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Effect of Metformin in Combination With Trametinib and Paclitaxel on Cell Survival and Metastasis in Melanoma Cells

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Abstract. *Background/Aim: Despite clinical benefit from treatment with dabrafenib and trametinib in melanoma patients with BRAF mutations, half relapse within months and one-third are unresponsive to treatment. We evaluated the anticancer potential of metformin in combination with trametinib plus paclitaxel, against four melanoma cell lines. Materials and Methods: Metformin with trametinib and paclitaxel was tested for effects on cell viability, signaling molecules in MAPK and mTOR pathways, factors involved in epithelial-mesenchymal transition (EMT), and cell motility. Results: The combination of metformin with trametinib and paclitaxel showed differential growth inhibitory effects; synergistic effects were observed in a cell line in which metformin suppresses ERK activity, whereas the combination showed antagonistic effects in a cell line with metformin-induced ERK activation. Trametinib or paclitaxel increased the expression of EMT regulators and melanoma cell motility, which were suppressed by combining metformin with trametinib and paclitaxel. Conclusion: The combined treatment of metformin with trametinib and paclitaxel showed divergent effects on melanoma cell viability. Metformin might be useful as a potential adjuvant against cell proliferation and metastatic activity in melanoma patients.*

Cutaneous melanoma is one of the most aggressive malignancies with high metastatic potential. The incidence of this cancer has been increasing rapidly and the International Agency for Research on Cancer (IARC) estimated that about 300,000 new cases were diagnosed worldwide in 2018 (1). While surgical resection is often curative in its early stage, melanoma remains a fatal disease with 5-year survival rate of 25% for patients with distant metastasis (2). By virtue of major advances in the area of molecular oncology, however, remarkable progress has been made in recent years with improved prognosis.

In most melanomas, the MAPK (mitogen-activated protein kinase) pathway (also known as RAS/RAF/MEK/ERK pathway) is constitutively active through mutations in BRAF (40-60%), NRAS (15-20%) and through autocrine growth factor stimulation, and consequently these have become primary therapeutic targets and led to the development of novel targeted agents during the last decade (3, 4). Targeted therapies with BRAF inhibitors, such as dabrafenib and vemurafenib, have shown significant clinical activity for melanoma patients harboring BRAF mutations, with higher response rates of about 50% compared to chemotherapeutic agents such as carboplatin and paclitaxel with 10% or less (5, 6). Most melanoma patients with BRAF mutations, however, develop resistance to single agents dabrafenib and vemurafenib within months. The acquired resistance to BRAF inhibitor monotherapy is mainly attributed to reactivation of the MAPK pathway through BRAF gene amplification, aberrant BRAF splicing and secondary mutations in NRAS (7). Therefore, an approach combining dabrafenib with MEK inhibitor trametinib was evaluated in clinical trials and showed remarkable clinical benefit with response rates of about 70% (8). Currently, oncologists typically use the combination of dabrafenib with trametinib as a first-line treatment for unresectable or metastatic BRAF-mutant melanoma.

In spite of significant advances in clinical outcomes and long-term durability of responses, half of patients treated with dabrafenib plus trametinib experience tumor progression at 9-10 months following initial treatment. Moreover, about one-third of BRAF-mutant metastatic melanoma patients do not even respond to the combination, and in this situation therapeutic options for these unresponsive patients remain limited (9). Mechanistic factors underlying the lack of response or the intrinsic resistance to combination therapy are diverse and include compensatory activation of parallel signaling cascades such as PI3K/mTOR (phosphoinositide 3-kinase/mammalian target of rapamycin)

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pathway along with loss of suppressive feedback regulation (10). Preclinical studies have shown concomitant targeting of both pathways is more effective than inhibition of MAPK pathway alone in melanoma cells with BRAF or NRAS mutation (11, 12). However, the most significant challenge is the severe drug-related toxicities of such cross-pathway targeted combination regimens, preventing delivery of drugs at optimal therapeutic concentrations (13).

Metformin belongs to a family of biguanides and has been most commonly prescribed to diabetic patients for a period of over 60 years. It lowers blood glucose levels by inhibiting gluconeogenesis in liver and increasing uptake and utilization of glucose by skeletal muscles (14). In addition to its hypoglycemic properties, retrospective studies suggested that metformin treatment is associated with a reduced risk of cancer, compared to other antidiabetic medications in diabetic patients (15, 16). *In vitro* and *in vivo* preclinical studies using melanoma cell lines have shown that metformin alone or in combination with BRAF/MEK inhibitors suppresses cell proliferation and mouse xenograft growth (17, 18). Metformin interferes with PI3K/mTOR pathway through activation of AMP-activated protein kinase (AMPK), which results in cell-cycle arrest and apoptosis in various cancer cells, including melanoma (19-21). In addition, it was reported to suppress melanoma cell invasion and metastasis development by reducing the expression of proteins involved in epithelial-mesenchymal transition (EMT) (22). Recently, clinical trials have also indicated improvements in therapeutic responses by metformin monotherapy, or by combining metformin with targeted or chemotherapeutic agents in patients with various types of cancer (23-25).

In this preclinical study, we attempted to develop a rational combination therapy using agents with distinct anticancer mechanisms, with the goal of enhancing tumor responses and patient survival, but also for reducing drug-related toxicities in the management of melanoma. MEK inhibitor trametinib inhibits cell proliferation, but cells with adaptive resistance to trametinib through compensatory activation of PI3K/mTOR pathway are likely to escape from its anti-proliferative action. Metformin, a well-tolerated drug with good safety profile, was thought to be capable of capturing these unresponsive cells through suppression of mTOR activity, and reduce the incidence of side-effects compared to other inhibitors of the PI3K/mTOR pathway (13). However, both trametinib and metformin induce G_0/G_1 cell-cycle arrest in melanoma cells (17, 20, 26), and therefore unresponsive cells or cells resistant to a combination treatment of metformin and trametinib would enter G_2/M phase to advance cell proliferation. We, therefore, reasoned that combining trametinib and metformin with paclitaxel, an antimitotic agent targeting tubulin, could generate more synergistic antitumor activity in melanoma cells. In the present study, using melanoma cell lines with BRAF or NRAS mutation, we show that the combined treatment of metformin with trametinib plus paclitaxel displays synergistic effect on cell viability in a cell line in which metformin suppresses ERK activity, but antagonistic effects in a cell line showing metformin-induced ERK activation. Of note, trametinib and paclitaxel as single agents increased the expression of EMT regulators and enhanced melanoma cell motility, both of which are suppressed by combined treatment of metformin with trametinib plus paclitaxel. These results suggest that metformin might be used as a potential therapeutic option for the treatment of melanoma patients by suppressing metastatic activity as well as by inhibiting cellular growth.

Materials and Methods

Cell culture and reagents. The human melanoma cell lines A375, G361, SK-MEL-28 and SK-MEL-2 were purchased from the Korean Cell Line Bank (Seoul, Republic of Korea). The cells were cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) for G361 and SK-MEL-2; or Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) for A375 and SK-MEL-28, supplemented with 10% (v/v) heat inactivated fetal bovine serum (Gibco BRL, Bethesda, MD, USA) and 1% streptomycin/penicillin, at 37˚C in a humidified atmosphere consisting of 5% CO_2 and 95% air. Cells were maintained mycoplasma free by treating with 5 μg/mL plasmocin (InvivoGen, San Diego, CA, USA). Trametinib (LC Laboratories, Woburn, MA, USA) and paclitaxel (Sigma-Aldrich) were initially dissolved in DMSO (Sigma-Aldrich) to a concentration of 1mM and further diluted in culture media. The final concentration of DMSO in the culture media did not exceed 0.1% (v/v). Metformin (Sigma-Aldrich) was prepared in PBS to a working concentration of 100 mM.

Cell viability assay. MTT assay was applied to measure cell viability as described previously (27). Briefly, cells were harvested and seeded in 24-well plates at a density of 5×10^4 cells/well for 24 h. Then, cells were treated with increasing concentrations of trametinib, paclitaxel, metformin or their combinations for 72 h. Experiments were performed in triplicate, and each conducted in quadruplicate. The IC_{50} values (concentrations of drugs resulting in 50% decrease in cell viability relative to controls) and combination index (CI) were calculated using CompuSyn software (ComboSyn Inc., Paramus, NJ, USA). The CI value is a quantitative measure of the degree to which drugs interacted. According to the recommendation of Chou-Talalay (28), CI<1 indicates synergistic effects of drugs; CI=1 indicates an additive effect; and CI>1 indicates antagonism.

Western Blot analysis. Western blotting assays were carried out as described earlier (27) to evaluate the effect of trametinib, metformin, paclitaxel and their combinations on the expression of cell signaling proteins or factors involved in cancer metastasis. Melanoma cells were incubated with respective drugs and their combinations for 24 h. Primary antibodies included pERK1/2 (Tyr204), ERK1/2, cyclin D1, pRSK (Ser380), RSK, SPARC, Twist1, Slug, N-cadherin, integrin αV, integrin β3, fibronectin, GAPDH, β-actin (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), and p4E-BP1 (Ser65), 4E-BP1, pS6 (Ser240/244), pS6

Table I. *Growth inhibitory effects of trametinib, paclitaxel and metformin in melanoma cell lines.*

Cell line	BRAF mutation	NRAS mutation	IC_{50} values		
			Trametinib (nM)	Paclitaxel (nM)	Metformin (mM)
A375	V600E	Wild type	13.3	2.5	2.2
G361	V600E	Wild type	1.2	4.9	1.5
SK-MEL-28	V600E	Wild type	2.1	16.2	3.9
SK-MEL-2	Wild type	Q61R	0.4	2.6	1

IC_{50} represents 50% inhibitory concentrations of cell viability relative to untreated controls.

(Ser235/236), S6, pAMPKα (Tyr172), AMPKα1/2 (all from Cell Signaling Technology, Danvers, MA, USA). Following incubation with secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology), immunoreactivity was detected with enhanced chemiluminescence (Santa Cruz Biotechnology).

Cell invasion and wound healing assay. For cell invasion assay, Boyden chambers (8.0μm pores, Transwell; Millipore, St. Louis, MO, USA) were coated with 0.4 mg/ml Matrigel (Corning Inc., Corning, NY, USA) and placed into 24-well cell culture chambers. In the lower chamber, complete medium supplemented with 10% FBS was added as chemoattractant. A375 melanoma cells (1×10^5 cells) were suspended in 100 μL serum-free medium, and loaded into the upper chamber. Following 24 hours of incubation, the inserts were removed and the non-invading cells on the upper surface were removed with a cotton swab. The invaded cells on the lower surface of filters were stained with 0.4% crystal violet after fixation with 4% paraformaldehyde. Photographs of five randomly chosen fields were taken and quantified by manual counting. Results represent the average of triplicate samples from three independent experiments.

For wound healing assay, A375 melanoma cells were grown to 80% confluence in 12-well culture plates in complete medium. After serum-starving cells for 24 hours, a 200-μl tip was used to create a consistent scratch in the cell monolayer. All wells were then carefully washed with culture medium and drug solutions in complete medium were added and incubated for 24 hours. Photographs were taken with an Olympus 1X70 microscope and an Olympus DP72 camera and DPController Software (Olympus Korea Inc., Seoul, Republic of Korea).

Statistical analysis. Data are expressed in the form of mean±SE. The statistical analysis was done by Student's *t*-test. Differences between means in each analysis were considered statistically significant when yielding $p\leq0.05$.

Results

Combining metformin with trametinib, or trametinib plus paclitaxel, shows differential antitumor effect in melanoma cell lines. To evaluate the growth inhibitory effects of trametinib, paclitaxel, metformin and their combinations, we used four melanoma cell lines; A375, G361, SK-MEL-28 and SK-MEL-2. The mutation status and IC_{50} values for each drug are shown in Table I. All cell lines had reduced cell viability in a dose-dependent manner following treatment with each drug (Figure 1). The IC_{50} values for trametinib and paclitaxel were at the nanomolar level, ranging from 0.4 to 13.3 nM for trametinib and from 2.5 to 16.2 nM for paclitaxel. IC_{50} for metformin was from 1 to 3.9 mM.

Next, we examined the growth inhibitory effects by combining the drugs in constant ratios to each other. To quantify the effects of drug combination, we employed the CompuSyn software to calculate the CI value for each combination therapy. Combining trametinib with paclitaxel resulted in synergistic growth inhibition in all four cell lines with CI values between 0.64 and 0.86. On the other hand, the combination of metformin with trametinib led to synergistic growth inhibition only in A375 cells (CI value=0.52). With this combination, G361 cell line exhibited an additive effect (CI value=0.97), whereas SK-MEL-28 and SK-MEL-2 cell lines displayed antagonistic effects having CI values 1.16 and 1.43, respectively. Finally, the combination of metformin with trametinib plus paclitaxel showed synergistic effect in A375, G361 and SK-MEL-28 cell lines with CI values of 0.64, 0.71 and 0.80 respectively, but antagonistic effect in SK-MEL-2 cell line with CI value of 1.13 (Figure 1).

Alterations in signaling molecules in response to trametinib, paclitaxel, metformin and their combinations. To characterize the underlying molecular basis for the differential antitumor effects found in cell viability assays, we next performed western blotting for the effector proteins and their activated forms in MAPK and mTOR signaling pathways following treatments with drugs and their combinations. To this end, we selected A375 and SK-MEL-2 cell lines, since these two showed distinct divergent effects on cell viability in response to the combined treatments of metformin with trametinib or with trametinib plus paclitaxel, resulting in synergism and antagonism, respectively.

Metformin is known to induce AMPK activation resulting in mTOR inhibition through activation of TSC2 and subsequent inhibition of Rheb. mTOR regulates cell growth and survival by phosphorylating the eukaryotic initiation

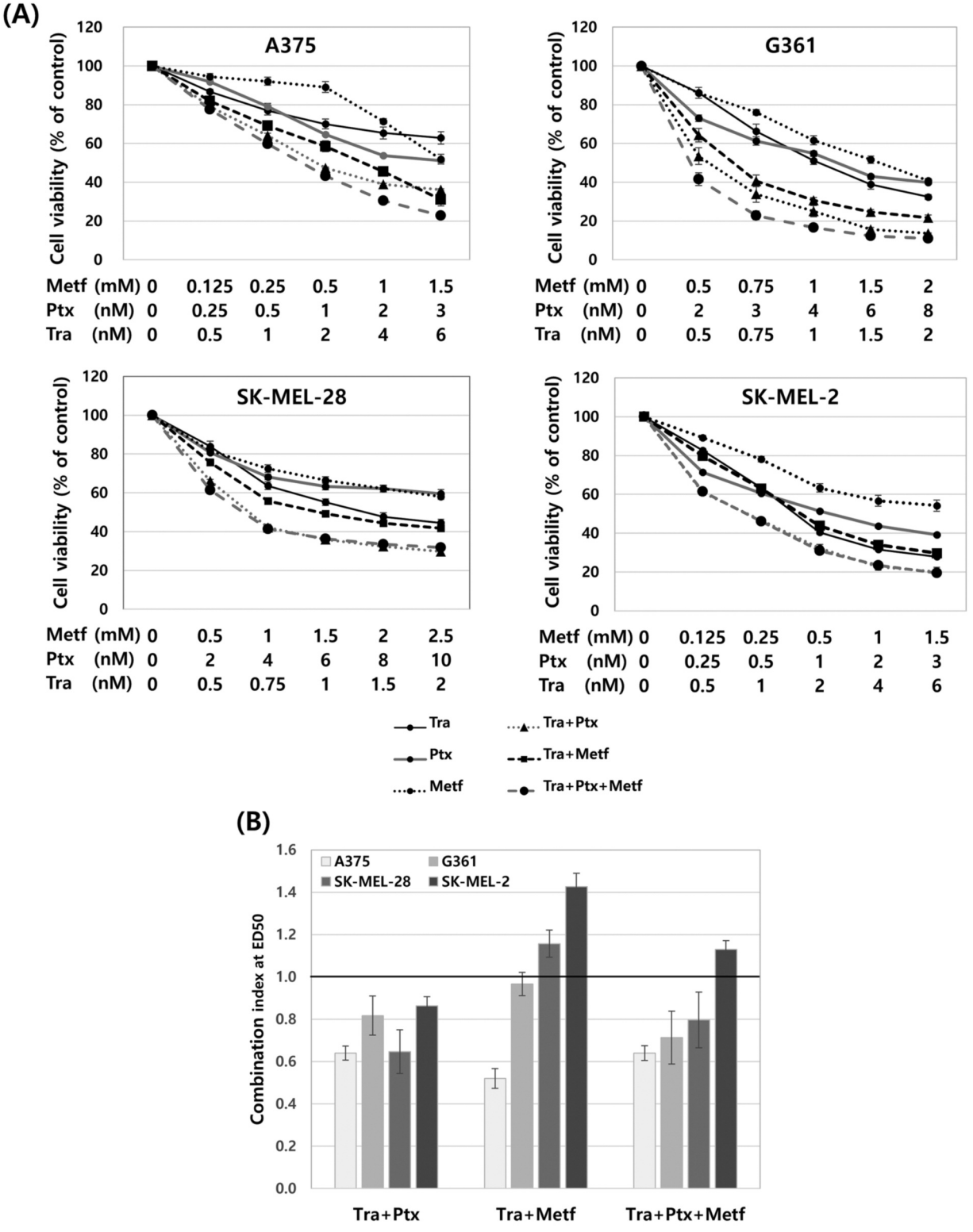

Figure 1. *Effects of trametinib (Tra), paclitaxel (Ptx), metformin (Metf) or their combinations on cell viability. (A) Dose-response curves on four melanoma cell lines (A375, G361, SK-MEL-28 and SK-MEL-2) following treatment of each drug alone or in combinations for 72 h (n=3, error bars represent SE). (B) Combination index (CI) values from A375, G361, SK-MEL-28 and SK-MEL-2 cell lines treated with drugs and their combinations. The CI values were calculated from the CompuSyn program by combining each drug in constant ratios. ED50 represents the doses effecting 50% of growth inhibition compared to control.*

factor 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 at both Ser240/244 and Ser235/236 *via* p70 rpS6 kinase (S6K) (19, 21). Interestingly, as shown in Figure 2A, treatment with metformin alone had differential effects on the two cell lines; it led to reductions in the levels of pERK, pRSK and pS6 (Ser235/236) in A375 cells but increases in SK-MEL-2 cells. These effects were dose-dependent. In contrast, metformin exerted similar effects on both cell lines by inducing AMPK activation and suppressing the phosphorylation of 4E-BP1 and S6 (Ser240/244). As for the effect of treatment with trametinib, A375 and SK-MEL-2 cells exhibited similar results, in which it inhibited the phosphorylation of ERK, RSK, 4E-BP1 and S6 dose-dependently. Notably, treatment with trametinib resulted in a weaker inhibitory effect in SK-MEL-2 cells compared to A375, implying that MEK inhibition is more sensitive in A375 cells harboring BRAF mutation, than SK-MEL-2 cells with NRAS mutation. Of further note, trametinib led to activation of AMPK in both cell lines. Paclitaxel had little or minimal effect on MAPK and mTOR signaling pathways in both cell lines (Figure 2A).

In the study of combined treatments, the combination of metformin with trametinib, or metformin with trametinib plus paclitaxel, led to more pronounced reductions in the levels of pERK, pS6 (Ser235/236), pS6 (Ser240/244) and p4EBP-1 in both cell lines. However, in contrast to the results observed in A375 cell line, the combined treatment of metformin with trametinib or trametinib plus paclitaxel did not completely abolish the metformin-induced increase of pRSK in SK-MEL-2 cells, which remained at even higher level than following treatment with trametinib alone. Trametinib plus paclitaxel exerted a similar effect on signaling molecules in both cell lines (Figure 2B).

Effects on expression of proteins involved in EMT following treatments with trametinib, paclitaxel, metformin and their combinations. Metformin has been reported to suppress cancer cell motility and metastasis development (22, 29, 30). On the other hand, recent studies have shown that MEK inhibition and low-dose paclitaxel enhance metastatic properties in melanoma and breast cancer cells, which could lead to the promotion of malignancy and treatment failure (31-34). To further investigate, we performed western blot assay on A375 cells to determine whether metformin, trametinib, paclitaxel or their combinations affect the expression of proteins implicated in EMT. As shown in Figure 3A, treatment with trametinib or paclitaxel as single agents increased the expression of Twist1, Slug and fibronectin in dose-dependent manner and exerted little effect on expression of SPARC, N-cadherin, integrin αV and integrin β3. In contrast, metformin remarkably reduced the levels of SPARC, Twist1, Slug, N-cadherin, integrin αV, integrin β3, and fibronectin, dose-dependently. We also observed that the combination of metformin with trametinib, or metformin with trametinib plus paclitaxel, almost completely extinguished the expression of SPARC, Twist1, Slug and fibronectin, and markedly reduced N-cadherin compared to results observed in treatments of trametinib and/or paclitaxel (Figure 3B).

Effects on cell invasion and migration following treatment with trametinib, paclitaxel, metformin and their combinations. Next, to investigate whether the molecular changes seen in the expression of EMT markers after treatment with trametinib, paclitaxel or metformin lead to functional alterations, we monitored cell invasion and migration in A375 cell line using trans-well invasion and wound healing assays, respectively. In a system using Boyden chambers coated with Matrigel, we found that metformin and paclitaxel as single agents significantly inhibited cell invasion in a dose-dependent manner after 24 h. In contrast, trametinib markedly increased cell invasion (Figure 4A). Combined treatment of metformin with trametinib resulted in suppression of the trametinib-induced pro-invasive effects, and the combination of metformin with trametinib plus paclitaxel further suppressed the invasion capacity as compared to treatment with trametinib plus metformin (Figure 4B).

In wound healing assay to monitor melanoma cell migration, treatment with metformin alone neither promoted nor inhibited cell migration compared to control. Of note, a dose-specific effect of trametinib or paclitaxel on cell migration was observed; cell migration was significantly promoted by low doses of trametinib or paclitaxel, but high doses exerted little effect on cell migration (Figure 5A). Combined treatments of metformin with trametinib or trametinib plus paclitaxel suppressed the trametinib or paclitaxel-induced cell migration (Figure 5B).

Discussion

Although the treatment of advanced melanoma with combinations of anticancer drugs has displayed more promising clinical benefits than monotherapy, a substantial proportion of patients are unresponsive to combination therapies and develop resistance with severe toxicities (6-10). Therefore, the development of rational combination strategies is urgently needed to improve clinical efficacy as well as to minimize the incidence of side-effects, with concomitant reductions in resistance and dose. The present study was designed to search for more effective drug combinations for the management of melanoma. Our strategy was based on dual inhibition of key proliferation signaling cascades, MAPK and PI3K/mTOR pathways, and dual cell cycle arrest at G_0/G_1 and G_2/M phase simultaneously. The strategy also aimed to minimize side-effects. For this purpose, we employed trametinib, paclitaxel and metformin,

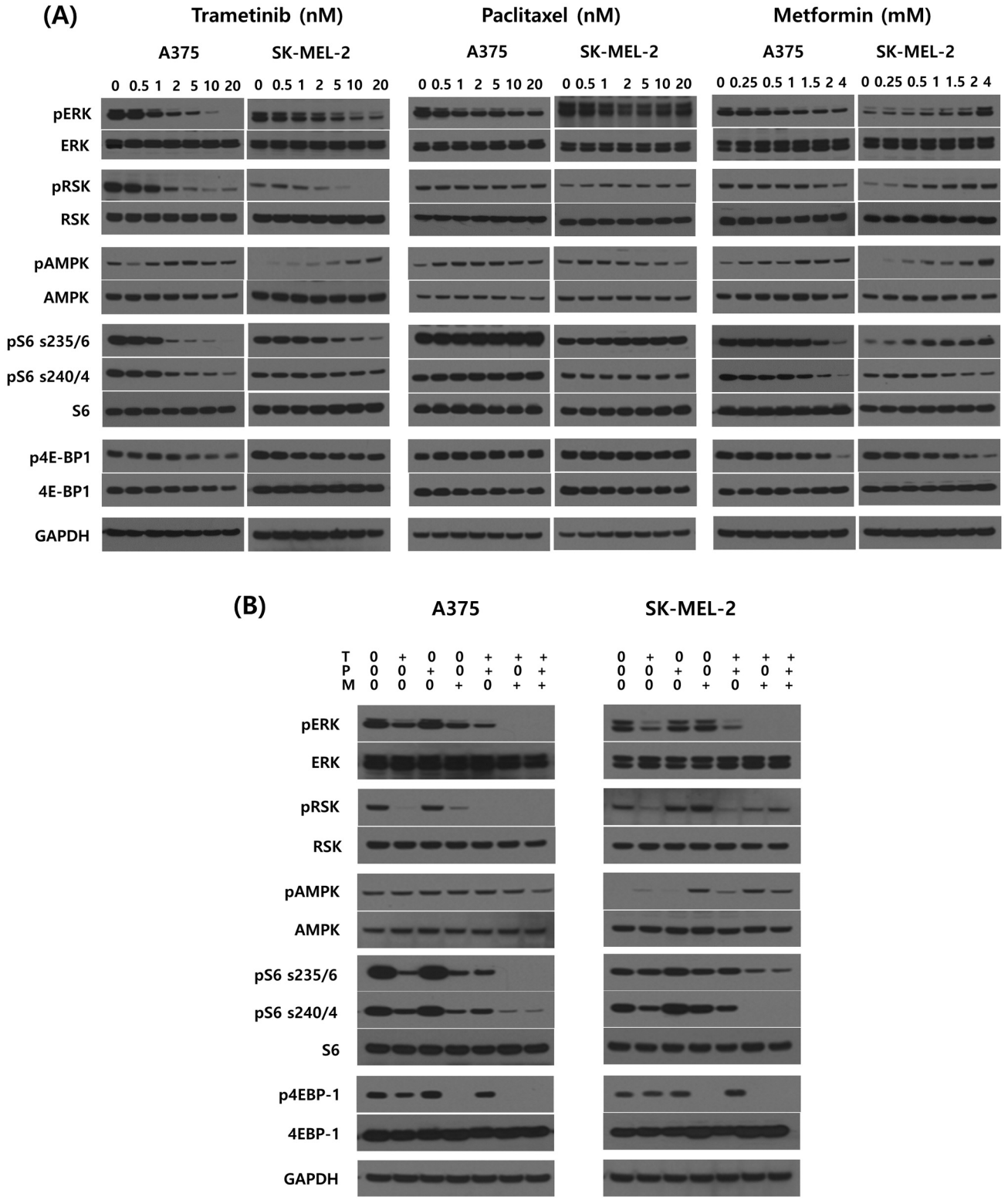

Figure 2. *Western blot analysis for downstream effector proteins of MAPK and mTOR signaling pathways on melanoma cell lines after 24-h treatment. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (A) A375 and SK-MEL-2 cell lines were treated with increasing concentrations of trametinib, paclitaxel or metformin. (B) A375 and SK-MEL-2 cell lines were treated with trametinib (T, 5 nM), paclitaxel (P, 5 nM), metformin (M, 2 mM) or their combinations.*

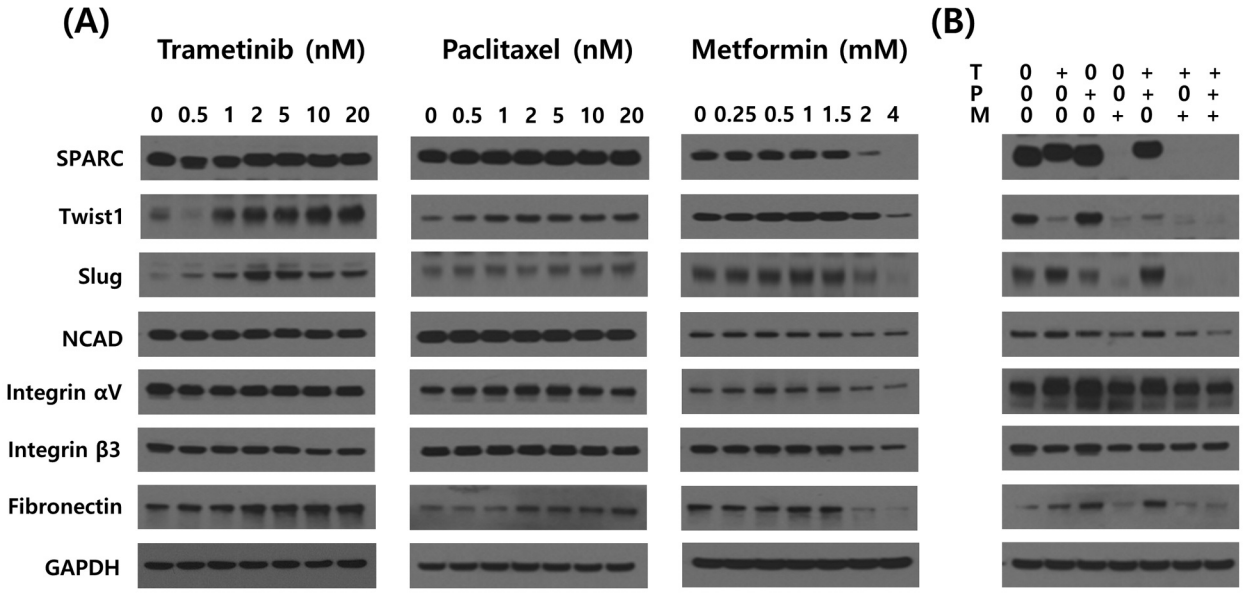

Figure 3. *Western blotting for EMT markers in A375 cell line after 24-h treatment. (A) Cells were treated with increasing concentrations of trametinib, paclitaxel, metformin or their combinations. (B) Cells were treated with trametinib (T, 5 nM), paclitaxel (P, 5 nM), metformin (M, 2 mM) or their combinations. Note that EMT markers (SPARC, Slug, NCAD and fibronectin) were markedly suppressed by combined treatment of metformin with trametinib or trametinib plus paclitaxel.*

each with distinct anticancer activities (8, 21, 27). In the area of oncology, the most significant obstacles to multidrug treatment are adverse events, which often lead to interruption of treatment and subsequent progression of disease. Antidiabetic drug metformin has been proven to be a well-tolerated drug with good safety profile and little drug resistance (35). Several clinical trials for the combined treatment of metformin with chemotherapeutic and/or targeted agents also revealed that metformin can be safely given to patients at up to 1,000 mg, two or three times a day which reaches plasma levels within the therapeutic range for diabetic patients (23-25, 36). In addition, Urbonas and his collaborators recently reported that adding trametinib to weekly paclitaxel at full monotherapy dose is tolerable and improves progression-free survival and objective response rates for melanoma patients (37). These clinical studies led us to try the combination of metformin with trametinib plus paclitaxel in melanoma cells.

In the present study, we had anticipated that the combination of metformin with trametinib plus paclitaxel would give synergistic antitumor activity, since simultaneous inhibition of MAPK and PI3K/mTOR pathways with trametinib and metformin blocks the compensatory effect between these two pathways and induces G_0/G_1 cell cycle arrest, and thus unresponsive or resistant cells evading G_0/G_1 arrest would then be captured at G_2/M phase with paclitaxel. However, our experimental results revealed divergent effects of the combination on melanoma cell viability, in particular showing synergism in the A375 cell line and antagonism in the SK-MEL-2 cell line.

In our western blot assays analyzing the differential growth inhibitory effect in the two cell lines, trametinib-induced reductions of ERK activity correlated with a decrease in RSK phosphorylation, and this reduction is likely to activate AMPK through the restoration of LKB1-AMPK complexes, which remain uncoupled and inactive in the face of hyperactive ERK/RSK signaling (38). Of note, especially in A375 cells, treatment with trametinib induced a remarkable reduction in pS6 (Ser235/236 and Ser240/244) and p4E-BP1, the main downstream effectors of mTOR. Indeed, these results are consistent with the notion that oncogenic ERK/RSK signaling promotes mTOR-dependent functions and melanoma cells are highly dependent on ERK/RSK signaling for their growth and proliferation (39).

Our study also showed that metformin treatment leads to suppression of 4E-BP1 and S6 phosphorylation in A375 cells. This is inconsistent with the report of Martin and his colleagues (40) in which metformin did not suppress the phosphorylation of 4E-BP1 and S6, but rather accelerated cell proliferation by increasing RSK activity in A375 cells. The reason for this apparent contradiction is unclear, but we assume that it might be attributable to different treatment regimens of metformin between the two studies. They used metformin at 2 mM, whereas we applied the incremental

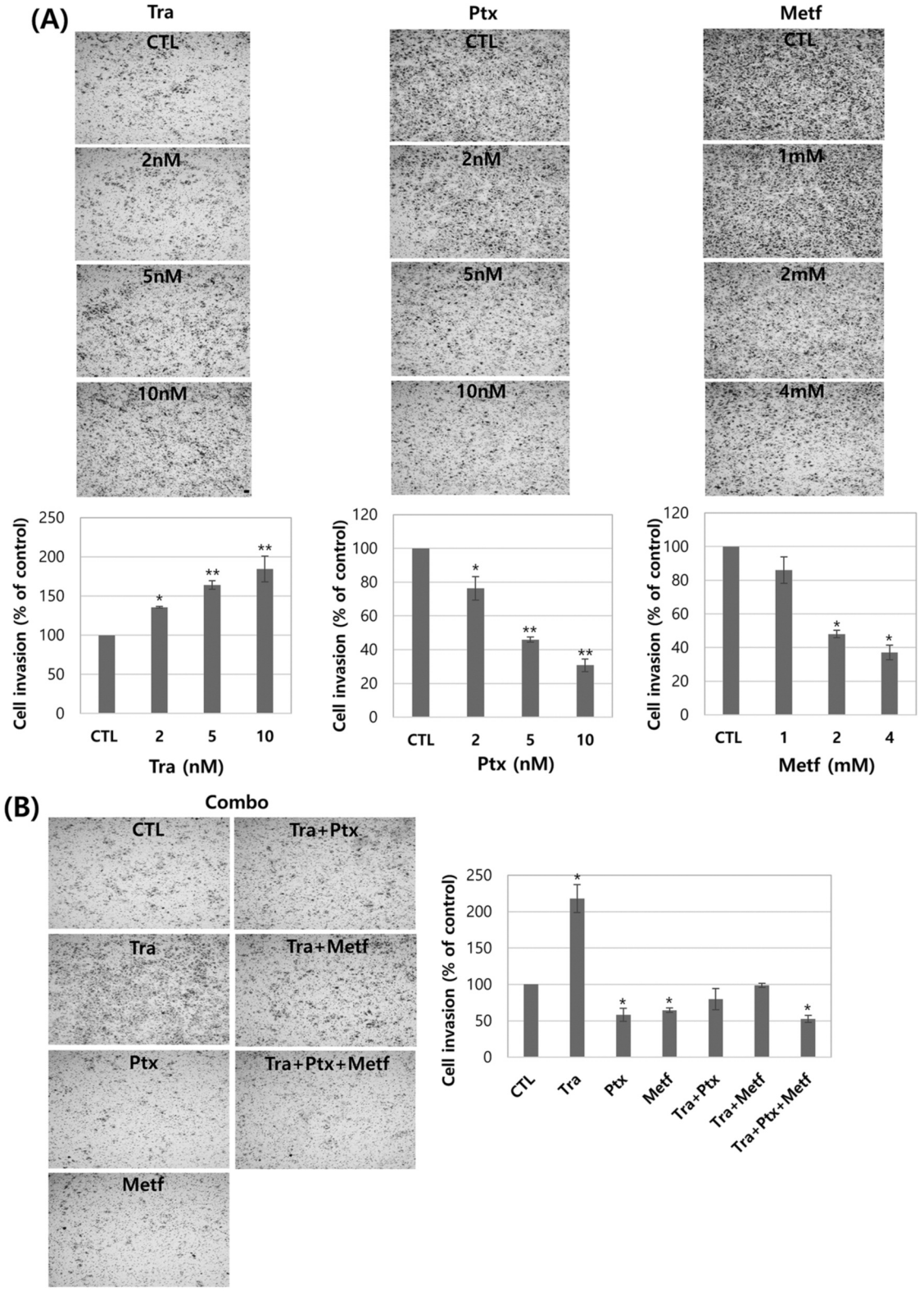

Figure 4. *Effects of trametinib (Tra), paclitaxel (Ptx), metformin (Metf) or their combinations on cell invasiveness of A375 cells. Cell invasion was evaluated using Boyden chambers coated with Matrigel after 24-h treatment. Representative images are shown. Bar graphs show the normalized values of the number of invading cells against controls. (A) Cells were treated with increasing doses of trametinib, paclitaxel or metformin. (B) Cells were treated with trametinib (5 nM), paclitaxel (5 nM), metformin (2 mM) or their combinations. n=3, error bars represent SE. *p<0.05, **p<0.01.*

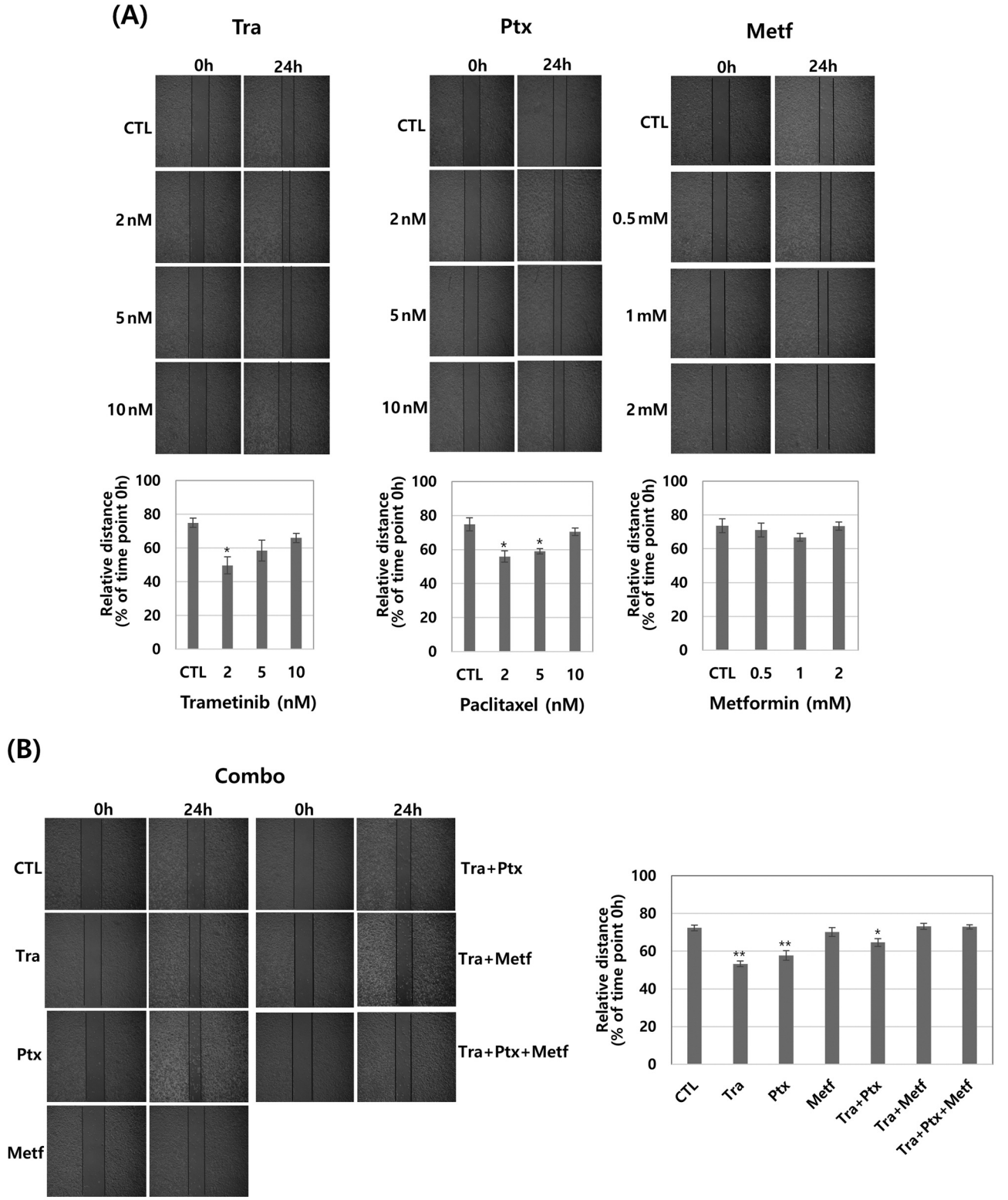

Figure 5. *Wound healing assay to measure the effects of trametinib (Tra), paclitaxel (Ptx), metformin (Metf) or their combinations on cell migration in A375 cells after 24-h treatment. Representative images taken at 0 h and 24 h are shown. Bar graphs show the relative gap distance against controls of time point zero. (A) Cells were treated with increasing doses of trametinib, paclitaxel or metformin. (B) Cells were treated with trametinib (5 nM), paclitaxel (5 nM), metformin (2 mM) or their combinations. n=3, error bars represent SE. *p<0.05, **p<0.01.*

concentrations of metformin up to 4 mM and found the levels of p4E-BP1 and pS6 to be reduced in a dose-dependent manner. Moreover, our data showed that metformin suppresses the phosphorylation of ERK and RSK, which correlates with the decrease in levels of p4E-BP1 and pS6 as a result of ERK-RSK-mTOR signaling (39).

Another interesting finding in this study was that metformin exerted differential effects on the ERK activity between melanoma cell lines. In contrast to A375 cells which showed decreased levels of pERK, metformin treatment in SK-MEL-2 cells activated ERK. Indeed, the effect of metformin on MAPK pathway has shown conflicting results in cancer cells (40-42). It has been shown that AMPK activators down-regulate MAPK signaling *via* AMPK-induced BRAF phosphorylation at Ser729 which inhibits its kinase activity through binding to 14-3-3 proteins (43). Thus, pERK downregulation after metformin exposure in A375 cells seems to be mediated by activation of AMPK, which then leads to inhibition of BRAF kinase activity. On the other hand, the molecular mechanism underlying the metformin-induced activation of ERK is less clear. Inhibition of mTOR is known to activate MAPK pathway through S6K-PI3K-RAS negative feedback regulation (44). As well, Morgillo and colleagues suggested that metformin treatment leads to phosphorylation of ERK through AMPK-induced heterodimerization of BRAF and CRAF in non-small cell lung cancer cells (NSCLC) (41). The enhanced activation of ERK *via* BRAF/CRAF heterodimerization has been reported to occur in melanoma cells with activated RAS (45). Thus, it is probable that ERK activation following metformin treatment in SK-MEL-2 cells with the NRAS mutation is mediated by AMPK-induced heterodimerization of BRAF and CRAF and/or activation of MAPK pathway resulting from abrogation of S6K-PI3K-RAS negative feedback loop by AMPK-induced mTOR inhibition. However, metformin-induced ERK activation is also observed in cancer cells with wild-type RAS (42) and with coexistent mutations of NRAS and BRAF (46). Moreover, using melanoma cells with BRAF mutation, metformin-induced AMPK activation targets and reduces the DUSP6 protein, a phosphatase acting as ERK-negative regulator, which results in increased ERK activity and acceleration of cell growth (40). Therefore, ERK activation in response to metformin seems to have occurred irrespective of the mutation status of the cancer cells. Further studies will be needed to elucidate the cellular context and molecular mechanism underlying this issue.

ERK activation after exposure to metformin could be therapeutically relevant since metformin as sole agent or in combination with other drugs could facilitate cell survival and proliferation by enhancing proliferative signals through MAPK pathway (41). Here, we showed that SK-MEL-2 cells with ERK activation following metformin treatment provides antagonistic growth inhibition in combinations of metformin with trametinib or with trametinib plus paclitaxel, whereas A375 cell line showing metformin-induced decrease of pERK displays a synergistic effect. Thus, it is plausible that this synergism in A375 cells could be due to a more enhanced blockade of MAPK signaling by combined treatment of metformin with trametinib or with trametinib plus paclitaxel, as evidenced by almost complete inhibition of pERK, pRSK, pS6 (Ser235/236 and Ser240/244), and p4E-BP1 in our western blots. In SK-MEL-2 cells, however, even though ERK activity was abolished by these combination treatments, there remains much higher levels of pRSK compared to treatment by trametinib alone, suggesting that metformin-induced elevation of RSK phosphorylation could not be completely inhibited by trametinib. RSK as a key ERK substrate promotes cell growth and proliferation by modulating S6 phosphorylation using dual mechanisms (39). In the mTOR-dependent mechanism, RSK inhibits TSC1/2, which then activates mTOR and accordingly, phosphorylates S6 at both Ser240/244 and Ser235/236. In the mTOR-independent mechanism, RSK phosphorylates S6 directly and exclusively at Ser235/236. Thus, the sustained RSK activity seems to directly phosphorylate S6 at Ser235/236 as revealed in our Western blot (Figure 2B), which could compromise anticancer effects in the combination treatments and lead to antagonistic effects in SK-MEL-2 cells. Therefore, we tentatively suggest that cancer cell-specific differential activity of ERK/RSK in response to metformin could serve as one predictive biomarker for development of effective therapeutic regimens when using metformin in combination with other anticancer agents.

As stated initially, melanoma is notorious for its aggressive metastatic behavior and metformin has been documented to suppress cell invasion and metastasis through reducing the expression of transcription factors driving EMT (22, 29, 30). Our present data also showed that metformin remarkably reduced the levels of SPARC, Twist1, Slug, N-cadherin, integrin αV, integrin β3, and fibronectin, dose-dependently. On the other hand, MEK inhibition can induce cell invasion and migration in breast cancer and melanoma cells (32, 34), and low-doses of paclitaxel directly induces EMT and enhances metastasis of breast cancer cells by increasing the expression of EMT regulators (31, 33). These reports are in agreement with our present findings, showing that trametinib and paclitaxel as single agents increased the expression of fibronectin and EMT core regulators such as twist1 and slug, and these molecular changes functionally correlated with the enhancement of melanoma cell migration or invasion. Undoubtedly, the pro-metastatic activities could be a serious obstacle to the effective cure of melanoma. Therefore, we extended the observations to the combined treatments of metformin with trametinib and trametinib plus paclitaxel, and found that metformin counteracts the cell migratory and invasive activities of these agents. Furthermore, the functional analyses using cell invasion or

migration assay are supported by the western blot results in which trametinib or paclitaxel-induced increases in the levels of twist1, slug and fibronectin are extinguished by the addition of metformin. These results reinforce the premise that metformin might be used as a potential adjuvant for melanoma patients not only *via* inhibition of cell proliferation but also by suppression of metastasis. Further *in vivo* studies using mouse xenograft models and clinical trials are required to better understand the combined effect of metformin with trametinib and paclitaxel on metastatic activity in melanoma.

Taken together, our study provides the first *in vitro* results and a rationale for the concept that combining metformin with trametinib plus paclitaxel might be a promising therapeutic option for treatment of patients with melanoma. In particular, our data suggest that alteration of ERK activity in response to metformin could serve as a predictive biomarker for the efficacy of metformin monotherapy or combination therapies with other anticancer agents. In addition, our data show for the first time that metformin inhibits the cell migratory and invasive activities of trametinib and paclitaxel. Future perspective studies are needed to identify the best context in which cancer patients can most benefit from combining metformin with molecular targeted or/and chemotherapeutic agents.

Conflicts of Interest

The Authors have no conflicts of interest regarding this study.

Authors' Contributions

Youngki Lee designed, performed the experiments and wrote the paper. Deokbae Park contributed to discussions and manuscript reading.

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