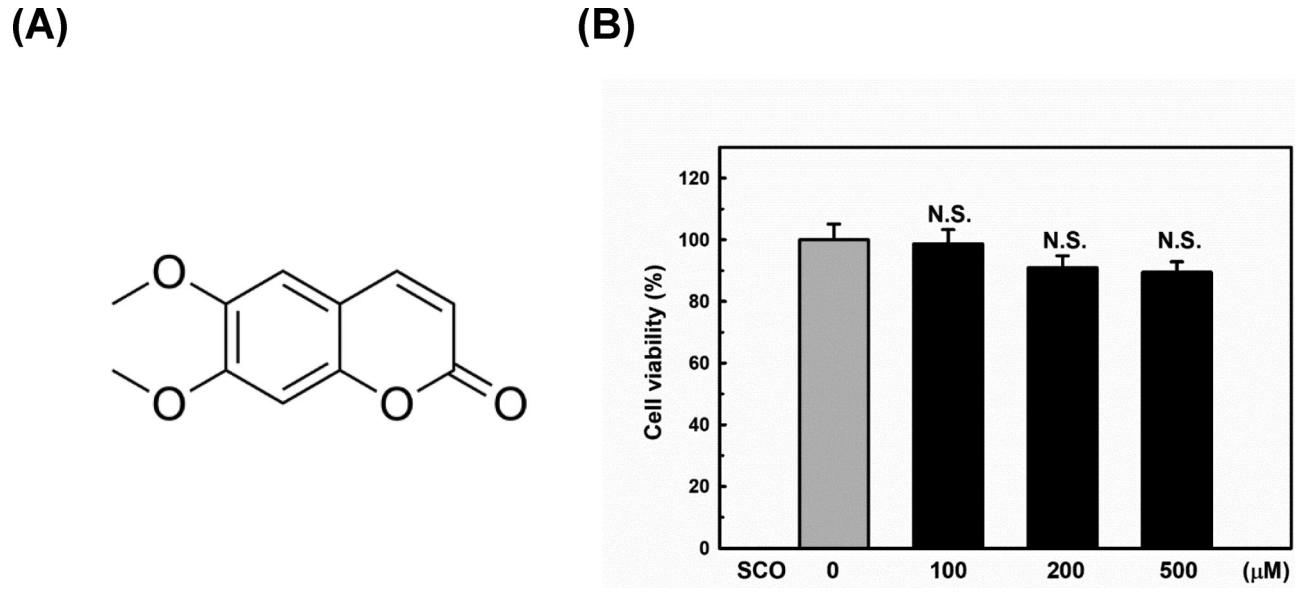

Figure 1. Chemical structure and effect of scoparone (SCO) on cell viability. (**A**) Chemical structure of SCO. (**B**) Effect of treatment with vehicle (DMSO) or SCO concentrations for 24 h on the viability of MDA-MB-231 cells, as evaluated by the cell counting kit-8 assay. The results are represented as the mean ± standard deviation of three independent experiments. N.S. indicates not significant ($p > 0.05$).

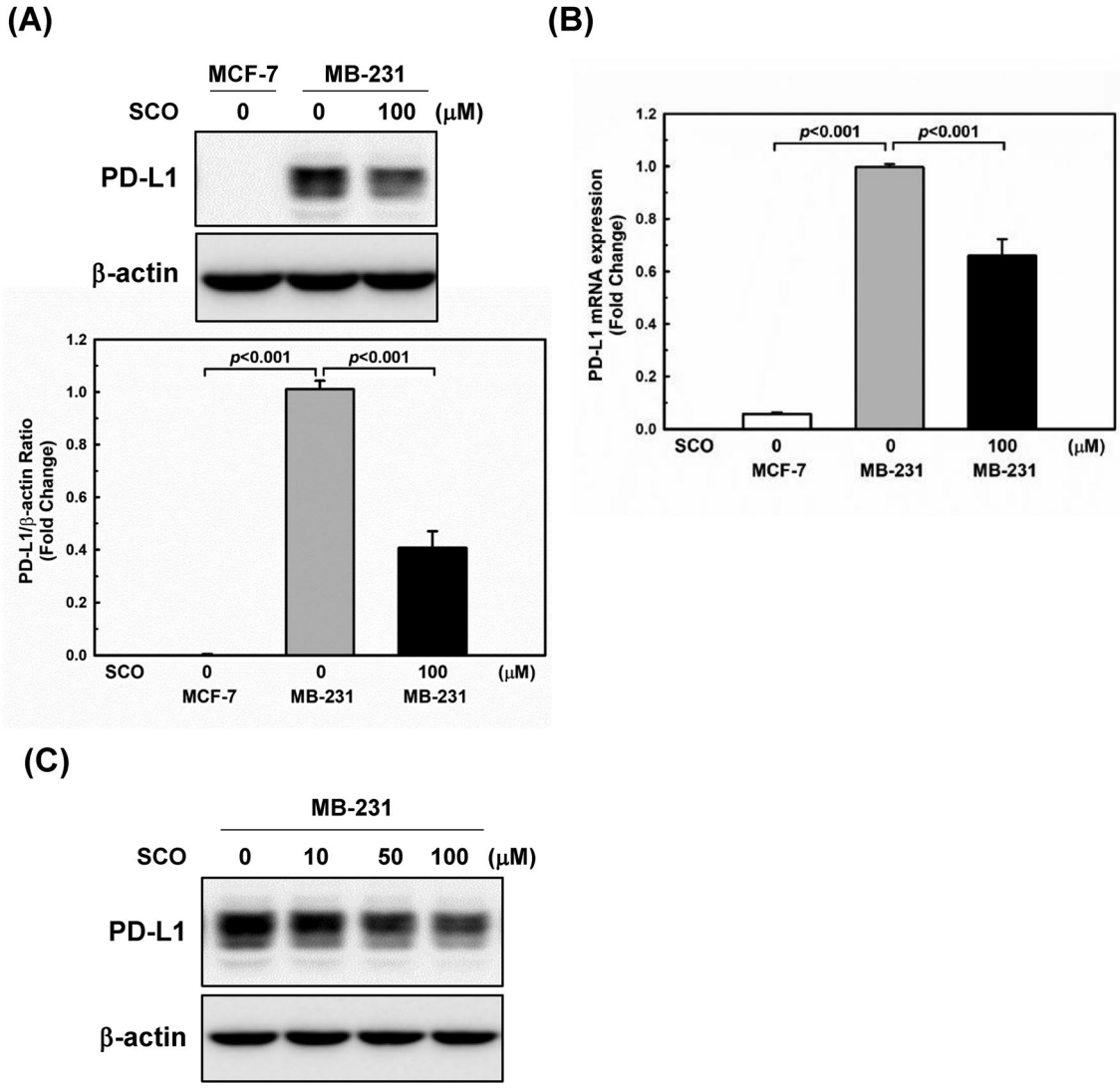

Figure 2. Scoparone (SCO) downregulates the expression of programed cell death-ligand 1 (PD-L1) in human breast cancer cells. MDA-MB-231 (MB-231) cells were treated with vehicle (DMSO) or SCO (100 μM) for 24 h. MCF-7 was used as a negative control for PD-L1. (**A**) PD-L1 expression was measured by western blotting analysis. The *β*-actin protein level was considered as a loading control. Data are represented as mean ± standard deviation ($n = 3$). (**B**) The mRNA expression of PD-L1 was detected by real-time polymerase chain reaction. 18S ribosomal RNA was used as an internal control. Data are represented as mean ± standard deviation. (**C**) MDA-MB-231 (MB-231) cells were treated with various concentrations of SCO for 24 h, followed by a western blot analysis with the indicated antibodies.

levels of PD-L1 in several cancers. Thus, we examined the changes induced by SCO treatment in various signaling pathways. As shown in Figure 3(A), among the signaling molecules that regulate PD-L1 expression, AKT phosphorylation decreased in a dose-dependent manner following SCO treatment. SCO treatment decreased AKT phosphorylation by 45% (Figure 3(B)).

The gene encoding for phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a major gatekeeper gene of the AKT signaling pathway. Additionally, it ranks among the most frequently deleted tumor suppressor genes in human cancer cells (Leslie and Downes 2004; Haddadi et al. 2018). MDA-MB-231 cells express wild-type PTEN (Hlobilkova et al. 2006; Wieland et al. 2021). In addition, Razmara et al. suggested that MKP-3 negatively modulates the PI3K-dependent AKT phosphorylation (Razmara et al. 2012). Therefore, we measured protein levels of PTEN and MKP-3 protein. PTEN expression remained unchanged, whereas MKP-3 expression increased with increasing SCO levels (Figure 4(A)). SCO treatment resulted in a 46% increase in MKP-3 protein levels (Figure 4(B)) and a 52% increase in MKP-3 mRNA levels (Figure 4(C)). To examine whether MKP-3 affected AKT phosphorylation in MDA-MB-231 cells, we conducted MKP-3 knockdown experiments. As shown in Figure 4(D), a 2.1-fold increase

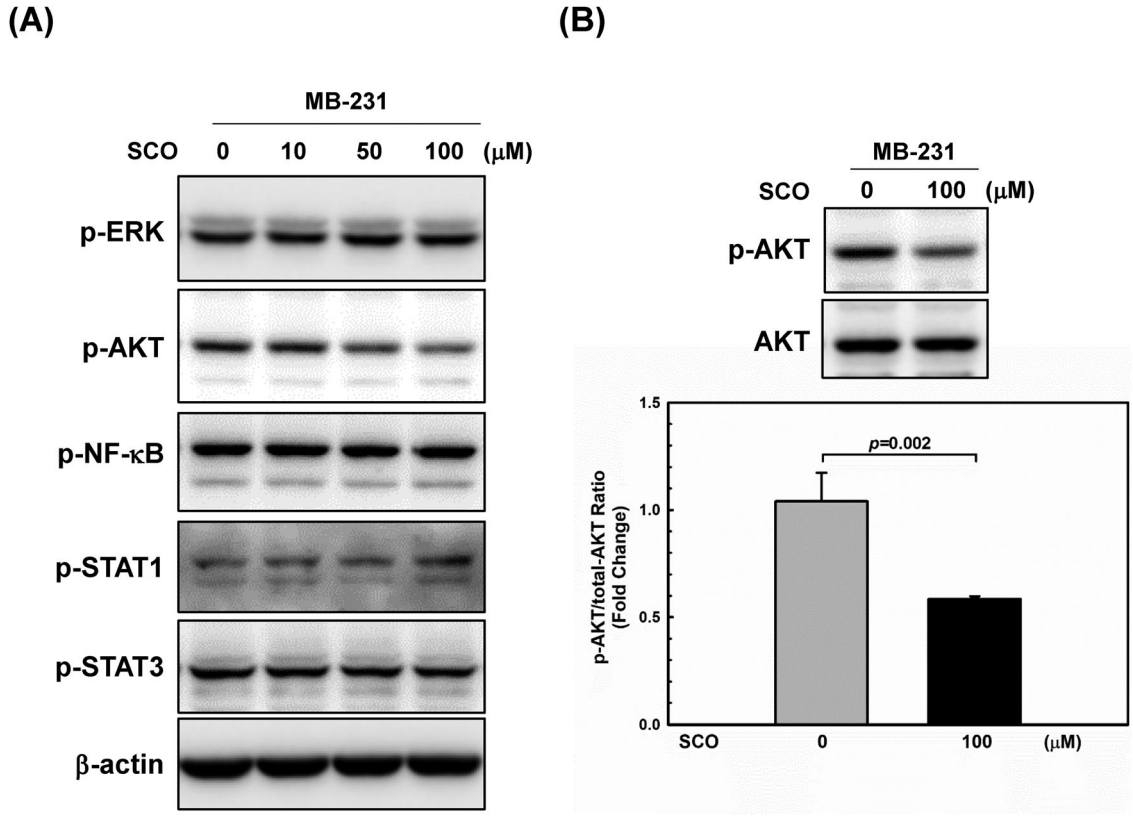

Figure 3. Scoparone (SCO) inhibits AKT phosphorylation in human breast cancer cells. (**A**) MDA-MB-231 (MB-231) cells were treated with various concentrations of SCO for 24 h, followed by using western blot analysis with the indicated antibodies. (**B**) Western blot was used to analyze the protein levels of p-AKT and AKT in MDA-MB-231 (MB-23) cells with or without SCO treatment for 24 h. The β-actin was used as a loading control. The data show the mean ± standard deviation for three independent experiments.

in phosphorylated AKT was observed in cells with MKP-3 knockdown. This result is consistent with the findings of Razmara et al (Razmara et al. 2012).

SCO-induced reduction of PD-L1 expression in human breast cancer cells is mediated by MKP-3

To further investigate whether MKP-3 is necessary for the SCO-mediated inhibition of PD-L1 expression, MDA-MB-231 cells were transfected with either control siRNA or MKP-3 siRNA and then treated with SCO. Even in untreated controls, MKP-3 knockdown led to a marked increase in the expression of PD-L1. In addition, PD-L1 inhibition by SCO was abolished when MKP-3 was knocked down (Figure 5(A)). These results suggested that MKP-3 is involved in the reduction of SCO-induced PD-L1 expression in human breast cancer cells.

Discussion

PD-L1 is an immunoglobulin-like molecule that is widely expressed in most human cancers. The binding of PD-L1 to its receptor, PD-1, on T cells results in immunosuppression, leading to immune evasion in cancer (Chen 2004; Tsushima et al. 2007). Overexpression of PD-L1 is associated with poor prognosis (Ghebeh et al. 2006; Muenst et al. 2014; Wang C et al. 2017; Wu Z et al. 2019), larger tumors, higher tumor grade, estrogen receptor-negative, progesterone receptor-negative, HER-2-positive status, cell proliferation, and an increased abundance of tumor-infiltrating lymphocytes (TILs) in patients with breast cancer (Bertucci et al. 2015; Twyman-Saint Victor et al. 2015). Tumors overexpressing PD-L1 are often accompanied by the infiltration of PD-1-positive TILs, which are associated with shortened overall survival, indicating a poor prognosis for breast cancer (Muenst et al. 2013; Sun et al. 2014). PD-L1 affects not only T cells but also tumor-associated macrophages, inducing tumor-promoting M2 polarization (Meng et al. 2022; Zhang R et al. 2022).

Although inhibition of the PD-L1/PD-1 axis has been developed as an important approach to cancer immunotherapy, PD-L1 also exhibits tumor-intrinsic functions. For instance, PD-L1 signaling promotes epithelial-to-mesenchymal transition in several cancer subtypes (Alsuliman et al. 2015; Wang Y et al. 2015). PD-L1 also acts as an anti-apoptotic receptor in response to Fas ligation. Furthermore, it is associated with cancer stem cell

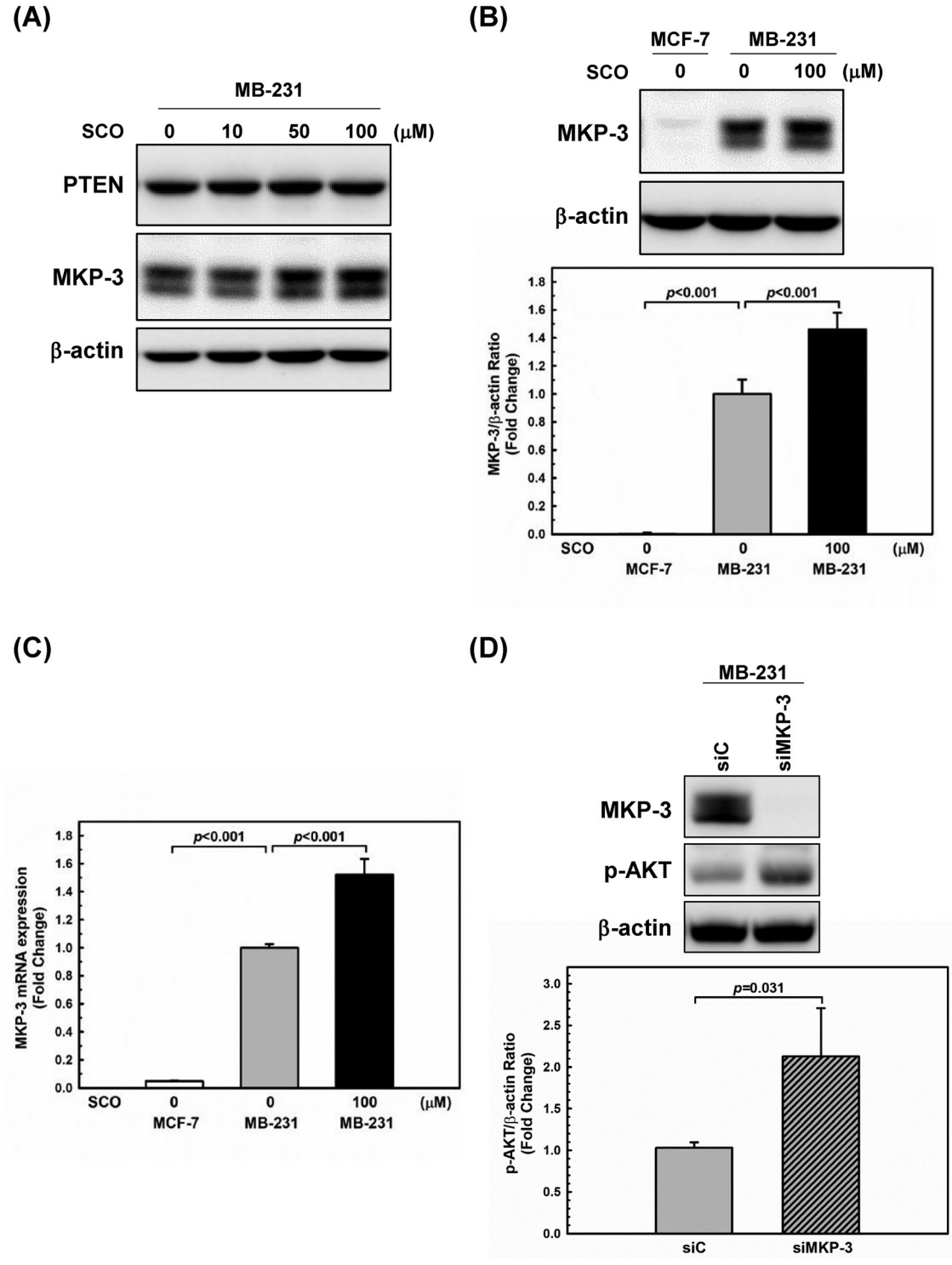

Figure 4. Scoparone (SCO)-induced expression of mitogen-activated protein kinase phosphatase-3 (MKP-3) inhibits AKT phosphorylation in human breast cancer cells. (**A**) MDA-MB-231 (MB-231) cells were treated with various concentrations of SCO for 24 h, followed by using western blot analysis with the indicated antibodies. (**B**) MDA-MB-231 (MB-231) cells were treated with vehicle (DMSO) or SCO (100 μM) for 24 h. MKP-3 expression was measured using western blotting analysis. The *β*-actin protein level was considered as a loading control. (**C**) The mRNA expression of MKP-3 was detected by real-time polymerase chain reaction. 18S ribosomal RNA was used as an internal control. (**D**) MDA-MB-231 (MB-231) cells were transfected with either non-targeting siRNA (siC) or siRNA directed against MKP-3 (siMKP-3) for 48 h. MKP-3, p-AKT, and β-actin levels were assessed using western blot analysis. All Data are represented as the mean ± standard deviation ($n = 3$).

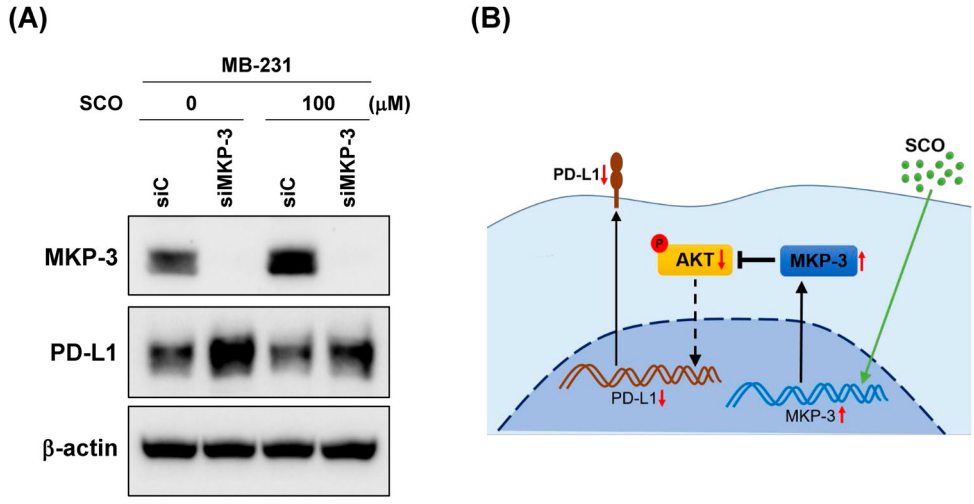

Figure 5. Mitogen-activated protein kinase phosphatase-3 (MKP-3) is necessary for scoparone (SCO)-mediated inhibition of programed cell death-ligand 1 (PD-L1) expression. (**A**) MDA-MB-231 (MB-231) cells were transfected with either non-targeting siRNA (siC) or siRNA directed against MKP-3 (siMKP-3) for 24 h and subsequently exposed to vehicle (DMSO) or SCO (100 μM) for 24 h**.** MKP-3, PD-L1, and β-actin levels were assessed using western blot analysis. (**B**). Possible mechanisms underlying the reduction in the activity of PD-L1 mediated by SCO. SCO-induced activation of MKP-3 reduced PD-L1 expression via inhibition of AKT.

proliferation (Azuma et al. 2008; Yang et al. 2015). PD-L1 can enhance the proliferation and survival of acute myeloid leukemia cells by upregulating the pentose phosphate pathway, which is crucial for cell proliferation and the *β*-oxidation of fatty acids, which promotes cell survival (Soltani et al. 2023). PD-L1 suppresses tumor autophagy by promoting basal mTORC1 signaling in melanoma and ovarian cancer models (Clark et al. 2016). Therefore, in addition to targeting the disruption of PD-L1/PD-1, it is important to identify agents that impede the expression of PD-L1.

SCO, also known as 6,7-dimethoxycoumarin, has been proposed to exert antitumor activity by regulating signaling pathways involving AKT (Li N et al. 2021; Huang S et al. 2023), NF-kB (Wu X et al. 2023), and STAT3 (Kim JK et al. 2013). Since PD-L1 expression in cancer cells is also regulated by signaling pathways, such as AKT (Fresno Vara et al. 2004; Haddadi et al. 2018), NF-κB (Antonangeli et al. 2020), and STAT3 (Marzec et al. 2008), we investigated whether SCO can regulate PD-L1 expression.

In this study, we identified SCO as a PD-L1 inhibitor in MDA-MB-231 cells – a highly aggressive, invasive, and poorly differentiated TNBC cell line (Figure 2). SCO decreases AKT phosphorylation, a crucial signaling pathway for PD-L1 expression (Fresno Vara et al. 2004), but does not upregulate PTEN. Instead, this decrease was attributed to an increase in MKP-3 levels (Figures 3 and 4). Although Razmara et al. suggested that MKP-3 inhibits AKT activity (Razmara et al. 2012), Rodrigues et al. reported that MKP-3 overexpression does not affect AKT phosphorylation (Rodrigues et al. 2017). Therefore, MKP-3-mediated AKT dephosphorylation might be an indirect effect. Interestingly, SCO treatment increased MKP-3 protein levels by 46% (Figure 4(B)) but did not affect the phosphorylation of ERK (Figure 3(A)), which is the preferred substrate of MKP-3. Regulation of protein phosphorylation requires a balance between the activities of protein kinases and phosphatases. Although MKP-3 is an important phosphatase for ERK, several other phosphatases – such as PP1 (Li R et al. 2013), PP2A (Yu et al. 2004), DUSP5 (Rushworth et al. 2014), DUSP7 (Keyse 2008), MKP-1 (Zhao et al. 2021), MKP-4 (Emanuelli et al. 2008), MKP-5 (Nomura et al. 2012), and alkaline phosphatase (ALP) (Sharma et al. 2014) – have also been reported to dephosphorylate ERK. Other kinases such as PI3 K, conventional protein kinase C (Grammer and Blenis 1997), and TGF-*β*-activated kinase 1 (TAK1) (Simard et al. 2015) play major roles in MEK-independent ERK activation. Therefore, further research is necessary on the upstream kinases and phosphatases that may regulate ERK phosphorylation.

The knockdown experiments further confirmed that the SCO-induced increase in MKP-3 expression was required for the suppression of PD-L1 expression (Figure 5(A)). MKP-3 knockdown alone led to a substantial increase in PD-L1 protein levels (Figure 5(A)). This implied that MKP-3 plays an important role in regulating the expression of PD-L1. To our knowledge, this is the first report describing the role of MKP-3 in reducing PD-L1 expression in cancer cells via AKT regulation, which presents a new therapeutic target for PD-L1 regulation.

Kim et al. reported that SCO inhibited the phosphorylation and transcriptional activity of STAT3 in DU145

prostate cancer cells (Kim JK et al. 2013); however, no inhibition of STAT3 was observed in the present study. Several drugs exhibited varying effects on STAT3 in MDA-MB-231 and DU145 cells: SH003 inhibited STAT3 in MDA-MB-231 cells, but not in DU145 cells (Choi YJ et al. 2016); the methylene chloride fraction of *Chrysanthemum indicum L.* exhibited a clear inhibitory effect on constitutive STAT3 activation in DU145 cells, but not in MDA-MB-231 cells (Kim C et al. 2013). Thus, SCO may have different signaling effects on different types of cancer cells.

Collectively, the findings of the present study suggest that SCO attenuates PD-L1 expression in human breast cancer cells via MKP-3 upregulation (Figure 5(B)).

Recently, curcumin was reported to promote PD-L1 degradation and sensitize breast cancer cells to anti-cytotoxic T-lymphocyte-associated antigen 4 therapy (Lim et al. 2016). Furthermore, extracellular release of PD-L1 with platycodin D increased interleukin-2 production in activated Jurkat T cells (Huang MY et al. 2019), suggesting that phytochemicals, including SCO, can selectively reduce PD-L1 expression on the cell membrane, leading to an immunotherapeutic effect. As mentioned previously, PD-L1 is involved in various intrinsic tumor functions. Therefore, further studies are required to investigate the effects of SCO on epithelial-to-mesenchymal transition, proliferation, survival, and autophagy.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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