

# Dual Inhibition of MAPK and JAK2/STAT3 Pathways Is Critical for the Treatment of BRAF Mutant Melanoma

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**BRAF and MEK inhibitors significantly prolong progression-free survival in patients with BRAF mutant melanoma. However, most patients quickly develop drug resistance. The mechanism of drug resistance is complicated and remains to be further explored. Here, we found that inhibition of the MAPK pathway activates the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway, whereas JAK2 inhibitors that inhibit the JAK2/STAT3 pathway activate the MAPK pathway, suggesting a crosstalk between these two pathways in BRAF mutant melanoma cells. Reactivation of the MAPK pathway occurs in most drug-resistant patients with BRAF mutations. Therefore, dual inhibition of the MAPK and JAK2/STAT3 pathways is critical for the treatment of BRAF mutant melanoma. However, we found that the combination of BRAF, MEK inhibitors, and JAK2 or STAT3 inhibitors could not simultaneously inhibit the MAPK and JAK2/STAT3 pathways in BRAF mutant melanoma cells. Subsequently, we found that a combination of all three MAPK pathway inhibitors—BRAF, MEK, and ERK inhibitors—with JAK2 or STAT3 inhibitors can dually inhibit the MAPK and JAK2/STAT3 pathways, showing a significant inhibition of the growth of BRAF mutant melanoma cells compared with either treatment alone. Therefore, dual inhibition of MAPK and JAK2/STAT3 pathways may be a novel strategy for the treatment of BRAF mutant tumors.**

## INTRODUCTION

Approximately 7% of all human tumors have BRAF mutations.<sup>1</sup> BRAF mutations are common in melanoma (50%), papillary thyroid cancer (30%–70%), ovarian cancer (15%–30%), and colorectal cancer (5%–20%).<sup>2</sup> The mutant BRAF protein continuously activates the mitogen-activated protein kinase (MAPK) pathway (also known as the RAS-RAF-MAPK kinase [MEK]-extracellular signal-regulated kinase [ERK] pathway) to promote tumor cell proliferation and survival.<sup>3,4</sup> PLX4032 (vemurafenib) is a specific and potent BRAF inhibitor that was approved by the US Food and Drug Administration (FDA) for unresectable metastatic melanoma in 2011. PLX4032-targeted therapy significantly prolongs progression-free survival in melanoma patients.<sup>5–7</sup> Combination therapy with MEK and BRAF inhibitors showed more durable and greater tumor responses than BRAF

monotherapy.<sup>8,9</sup> Clinical results indicated that BRAF mutant melanoma patients had a response rate of approximately 70% for BRAF inhibitors combined with MEK inhibitors, whereas 50% for BRAF monotherapy.<sup>7</sup> However, most patients develop tumor recurrence after 11–14 months of targeted therapy.<sup>8,10</sup> Therefore, it is urgent to explore new strategies to improve the treatment of melanoma.

The Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway plays an important role in cell proliferation and survival. It is hyperactive in many tumors, including melanoma.<sup>11</sup> Most of drug-resistance mechanisms currently discovered involve the reactivation of MAPK pathway and activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway.<sup>1,7,8,10,12–14</sup> MAPK, PI3K/AKT, and JAK2/STAT3 pathways are all regulated by the receptor tyrosine kinases (RTKs) and nonreceptor tyrosine kinases (NRTKs). Whether the JAK2/STAT3 pathway is involved in the resistance of BRAF mutant tumors to BRAF inhibitors remains unclear.

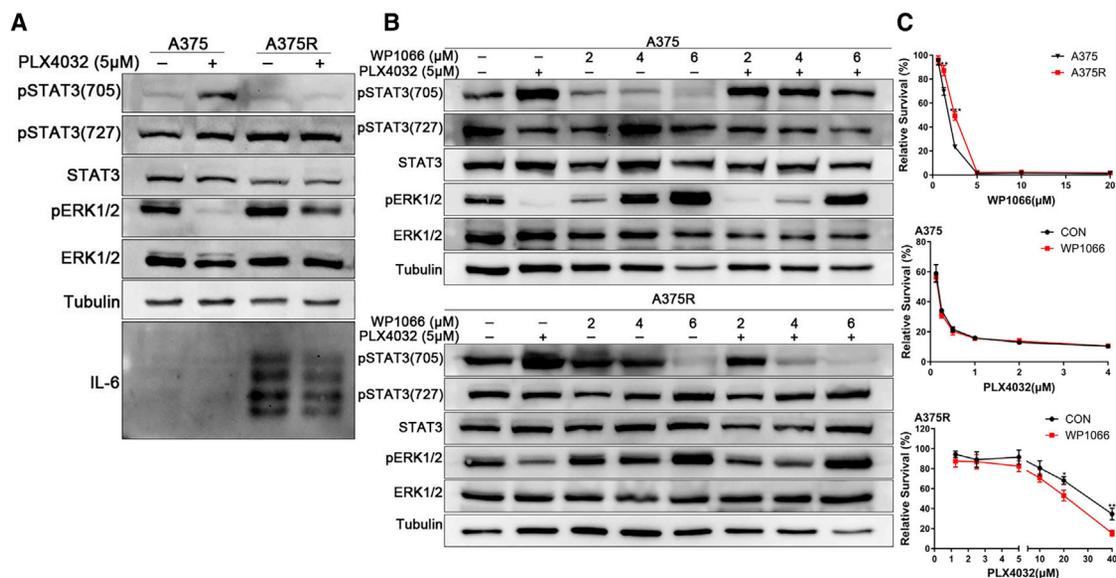
Vascular endothelial growth factor (VEGF) plays important roles in angiogenesis, cell proliferation, and metastasis.<sup>15,16</sup> Many drugs that target VEGF have been approved for the treatment of various diseases. VEGF is a downstream effector of the JAK2/STAT3 pathway. The silencing of STAT3 in B16.F10 melanoma significantly inhibits VEGF expression.<sup>17</sup> It is unclear whether VEGF promotes BRAF mutant tumor cells to resist BRAF inhibitors.

In this article, we found a crosstalk between MAPK and JAK2/STAT3 pathways in BRAF mutant tumor cells. However, the combination of

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**Figure 1. Crosstalk between the JAK2/STAT3 and MAPK Pathways in A375 and A375R Cells**

(A and B) A375 and A375R cells were treated with PLX4032 (A), PLX4032 and WP1066 (B) for 6 h. Phospho-STAT3 (705), phospho-STAT3 (727), STAT3, phospho-ERK1/2, ERK, and IL-6 (A only) levels were analyzed by western blotting, and tubulin served as a loading control. (C) A375 and A375R cells were treated with different concentrations of WP1066 or PLX4032 and WP1066 (2 μM) for 72 h. Cell viability was determined by CCK8 assays. Data are mean  $\pm$  SD. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; Student's *t* test.

BRAF, MEK, and JAK2 or STAT3 inhibitors cannot simultaneously inhibit the MAPK and JAK2/STAT3 pathways, while the combination of all three MAPK pathway inhibitors, BRAF, MEK, ERK inhibitors and JAK2 or STAT3 inhibitors can simultaneously inhibit these two pathways and achieve much better therapeutic effects in BRAF mutant melanoma cells.

## RESULTS

### Dual Inhibition of the MAPK and JAK2/STAT3 Pathway Is Essential to Inhibit the Growth of BRAF Mutant Melanoma Cells

Studies have found that autocrine interleukin 6 (IL-6) activates the JAK2/STAT3 and MAPK pathways to resist BRAF inhibitors in BRAF mutant melanoma cells.<sup>18</sup> To investigate whether IL-6 activates the JAK2/STAT3 pathway to resist BRAF inhibitors in BRAF mutant melanoma cells, we treated drug sensitive (A375) and resistant (A375R) cells with PLX4032 (a BRAF inhibitor) or dimethyl sulfoxide (solvent). The results showed that IL-6 did not activate the JAK2/STAT3 pathway in A375R cells (Figure 1A). Furthermore, we found that PLX4032 promoted STAT3 activation in A375 cells without IL-6 expression (Figure 1A).

To verify whether the JAK2/STAT3 pathway is involved in the BRAF mutant tumor cells against BRAF inhibitors, we treated A375 and A375R cells with PLX4032, alone or in combination with WP1066 (a JAK2 inhibitor). The results showed that PLX4032 suppressed ERK activation but promoted STAT3 activation in A375 and A375R cells. In contrast, WP1066 inhibited STAT3 activation but promoted ERK activation (Figure 1B). Additionally, treatment of A375 cells with PLX4032 plus WP1066 reactivated the MAPK and JAK2/STAT3 pathways, whereas treatment of A375R cells with

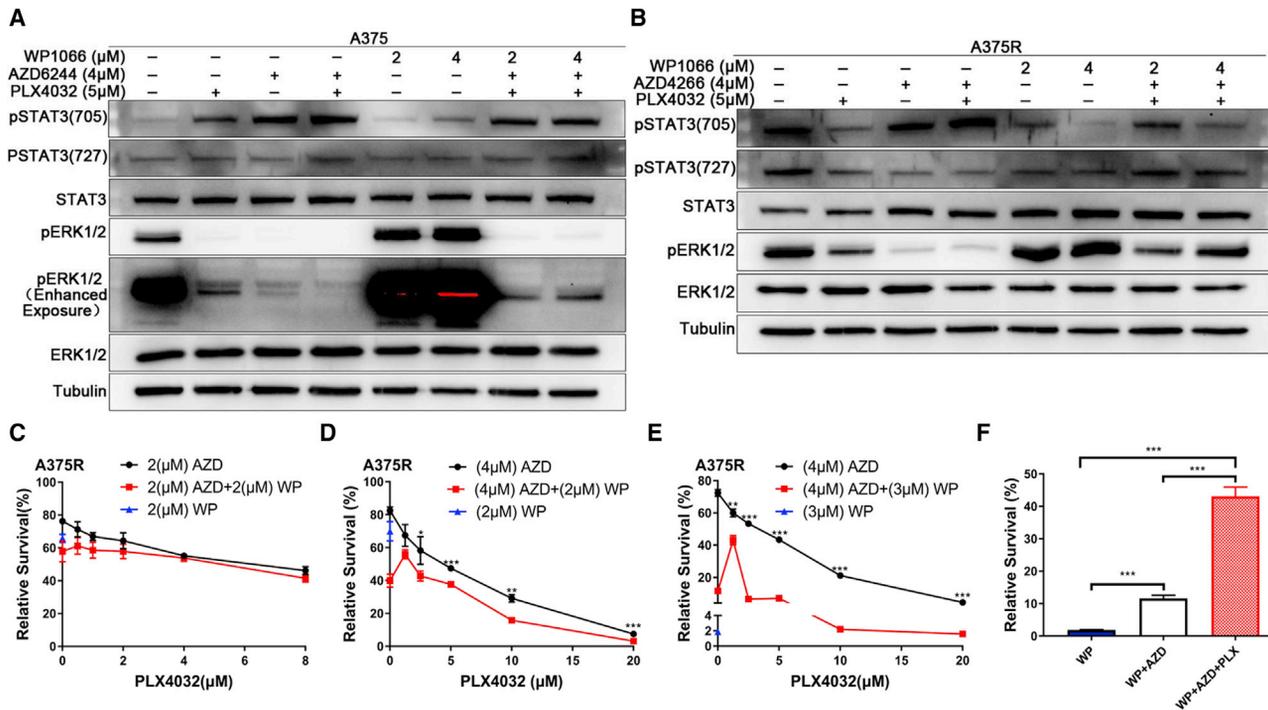
PLX4032 plus WP1066 reactivated the MAPK pathway but inhibited the JAK2/STAT3 pathway (Figure 1B). These results demonstrated that there is a crosstalk between MAPK and JAK2/STAT3 pathways in both A375 and A375R cells.

Next, we investigated the inhibitory effects of combined BRAF and JAK2 inhibitors in A375 and A375R cells. We first examined the cytotoxicity of JAK2 inhibitors in A375 and A375R cells. The results showed that A375R cells were more tolerant to JAK2 inhibitors than parental A375 cells (Figure 1C). Then, we treated A375 and A375R cells with PLX4032, alone or in combination with WP1066. There was no significant difference in growth inhibition of A375 cells between the combination treatment group and the single treatment group (Figure 1C). However, the combination of PLX4032 and WP1066 significantly inhibited the growth of A375R cells compared with PLX4032 alone (Figure 1C). These results were consistent with the results of western blotting.

In summary, we found that inhibition of the MAPK pathway activated the JAK2/STAT3 pathway, and vice versa, in BRAF mutant melanoma cells. There is a crosstalk between the MAPK and JAK2/STAT3 pathways in BRAF mutant melanoma cells. Dual inhibition of the MAPK and JAK2/STAT3 pathways is critical for the treatment of BRAF mutant melanoma. However, it is not feasible to treat BRAF mutant melanoma with BRAF and JAK2 inhibitors.

### The Combination of BRAF, MEK, and JAK2 Inhibitors Does Not Simultaneously Inhibit the MAPK and JAK2/STAT3 Pathways in BRAF Mutant Melanoma Cells

The combination of BRAF and MEK inhibitors was approved for advanced BRAF mutant melanoma in 2014. To explore whether



**Figure 2. JAK2 Inhibitors Reactivated the MAPK Pathway Silenced by BRAF and MEK Inhibitors in A375 and A375R Cells**

(A and B) A375 (A) and A375R (B) cells were treated with different concentrations of PLX4032, AZD6244, and WP1066 for 6 h. Phospho-STAT3 (705), phospho-STAT3 (727), STAT3, phospho-ERK1/2, and ERK levels were analyzed by western blotting, and tubulin served as a loading control. (C–E) A375R cells were treated with the same combination of WP1066, PLX4032, and AZD6244 inhibitors but at different concentrations respectively for 72 h. Cell viability was determined by CCK8 assays. (F) Data in the WP1066 treatment group, WP1066 plus AZD6244 group, and WP1066 plus AZD6244 and 1.25 μM PLX4032 group in (E) are summarized. Data are mean  $\pm$  SD. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; Student's *t* test.

combined BRAF, MEK, and JAK2 inhibitors can simultaneously inhibit MAPK and JAK2/STAT3 pathways, we treated A375 and A375R cells with PLX4032 (a BRAF inhibitor), AZD6244 (a MEK inhibitor), and WP1066 (a JAK2 inhibitor), separately or in combination. The results showed that WP1066 promoted ERK reactivation inhibited by PLX4032 and AZD6244 in A375 and A375R cells (Figures 2A and 2B). Additionally, we also confirmed that inhibition of the MAPK pathway promoted the activation of the JAK2/STAT3 pathway in BRAF mutant melanoma cells (Figures 2A and 2B; Figures S1A and S1B).

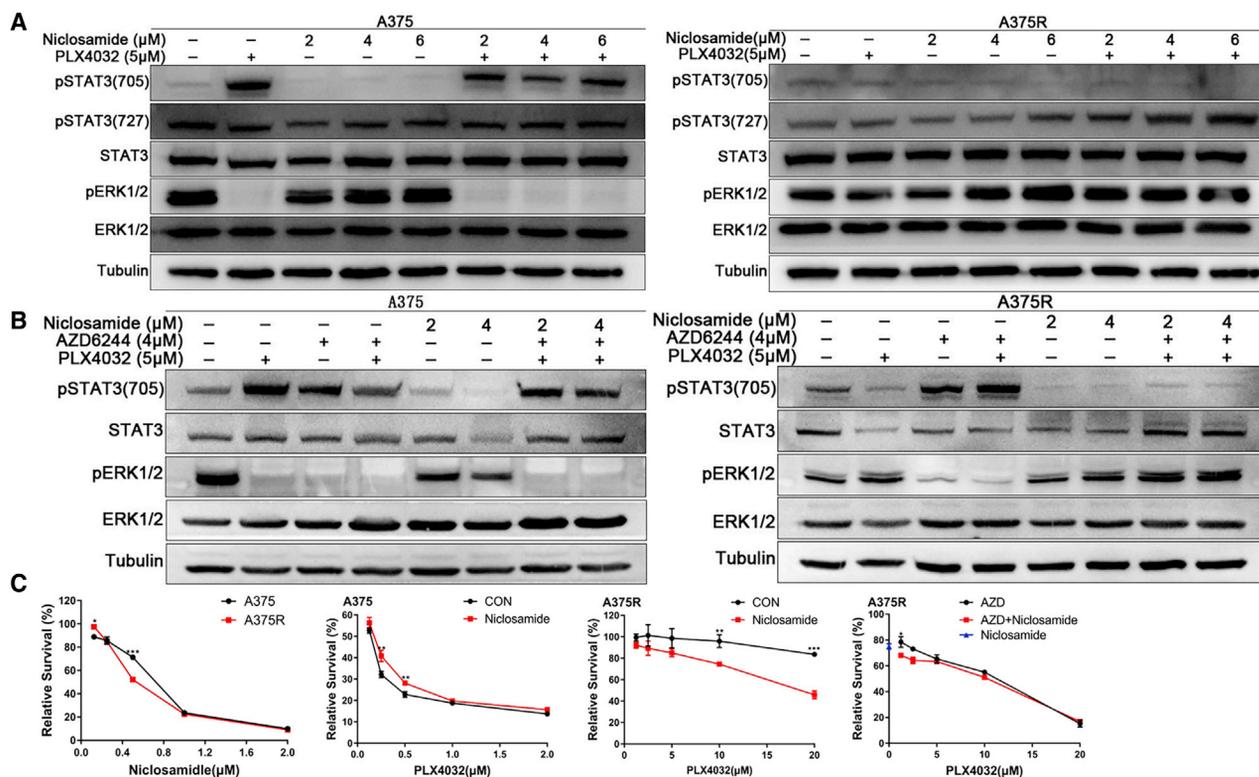
Then, we explored the inhibitory effects of combined MEK and JAK2 inhibitors on cells. The results showed that AZD6244 combined with WP1066 significantly inhibited the growth of A375 and A375R cells compared with AZD6244 alone (Figure S1C). Next, we compared the inhibitory effects of PLX4032, AZD6244, and WP1066 on A375R cells with those of PLX4032 and AZD6244. The results showed that PLX4032, AZD6244 (2 μM), and WP1066 (2 μM) had better inhibitory effects on A375R cells than PLX4032 and AZD6244 (2 μM); however, there were no significant differences (Figure 2C). As the concentration of AZD6244 increased, the inhibitory effect of BRAF, MEK, and JAK2 inhibitors on A375R cells was significantly enhanced (Figure 2D). To achieve better inhibition of tumor cell growth, we increased the concentra-

tion of WP1066. We found that BRAF and MEK inhibitors interfered with the inhibitory effects of JAK2 inhibitors on A375R cells (Figures 2E and 2F).

Therefore, JAK2 inhibitors can reactivate the MAPK pathway inhibited by BRAF and MEK inhibitors in A375 and A375R cells. The combination of BRAF, MEK, and JAK2 inhibitors is not suitable for the treatment of BRAF mutant melanoma.

#### The Combination of BRAF, MEK, and STAT3 Inhibitors Also Fails to Inhibit Simultaneously the MAPK and JAK2/STAT3 Pathways in BRAF Mutant Melanoma Cells

JAK2 inhibitors reactivate MAPK pathways silenced by BRAF and MEK inhibitors. To explore possible therapeutic strategies, we combined BRAF, MEK, and STAT3 inhibitors to treat A375 and A375R cells. Niclosamide is a specific STAT3 inhibitor that significantly inhibits STAT3 activation.<sup>19</sup> We treated A375 and A375R cells with PLX4032, alone or in combination with niclosamide. The results showed that niclosamide did not promote ERK activation in A375 and A375R cells (Figure 3A). However, we found that the JAK2/STAT3 pathway was reactivated in A375 cells treated with PLX4032 and niclosamide (Figure 3A). Next, we treated A375 and A375R cells with BRAF, MEK, and STAT3 inhibitors. The results indicated that PLX4032 and AZD6244 could reactivate STAT3



**Figure 3. STAT3 Inhibitors Also Reactivate the MAPK Pathway Inhibited by BRAF and MEK Inhibitors in A375R Cells**

(A and B) A375 and A375R cells were treated with different concentrations of PLX4032 and niclosamide (A), PLX4032, AZD6244, and niclosamide (B) for 6 h. Phospho-STAT3 (705), phospho-STAT3 (727), STAT3, phospho-ERK1/2, and ERK levels were analyzed by western blotting, and tubulin served as a loading control. (C) A375 and A375R cells were treated with different concentrations of niclosamide, PLX4032, and AZD6244 for 72 h. Cell viability was determined by CCK8 assays. Data are mean  $\pm$  SD. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; Student's *t* test.

inhibited by niclosamide in A375 cells (Figure 3B). In A375R cells, niclosamide reactivated ERK inhibited by PLX4032 and AZD6244 (Figure 3B).

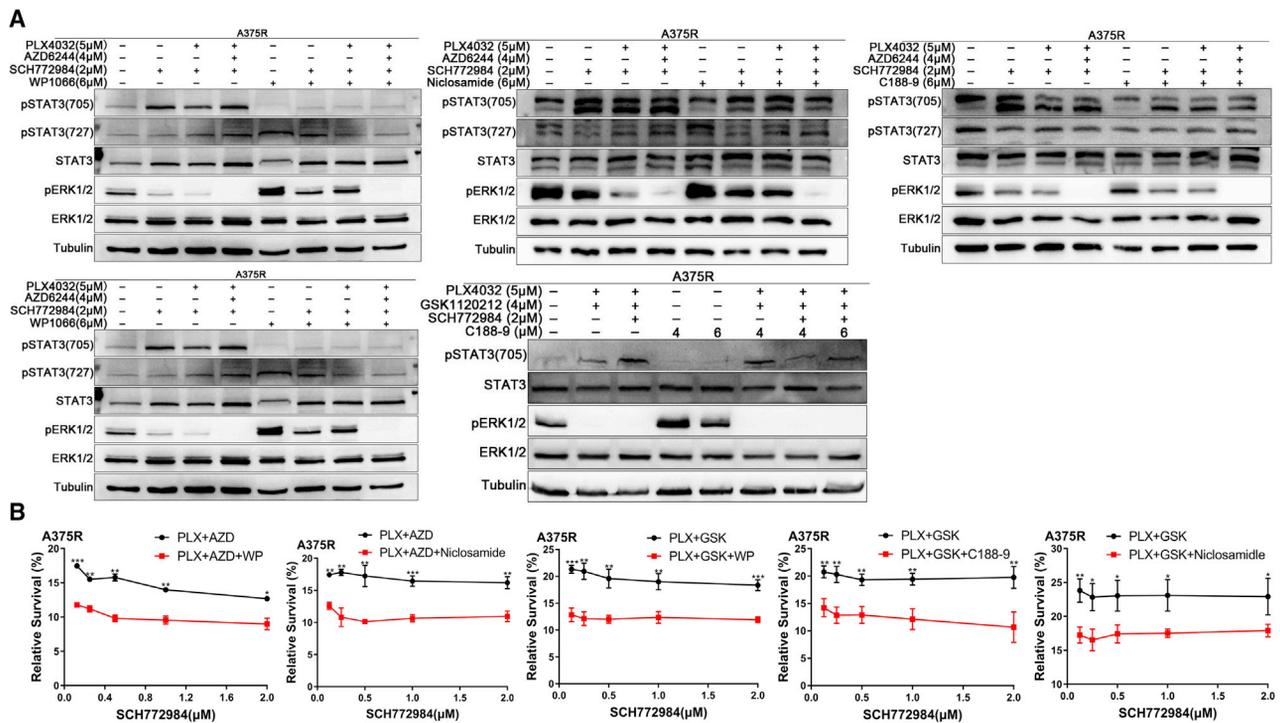
Next, we explored the inhibitory effects of combined BRAF, MEK, and STAT3 inhibitors on A375 and A375R cells. We tested the cytotoxicity of niclosamide in A375 and A375R cells (Figure 3C). Then, we treated A375 and A375R cells in combination with PLX4032 and niclosamide. The results showed that there was no significant difference in the inhibition of A375 cell growth between PLX4032 plus niclosamide and PLX4032 alone (Figure 3C). However, the growth of A375R cells was significantly inhibited by combined PLX4032 and niclosamide compared with PLX4032 alone (Figure 3C). Finally, we treated A375R cells in combination with PLX4032, AZD6244, and niclosamide. The inhibitory effects of combined PLX4032, AZD6244, and niclosamide on A375R cells were not significantly different from those of combined PLX4032 and AZD6244 (Figure 3C).

These results indicated that combined BRAF, MEK, and STAT3 inhibitors also failed to inhibit both MAPK and JAK2/STAT3 pathways in A375 and A375R cells. Treatment of BRAF mutant melanoma with

BRAF, MEK, and STAT3 inhibitors was not appropriate due to the crosstalk between MAPK and JAK2/STAT3 pathways.

#### The Combination of BRAF, MEK, and ERK Inhibitors and JAK2 or STAT3 Inhibitors Simultaneously Inhibits the MAPK and JAK2/STAT3 Pathways and Significantly Inhibits the Growth of BRAF Mutant Melanoma Cells

To simultaneously inhibit MAPK and JAK2/STAT3 pathways, we attempted to treat melanoma cells with ERK and JAK2 inhibitors. WP1066 (a JAK2 inhibitor) reactivated the MAPK pathway inhibited by SCH772984 (an ERK inhibitor) in A375R cells (Figure 4A). In A375 cells, SCH772984 blocked the activation of the MAPK pathway in the presence of WP1066 (Figure S2A). Next, we treated A375R cells with SCH772984 alone or in combination with other MAPK pathway inhibitors and JAK2/STAT3 pathway inhibitors. We found that WP1066 also reactivated MAPK pathway inhibited by SCH772984 and PLX4032 in A375R cells (Figure 4A). However, the combination of PLX3032, AZD6244, and SCH772984 in A375R cells completely inhibited ERK activation in the presence of WP1066 (Figure 4A). Moreover, STAT3 inhibitors (niclosamide or C188-9) no longer reactivated the MAPK pathway inhibited by BRAF (PLX4032), MEK (AZD6244 or



**Figure 4. Dual Inhibition of the JAK2/STAT3 and MAPK Pathways Significantly Inhibited the Growth of A375R Cells Compared with Inhibition of the MAPK Pathway Alone**

(A) A375R cells were treated with BRAF inhibitors (PLX4032), MEK inhibitors (AZD6244, GSK1120212), ERK inhibitors (SCH772984), and JAK2 inhibitors (WP1066) or STAT3 inhibitors (niclosamide, C188-9) for 6 h. Phospho-STAT3 (705), STAT3, phospho-ERK1/2, and ERK levels were analyzed by western blotting, and tubulin served as a loading control. (B) A375R cells were treated with different concentrations of WP1066, C188-9, PLX4032, AZD6244, GSK1120212, and SCH772984 for 72 h. Cell viability was determined by CCK8 assays. Data are mean  $\pm$  SD. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; Student's *t* test.

GSK1120212), and ERK (SCH772984) inhibitors in A375R cells (Figure 4A). At the same time, we found that STAT3 activation in A375R cells treated with MAPK pathway inhibitors (BRAF, MEK, and ERK inhibitors) and JAK2/STAT3 pathway inhibitors was significantly lower than that of MAPK pathway inhibitors alone (Figure 4A).

To investigate the inhibitory effect of combined MAPK and JAK2/STAT3 pathway inhibitors on BRAF mutant melanoma cells, we first treated A375 and A375R cells with SCH772984. We found that A375R cells were more tolerant to SCH772984 than A375 cells (Figure S2B). Then, we treated A375R cells with BRAF, MEK, and ERK inhibitors and JAK2 or STAT3 inhibitors. We found that the combination of BRAF (PLX4032), MEK (AZD6244 or GSK1120212), and ERK (SCH772984) inhibitors and JAK2 (WP1066) or STAT3 (niclosamide or C188-9) inhibitors showed significant inhibition of BRAF mutant melanoma cell growth compared with the combination of BRAF, MEK, and ERK inhibitors (Figure 4B).

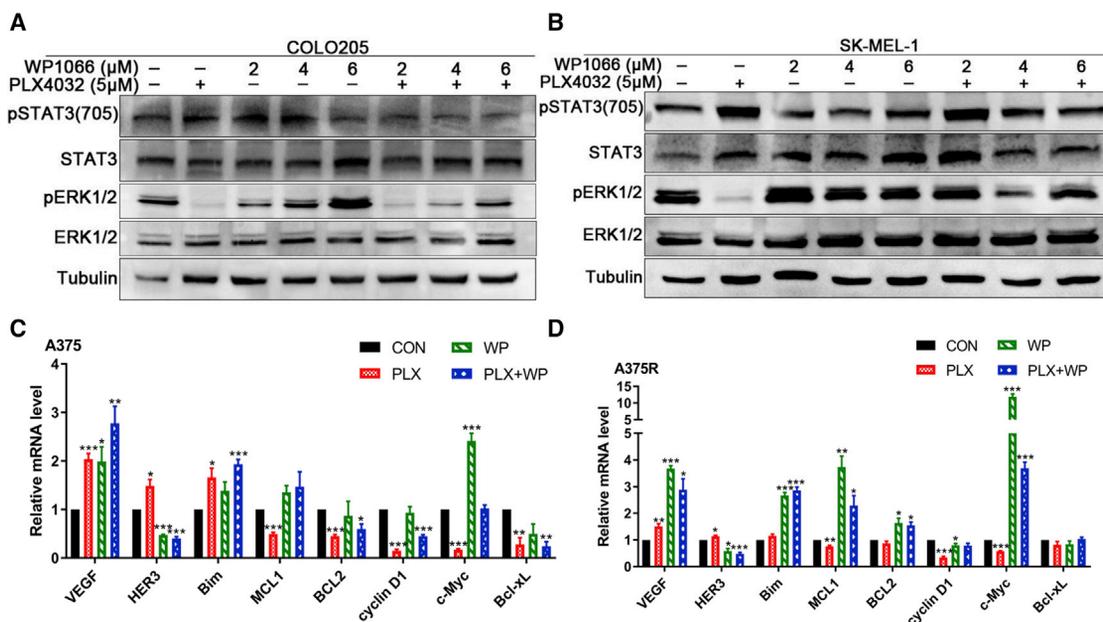
In summary, combined BRAF, MEK, and ERK inhibitors and JAK2 or STAT3 inhibitors can simultaneously inhibit MAPK and JAK2/STAT3 pathways in BRAF mutant melanoma cells. Dual inhibition of MAPK and JAK2/STAT3 pathways significantly inhibited the

growth of BRAF mutant melanoma cells compared with inhibition of either of them.

#### Crosstalk between MAPK and JAK2/STAT3 Pathways Occurs in Other BRAF Mutant Tumor Cells

To investigate whether there is a crosstalk between MAPK and JAK2/STAT3 pathways in other BRAF mutant tumor cells, we treated COLO205 (colon cancer) and SK-MEL-1 (melanoma) cells with PLX4032 alone or in combination with WP1066. The results demonstrated that WP1066 also reactivated the MAPK pathway inhibited by PLX4032 in SK-MEL-1 and COLO205 cells (Figures 5A and 5B), suggesting the crosstalk between JAK2/STAT3 and MAPK pathways is also present in other BRAF mutant tumor cells.

To investigate the mechanisms through which BRAF mutant tumor cells acquire resistance to BRAF and JAK2 inhibitors, we detected possible downstream genes of MAPK and JAK2/STAT3 pathways. We found that both BRAF and JAK2 inhibitors significantly promoted VEGF expression in A375 and A375R cells (Figures 5C and 5D). At the same time, the combination of BRAF inhibitors and JAK2 inhibitors significantly promoted the expression of VEGF in A375 cells compared with either of them alone



**Figure 5. Crosstalk between the JAK2/STAT3 and MAPK Pathways Occurs in COLO205 Cells and SK-MEL-1 Cells**

(A and B) COLO205 (A) and SK-MEL-1 (B) cells were treated with different concentrations of PLX4032 and WP1066 for 6 h. Phospho-STAT3 (705), STAT3, phospho-ERK1/2, and ERK levels were analyzed by western blotting, and tubulin served as a loading control. (C and D) A375 (C) and A375R (D) cells were treated with PLX4032 and WP1066 alone or in combination for 6 h, and then the downstream genes of the JAK2/STAT3 pathway were detected by RT-PCR. GAPDH was used as an internal reference. Data are mean  $\pm$  SD. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; Student's *t* test.

(Figure 5C). We speculated that VEGF is dually regulated by MAPK and JAK2/STAT3 pathways in A375 cells.

#### BRAF Mutant Cancer Cells Secrete VEGF to Resist BRAF Inhibitors

Previous studies have shown that VEGF plays an important role in cell proliferation, pathological angiogenesis, and metastasis in many types of tumors.<sup>15,17,20</sup> To further verify whether BRAF and JAK2 inhibitors induce VEGF protein expression in BRAF mutant tumor cells, we treated A375 and COLO205 cells with PLX4032 and WP1066. The results showed that VEGF expression was significantly increased in A375 and COLO205 cells after treatment with PLX4032 or WP1066 (Figures 6A and 6B).

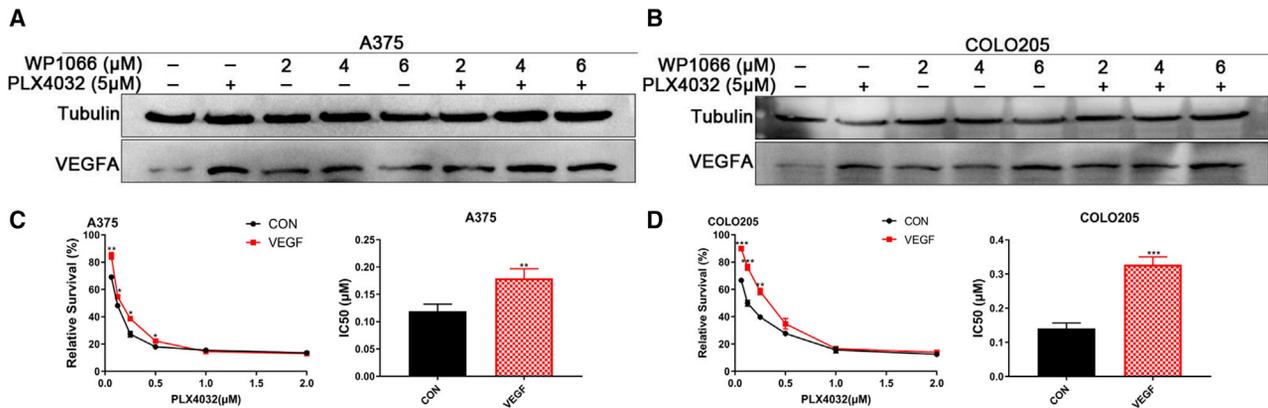
To investigate whether VEGF can promote the resistance of BRAF mutant tumor cells to BRAF inhibitors, we treated A375 and COLO205 cells with VEGF protein and PLX4032. We found that VEGF significantly promoted cell growth when treated with low concentration ( $<1 \mu\text{M}$ ) of PLX4032 (Figures 6C and 6D). In contrast, when the concentration of PLX4032 was higher than  $1 \mu\text{M}$ , VEGF protein did not affect the growth of BRAF mutant tumor cells. This is consistent with previous results that BRAF inhibitors promoted VEGF expression in A375 and COLO205 cells. By comparing the half-maximal inhibitory concentration ( $\text{IC}_{50}$ ) values, we demonstrated that VEGF promotes BRAF mutant tumor cells to resist BRAF inhibitors (Figures 6C and 6D).

In summary, we found that BRAF inhibitors enhanced the expression of VEGF in BRAF mutant tumor cells, and VEGF promoted the resistance of BRAF mutant tumors to BRAF inhibitors.

#### DISCUSSION

The MAPK pathway plays important roles in cell growth, development, and proliferation.<sup>21,22</sup> BRAF and MEK inhibitors can block activation of the MAPK pathway and prolong the survival of patients with BRAF mutant melanoma.<sup>5,10,23,24</sup> However, most patients develop tolerance to BRAF and MEK inhibitors after a period of treatment.<sup>7,10</sup> Reactivation of the MAPK pathway occurs in most melanoma patients who are tolerant to BRAF and MEK inhibitors.<sup>8</sup> Inhibition of MAPK pathway activation is critical for the treatment of BRAF mutant melanoma.

The JAK2/STAT3 pathway is important for cell proliferation, differentiation, and immune regulation. Liu et al.<sup>25</sup> reported that PLX4032 inhibited STAT3 activation in A375 cells. Different from previous reports, we verified that PLX4032 induced STAT3 activation in BRAF mutant cancer cells (e.g., A375, COLO205, and SK-MEL-1 cells). At the same time, we found that MEK or ERK inhibitors also promote the activation of the JAK2/STAT3 pathway in BRAF mutant tumor cells. These results indicated that inhibition of the MAPK pathway activates the JAK2/STAT3 pathway. Thus, the JAK2/STAT3 pathway may be an alternative pathway for the MAPK pathway in BRAF mutant cancer cells. On the other hand, we found that the JAK2/STAT3 pathway, inhibited by JAK2 inhibitors, significantly promoted



**Figure 6. BRAF Inhibitors Induced VEGF, Whereas VEGF Promoted Cell Resistance to BRAF Inhibitors in BRAF Mutant Tumor Cells**

(A and B) A375 (A) and COLO205 (B) cells were treated with PLX4032 and WP1066 alone or in combination for 6 h. The expression of VEGF was detected by western blotting, and tubulin served as a loading control. (C and D) A375 (C) and COLO205 (D) cells were treated with different concentrations of PLX4032 in the presence or absence of 50 ng/mL VEGF, and cell viability was determined by CCK8 assays after 3 days. Data are mean  $\pm$  SD. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; Student's *t* test.

the activation of the MAPK pathway in BRAF mutant tumor cells. These results indicated that simultaneous inhibition of the MAPK and JAK2/STAT3 pathways is essential for the treatment of BRAF mutant tumors. When we treated BRAF mutant cancer cells with BRAF and JAK2 inhibitors, the MAPK and JAK2/STAT3 pathways were both reactivated. These results suggested that there is a crosstalk between the MAPK and JAK2/STAT3 pathways in BRAF mutant tumor cells. Studies have found that BRAF inhibitors can relieve the negative feedback of ERK in BRAF mutant melanoma cells.<sup>10</sup> Both MAPK and JAK2/STAT3 pathways are regulated by RTKs and NRTKs. So, we speculated that the crosstalk between MAPK and JAK2/STAT3 pathways in BRAF mutant tumor cells may be due to eliminating the feedback inhibition of RTKs and NRTKs (Figure S3).

Various attempts have been made to inhibit simultaneously the MAPK and JAK2/STAT3 pathways in BRAF mutant melanoma cells. We found that JAK2 inhibitors can reactivate the MAPK pathway inhibited by BRAF and MEK inhibitors in A375 and A375R cells, but JAK2 or STAT3 inhibitors could not reactivate the MAPK pathway inhibited by BRAF, MEK, and ERK inhibitors, demonstrating that the combination of BRAF, MEK, and ERK inhibitors completely inhibited the MAPK pathway in BRAF mutant melanoma cells. Combining BRAF, MEK, and ERK inhibitors may be an effective strategy for the treatment of melanoma resistant to BRAF and MEK inhibitors. Furthermore, the combination of JAK2/STAT3 and MAPK pathway inhibitors significantly inhibited the growth of A375R cells compared with MAPK pathway inhibitors alone. Thus, these findings suggest that dual inhibition of MAPK and JAK2/STAT3 pathways may be a new strategy for the treatment of BRAF mutant melanoma.

VEGF plays an important role in tumor angiogenesis and cell proliferation.<sup>15,20</sup> We found that BRAF and JAK2 inhibitors significantly promoted VEGF expression in A375 or COLO205 cells. VEGF may be an intersection of MAPK and JAK2/STAT3 path-

ways. In addition, we found that VEGF promotes BRAF mutant tumor cells against BRAF inhibitors. Comunanza et al.<sup>26</sup> reported that blocking VEGF enhanced the effects of BRAF inhibitors on BRAF mutant tumors. Therefore, inhibition of the MAPK pathway and VEGF may be an effective strategy for the treatment of BRAF mutant melanoma.

One limitation of this research is that we did not reveal the mechanism of the crosstalk between the MAPK and JAK2/STAT3 pathways. Second, our study lacked clinical samples to demonstrate whether the crosstalk between those pathways also exists in patients.

Overall, for the first time, we confirmed a crosstalk between the MAPK and JAK2/STAT3 pathways in BRAF mutant tumor cells. We demonstrated that the combination of BRAF, MEK, and ERK inhibitors and JAK2 or STAT3 inhibitors can simultaneously inhibit both MAPK and JAK2/STAT3 pathways and significantly inhibit the growth of BRAF mutant melanoma cells. Our findings provided a potential new strategy for the treatment of BRAF mutant melanoma.

## MATERIALS AND METHODS

### Cell Lines, Reagents, Recombinant Protein

A375 and COLO205 cells were obtained from the Shanghai Institutes for Biological Sciences (Shanghai, China). SK-MEL-1 cells were obtained from the Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China). All cell lines were identified by STR Genotyping (Feiouer, Chengdu, China). A375 and SK-MEL-1 cells were cultured in Dulbecco's modified Eagle's medium (HyClone, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit HaEmek, Israel) and 1% penicillin/streptomycin (Thermo Fisher Scientific, MA, USA). COLO205 cells were cultured in RPMI-1640 (Corning, NY, USA), supplemented with 10% FBS and 1% penicillin/streptomycin. PLX4032, AZD6244, GSK1120212, C188-9, SCH72984, and WP1066 were purchased from Selleckchem

(Houston, TX, USA). Niclosamide was purchased from CSNpharm (Chicago, IL, USA). Recombinant VEGF protein was purchased from Sino Biological (Beijing, China). SYBR Green Master Mix was purchased from Vazyme (Nanjing, China). Radioimmunoprecipitation assay (RIPA) buffer and phosphatase inhibitors were obtained from CWBIO (Beijing, China) and protease inhibitor phenylmethylsulfonyl fluoride was from Beyotime (Shanghai, China).

### Resistant Cell Lines

PLX4032-resistant A375R cells were obtained by treating A375 cells with a very high concentration of PLX4032 for a short time. First, we treated A375 cells with 100  $\mu$ M PLX4032 for 24 h. Cells were then washed with normal saline to remove dead cells. The remaining cells were then cultured in fresh medium without PLX4032. After 21 days, PLX4032 was added to the medium again. Finally, the cells were cultured in medium with 5  $\mu$ M PLX4032. Cell sensitivity to PLX4032 was detected after 4 weeks of cell culture (A375:  $IC_{50}$  = 0.2114  $\mu$ M; A375R:  $IC_{50}$  = 24.36  $\mu$ M).

### Viability Assays

Cells were plated in 96-well plates with four replicate wells per group (1,000–3,000 cells/well). After adhering overnight, cells were treated with different concentrations of PLX4032 or DMSO. After 72 h of treatment, a Cell Counting Kit-8 (CCK8; Dojindo, Kumamoto, Japan) was used to detect cell viability, according to the manufacturer's instructions. Finally, we used nonlinear curve-fitting algorithms (Prism) to calculate the  $IC_{50}$ .

### Total RNA Isolation and Real-Time Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

We used TRIzol isolation reagent (Life Technologies, MD, USA) to extract total RNA from cells, according to the manufacturer's instructions. Next, we reverse transcribed the extracted RNA to cDNA using a high-capacity cDNA reverse transcriptase kit (Vazyme, Nanjing, China). Quantitative real-time PCR fluorescence was performed in triplicates using a Chromo4 cyclor (Bio-Rad, CA, USA) and SYBR Green PCR Master Mix Kit with specific primers (Table S1). The expression levels of all genes were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

### Western Blotting

Protein was extracted using RIPA lysis buffer. Briefly, 60  $\mu$ g protein was separated on 12.5% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% milk for 1 h and incubated overnight with primary antibodies. Membranes were washed, incubated with secondary antibodies, and imaged using a gel imager (Bio-Rad, CA, USA), according to the instructions. Western blotting was performed using the following antibodies: anti-IL-6 (1:1,000; CST, MA, USA), anti-ERK (1:2,000; CST, MA, USA), anti-phospho-ERK (1:2,000; CST, MA, USA), anti-phospho-STAT3 (705; 1:1,000; CST, MA, USA), anti-phospho-STAT3 (727; 1:1,000; CST, MA, USA), anti-STAT3 (1:1,000; CST, MA, USA), anti- $\alpha$ -tubulin

(1:2,000; ABclonal, Wuhan, China), and anti-VEGFA (1:1,000; BioWorld, Nanjing, China).

### Statistical Analysis

All experiments were repeated at least three times. Data are presented as the mean  $\pm$  standard error of means (SEM). The column and line charts were drawn using GraphPad Prism, version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA). The statistical significance of the results was assessed by Student's t test.

### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omto.2020.06.004>.

### AUTHOR CONTRIBUTIONS

Conceptualization, K.Z., J.C., W.Z.; Methodology, K.Z., W.Z.; Software, K.Z.; Investigation, K.Z.; Data Curation, K.Z.; Writing – Original Draft, K.Z.; Resources, Y.L., Y.C., J.C., W.Z.; Writing – Review & Editing, Y.L., Y.C., J.C., W.Z.; Validation, Y.L.; Formal Analysis, Y.L.; Visualization, Y.L., W.Z.; Supervision, Y.C., W.Z.; Funding Acquisition, J.C., W.Z.; Project Administration, W.Z.

### CONFLICTS OF INTEREST

The authors declare no competing interests.

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