

Combined BRAF^{V600E} and MEK blockade for BRAF^{V600E}-mutant gliomas

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Abstract BRAF^{V600E} is a common finding in glioma (about 10–60% depending on histopathologic subclassification). BRAF^{V600E} monotherapy shows modest preclinical efficacy against BRAF^{V600E} gliomas and also induces adverse secondary skin malignancies. Here, we examine the molecular mechanism of intrinsic resistance to BRAF^{V600E} inhibition in glioma. Furthermore, we investigate BRAF^{V600E}/MEK combination therapy that overcomes intrinsic resistance to BRAF^{V600E} inhibitor and also prevents BRAF^{V600E} inhibitor induced secondary malignancies. Immunoblotting and Human Phospho-Receptor Tyrosine Kinase Array assays were used to interrogate MAPK pathway activation. The cellular effect of BRAF^{V600E} and MEK inhibition was determined by WST-1 viability assay and cell cycle analysis. Flanked and orthotopic GBM mouse models were used to investigate the *in vivo* efficacy of BRAF^{V600E}/MEK combination therapy and the effect on secondary malignancies. BRAF^{V600E} inhibition

leads to recovery of ERK phosphorylation. Combined BRAF^{V600E} and MEK inhibition prevents reactivation of the MAPK signaling, which correlates with decreased cell viability and augmented cell cycle arrest. Similarly, mice bearing BRAF^{V600E} glioma showed reduced tumor growth when treated with a combination of BRAF^{V600E} and MEK inhibitor compared to BRAF^{V600E} inhibition alone. Additional benefit of BRAF^{V600E}/MEK inhibition was reflected by reduced cutaneous squamous-cell carcinoma (cSCC) growth (a surrogate for RAS-driven secondary malignancies). In glioma, recovery of MAPK signaling upon BRAF inhibition accounts for intrinsic resistance to BRAF^{V600E} inhibitor. Combined BRAF^{V600E} and MEK inhibition prevents rebound of MAPK activation, resulting in enhanced antitumor efficacy and also reduces the risk of secondary malignancy development.

Keywords BRAF^{V600E} · MEK · EGFR · Pediatric glioma · Secondary malignancy

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Introduction

BRAF^{V600E} mutation, which can constitutively activate the mitogen-activated protein kinase (MAPK) pathway, is one of the most common oncogenic mutations in human cancer, occurring in approximately 67% of melanoma, 36% of papillary thyroid cancer, 10% of colorectal cancer [1–3] and in a significant percentage of pediatric and adult gliomas (Supplementary Table 1). Small molecule kinase inhibitors developed specifically to target BRAF^{V600E}, such as PLX4032 (vemurafenib), have shown significant efficacy against BRAF^{V600E} melanomas [4, 5], raising the possibility that these drugs may show similar efficacy in treating BRAF^{V600E} gliomas.

The efficacy of these drugs, however, may be limited in tumors where receptor tyrosine kinase (RTK) signaling is prominent. Human epidermal growth factor receptor (HER) family member signaling is prominent in BRAF^{V600E} colorectal and thyroid cancer, and the presence of a negative feedback loop between BRAF^{V600E} and EGFR (HER1) or HER3 limits the efficacy of BRAF^{V600E} monotherapy in these tumors [6, 7]. Although EGFR signaling is also prominent in glioma [8, 9], our previous work showed that BRAF^{V600E} monotherapy has efficacy against BRAF^{V600E} glioma [10], and that BRAF^{V600E} inhibitor can be used in combination with a CDK4/6 inhibitor for achieving improved anti-tumor effect [11]. In addition to preclinical results, case reports have shown that BRAF^{V600E} gliomas can respond to BRAF^{V600E} inhibitor (vemurafenib) significantly [12–14]. Moreover, monotherapy against BRAF^{V600E} pediatric gliomas is currently being investigated clinically (ClinicalTrials.gov Identifier: NCT01748149).

Both acquired and intrinsic resistance to BRAF^{V600E} inhibitors has been described in BRAF^{V600E}-mutant malignancies [6, 7, 15, 16], both of which limit the duration of tumor response to inhibitor treatment. An additional concern over the use of BRAF^{V600E} inhibitors is their activation of wild-type RAF proteins, which promotes the development of secondary malignancies [17], and raises concern regarding the safety of BRAF^{V600E} inhibition, especially in young children.

To address these concerns, we investigated the relative efficacy of BRAF^{V600E} inhibition in BRAF^{V600E} gliomas compared to BRAF^{V600E} melanomas. We found that BRAF^{V600E} gliomas showed a relative intrinsic resistance to BRAF^{V600E} inhibition, and that this resistance could be overcome with the addition of a MEK inhibitor. Furthermore, this combination treatment also decreases the growth rate of RAS-driven secondary malignancies.

Materials and methods

Cell lines, xenografts, drugs, and primary tumors

Glioma cell lines (AM-38, DBTRG-05MG, NMC-G1) were obtained from ATCC or the Japanese Brain Tumor Repository; Melanoma cell lines (A375, WM793, WM9) were obtained from Dr. Martin McMahon (UCSF); B9 cSCC cell line were obtained from Dr. Alain Balmain (UCSF); BT40 pilocytic astrocytoma chunks were obtained under MTA from Nationwide Children's Hospital (Dr. Peter Houghton). PLX4720 was obtained from Plexxikon Inc. (Berkeley, CA, USA). PD0325901 was obtained from Pfizer Inc. (New York City, NY, USA). PLX4032 and GDC0973 were obtained from Genentech Inc. (South San Francisco, CA, USA).

Western blot analysis and human phospho-receptor tyrosine kinase (RTK) array analysis

Proteins were extracted using cell lysis buffer (Cell Signaling) supplemented with proteinase inhibitor cocktail (Roche). Western blots were developed as described previously [10]. Antibodies specific for p-ERK, and Beta-Actin were obtained from Cell Signaling Technologies. Antibodies specific for total ERK were purchased from Santa Cruz Biotechnology. The activation of phospho-receptor tyrosine kinase was tested using Human Phospho-RTK Array Kit (R&D Systems, Inc.)

Cell viability assay

Cells were seeded onto 48-well plates at 2500–3000 cells per well and treated with BRAF^{V600E} and/or MEK inhibitors, and media was changed every 3 days. Cell viability was determined by WST-1 assay (Roche), according to manufacturer's instructions. GI₅₀ value was analyzed using GraphPad Prism5 software.

Cell cycle analysis

Cells were harvested with 0.25% trypsin, washed with PBS, and then fixed with ice-cold 70% ethanol for minimum of 30 min. Cells were then washed with PBS once, and stained with propidium iodide (20 µg/ml) in PBS containing RNaseA (0.4 mg/ml) (Invitrogen). Cells were sorted using FACSCalibur (Becton, Dickinson), and data was analyzed using the ModFit software (Verity).

In vivo experiments using intracranial xenografts models

Five-week-old female athymic mice (nu/nu genotype, BALB/c background) purchased from Simonsen Laboratories were used. Animals were housed under aseptic conditions. The UCSF Institutional Animal Care and Use Committee approved all animal protocols. 1×10^5 modified AM-38 human glioma cells, which were transduced with lentivirus containing firefly luciferase, were intracranially injected into athymic mice as previously described [18]. In vivo bioluminescence imaging (BLI) was carried out using the Xenogen IVIS Lumina System coupled to LivingImage data-acquisition software (Xenogen Corp.). Mice were anesthetized with isoflurane and imaged 10 min after intraperitoneal (i.p.) injection of luciferin (D-luciferin potassium salt, 150 mg/kg, Gold Biotechnology). Signal intensity was quantified using LivingImage software.

Mice implanted with luciferase-modified AM-38 cells were randomized to four groups receiving treatment of vehicle control (DMSO i.p. injection and 0.5%

hydroxypropyl methylcellulose plus 0.2% Tween 80 oral gavage), PLX4720 and PD0325901 alone or in combination. PLX4720 treatment was carried out by i.p. injection using a daily dose of 20 mg/kg for consecutive days. PD0325901 treatment was carried out by oral gavage using a daily dose of 5 mg/kg with a schedule of 4 days on treatment and 4 days off. Treatment was initiated at day 7 post-implantation of tumor cells, once tumors were shown to be in log-phase of growth. In addition to monitoring by BLI to detect the luminescence signal, all the mice were checked every day for the development of symptoms related to tumor burden, and euthanized when they exhibited symptoms indicative of significant compromise to neurologic function. In addition to the mice used for survival analysis, two pre-symptomatic mice within each cohort were used for IHC analysis and western blot analysis. These mice were sacrificed 2 h after treatment on the third day, and their brains were resected and either placed in formalin and prepared for IHC, or dissected from surrounding normal brain and snap frozen in liquid nitrogen for western blot analysis. IHC was carried out as previously described [18], using Ki-67 antibody (Ventana Inc) and p-ERK1/2 antibody (Cell Signaling Technologies).

In vivo experiments using subcutaneous xenograft tumor models

Eight-week-old female scid mice (C.B-17 background) were purchased from Taconic Farms, Inc. Animals were housed under aseptic conditions, and UCSF Institutional Animal Care and Use Committee approve all protocols.

To investigate the efficacy of RO5185426 (PLX4032) and GDC0973 combined treatment against BRAF^{V600E}-mutant glioma, BT40 chunks were subcutaneously implanted into the right flank of scid mice. These mice were randomized to four groups receiving treatment of vehicle control (2% Hydroxypropylcellulose, and 0.5% hydroxypropyl methylcellulose plus 0.2% Tween 80 oral gavage), PLX4032 and GDC0973 alone or in combination. PLX4032 treatment was carried out by oral gavage using a dose of 25 mg/kg twice daily for 16 days with a schedule of 6 days on treatment and 2 days off. GDC0973 treatment was carried out by oral gavage using a daily dose of 5 mg/kg for 16 days with a schedule of 4 days on treatment and 4 days off. Two additional mice within each cohort were used for IHC analysis and western blot analysis. These mice were sacrificed 2 h after treatment on the fourth day in order to collect BT40 tumors. IHC and western blot analysis were carried out as those for intracranial BRAF^{V600E} MA xenografts above. Mice were monitored every two days and euthanized due to significant tumor burden (length >15 mm), weight loss >15%, or symptoms related to tumor burden.

To investigate the efficacy of PLX4720 and PD0325901 combined treatment against RAS-driven secondary malignancies, BT40 chunks were subcutaneously implanted into the right flank of scid mice. Then, after 10 days, 1×10^6 RAS-mutant B9 cutaneous squamous cells were subcutaneously injected to the left flank of scid mice (Fig. 5c). These mice were randomized to four groups receiving treatment of vehicle control (DMSO i.p. injection and 0.5% hydroxypropyl methylcellulose plus 0.2% Tween 80 oral gavage), PLX4720 and PD0325901 alone or in combination. PLX4720 treatment was carried out by i.p. injection using a daily dose of 10 mg/kg for 16 consecutive days. PD0325901 treatment was carried out by oral gavage using a daily dose of 5 mg/kg for 16 days with a schedule of 4 days on treatment and 4 days off. Mice were monitored every two days and euthanized due to significant tumor burden (length >20 mm), weight loss >15%, or symptoms related to tumor burden such as skin ulcer.

All of these treatments were initiated when tumor volume reached at 100 mm³. The maximum longitudinal diameter (length) and the maximum transverse diameter (width) of tumors were measured by caliper every 2 days, and tumor volume was calculated using the equation $1/2 \times (\text{length} \times \text{width}^2)$.

Statistical analysis

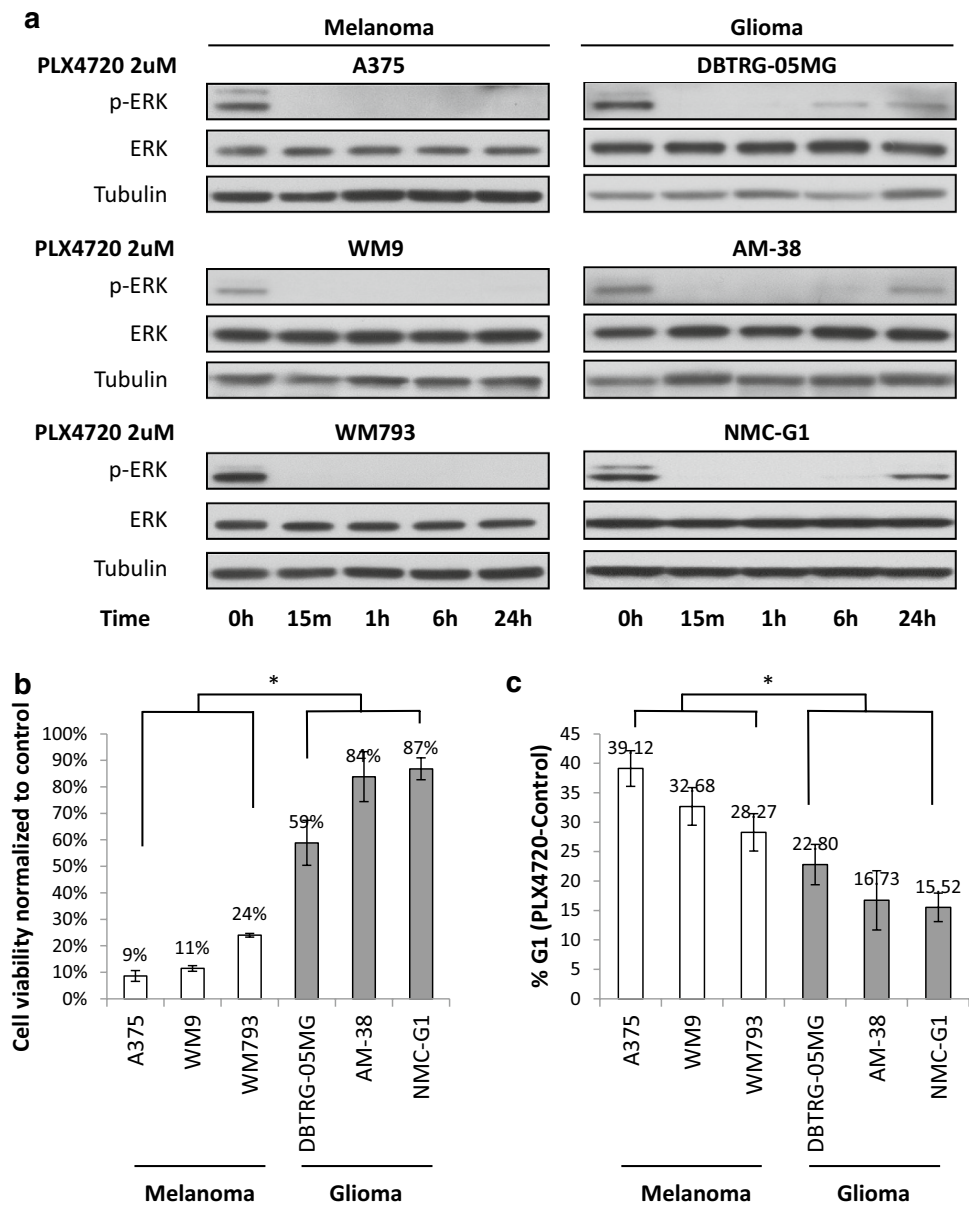
A 2-tailed unpaired *t* test was used for statistical comparison, with *p* values of <0.05 considered significant. A log-rank (Mantel-Cox) test was employed for survival analysis (GraphPad Prism5).

Results

BRAF^{V600E} inhibitor suppression of MEK-ERK activity in glioma cell lines is transient

We compared effects of PLX4720 (BRAF^{V600E} inhibitor, a tool compound analog of PLX4032) treatment in BRAF^{V600E} glioma (AM-38, DBTRG-05MG and NMG1) and melanoma (A375, WM793 and WM9) cell lines. Genetic characteristics of the cell lines tested are described in Supplementary Table 2 [19–23]. While phosphorylation of ERK was initially suppressed by PLX4720 in all cells, this phosphorylation partially recovered in glioma lines, but not in melanoma lines for the entire 24-h period of sample collection (Fig. 1a). This recovery was also observed functionally, as PLX4720 induced less inhibition of cell viability in glioma cell lines compared to melanoma cell lines (*p*=0.0082—Fig. 1b, Supplementary Fig. 1). While G1 phase arrest induced by PLX4720 was observed in all

Fig. 1 Efficacy of PLX4720 against BRAF^{V600E} melanoma and glioma cells. BRAF^{V600E}-mutant glioma cells (DBTRG-05MG, AM-38 and NMC-G1) and BRAF^{V600E}-mutant melanoma cells (A375, WM9 and WM793) were used. **a** Cells were cultured in 10% serum media, and treated with 2 μ M PLX4720 for 0 h, 15 m, 1, 6, and 24 h. Cell lysates were analyzed using western blot. Phosphorylation of ERK was inhibited by PLX4720 initially in all cell lines, but partially recovered in glioma cell lines compared to in melanoma cell lines. **b** Cells were treated with DMSO (control) or 2 μ M PLX4720 for 6 days. Media was changed once every 3 days. Cell viability was measured using WST-1 assay. Error bars indicate the variation between triplicate measurements. PLX4720 induced suppression of cell viability in all cell lines. But more cell viability was observed in glioma cell lines compared to in melanoma cell lines ($p=0.0082$). **c** Cells were treated with DMSO (control) or 2 μ M PLX4720 for 24 h for cell cycle analysis. PLX4720 induced G1 phase arrest in all cell lines. But less G1 phase arrest was observed in glioma cell lines compared to in melanoma cell lines ($p=0.0216$)



lines, arrest was less prominent in glioma cells compared to melanoma cells ($p=0.0216$ —Fig. 1c).

Co-inhibition of BRAF^{V600E} and MEK prevents recovery of MAPK signaling

Given the results above, we hypothesized that the combination of BRAF and MEK inhibitors may increase and/or extend the anti-tumor effect of small molecule inhibitors on glioma cell lines, relative to that of inhibitor monotherapy. The results show that combined use of PLX4720 and PD0325901 (MEK inhibitor) significantly reduced activation of ERK in both AM-38 and DBTRG-05MG glioma cells (Fig. 2a). The effect on ERK was accompanied by a more pronounced growth inhibitory activity of

combination treatment (Fig. 2b), and by modest, yet discernable increases in G1 phase cells in cultures treated with both inhibitors (all p values >0.05 —Fig. 2c, Supplementary Fig. 2).

Combined BRAF^{V600E} and MEK blockade shows increased anti-tumor efficacy against BRAF^{V600E}-mutant glioma in vivo

As the combination of BRAF^{V600E} and MEK inhibitors shows increased efficacy against cultured glioma cell lines, we next attempted to extend in vitro observations to in vivo intracranial BRAF^{V600E} glioma xenograft models. Athymic mice were injected intracranially with AM-38 cells modified with a luciferase reporter, and then treated with

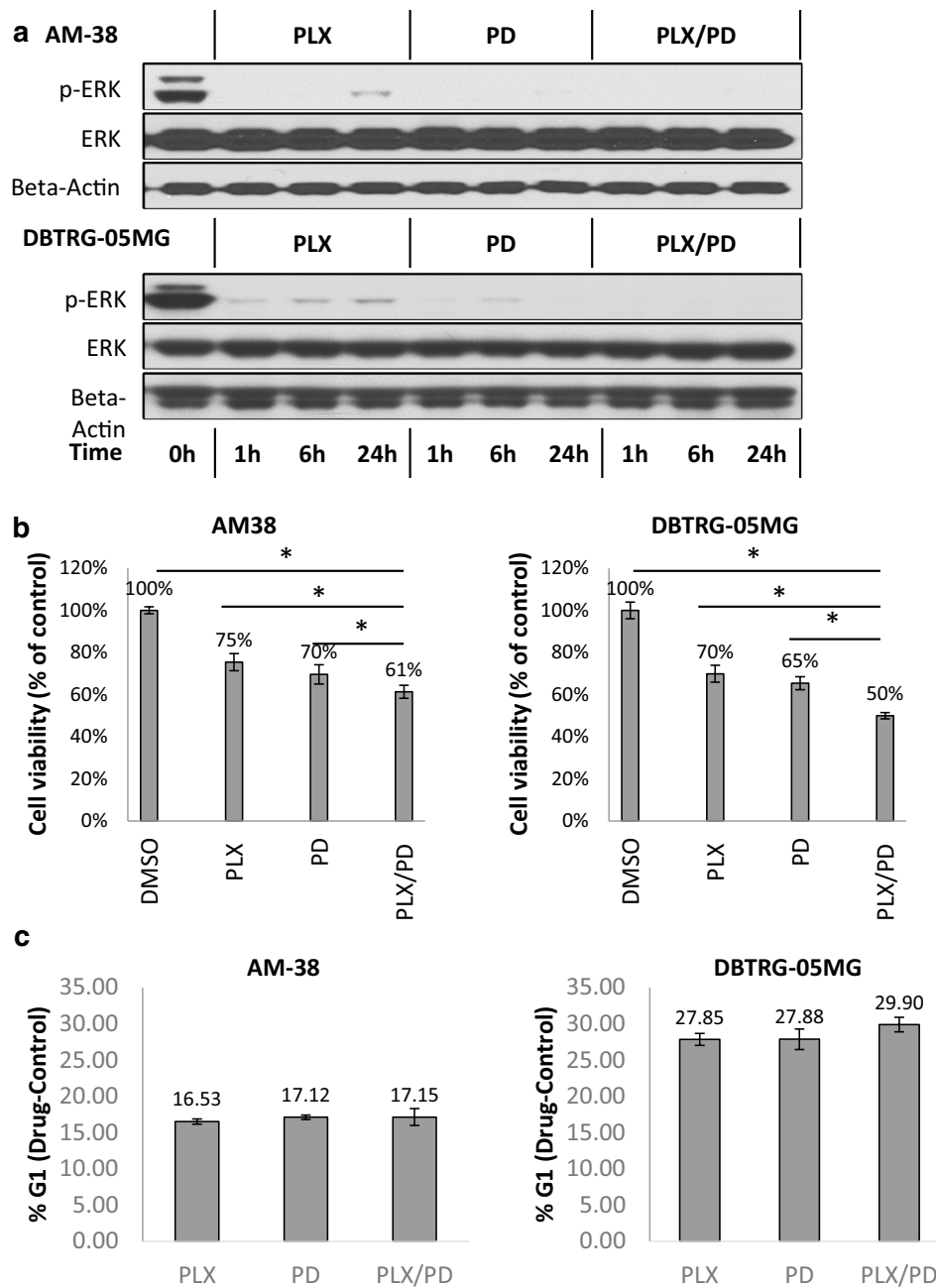


Fig. 2 Combined BRAF^{V600E} and MEK inhibition prevented ERK recovery, decreased cell viability, and increased G1 phase arrest in BRAF^{V600E} glioma cell lines. All treatments used DMSO (control), 2 μM PLX4720, 10 nM PD0325901 and their combination. **a** AM-38 and DBTRG-05MG cells were cultured in 10% serum media overnight, then treated for 0, 1, 6 and 24 h. Cell lysates were analyzed by western blot. In both AM-38 and DBTRG-05MG cell lines, combined treatment decreased the recovery of p-ERK induced by PLX4720. **b** AM-38 and DBTRG-05MG cells were treated for 5 days. Media was changed once every 3 days. Cell viability was measured using WST-1 assay. Error bars indicate the variation between triplicate measurements. PLX4720 and PD0325901 alone or in combination reduced cell viability significantly (p value of control vs. PLX4720 in AM-38=3.1339E-05, in DBTRG-05MG=4.0725E-

05; p value of control vs. PD0325901 in AM-38=1.5265E-05, in DBTRG-05MG=9.2805E-06; p value of control vs. combo in AM-38=5.5372E-07, in DBTRG-05MG=5.2469E-06). However, combined therapy led to the most significant cell viability reduction compared to either monotherapy in both AM-38 and DBTRG-05MG cell lines (p value of combo vs. PLX4720 in AM-38=0.0015, in DBTRG-05MG=0.0005; p value of combo vs. PD0325901 in AM-38=0.0237, in DBTRG-05MG=0.0005). **c** AM-38 and DBTRG-05MG cells were treated for 24 h for cell cycle analysis. In both of AM-38 and DBTRG-05MG cell lines, all three therapies increased G1 population cells, among which modest but discernable increases of G1 phase cells were induced by combined treatment compared to monotherapy (all p values >0.05)

PLX4720 or PD0325901 or both. Treatment with PLX4720 and PD0325901 alone reduced the growth of intracranial tumors, with further growth reduction evident from combination treatment (Fig. 3a). Consistently, all three therapies significantly prolonged the survival of mice compared to control, while combined treatment showed more benefit (statistically significant compared to PLX4720; modest

but discernable compared to PD0325901) (Fig. 3b). We also examined treatment effects on Ki-67 staining (used to indicate proliferating cells) and phosphorylation of ERK, in tumors obtained from mice euthanized while on therapy. Singular PLX4720 and PD0325901 treatments decreased Ki-67 positivity in AM-38 tumors, and combination therapy resulted in further reduced staining (Fig. 3c).

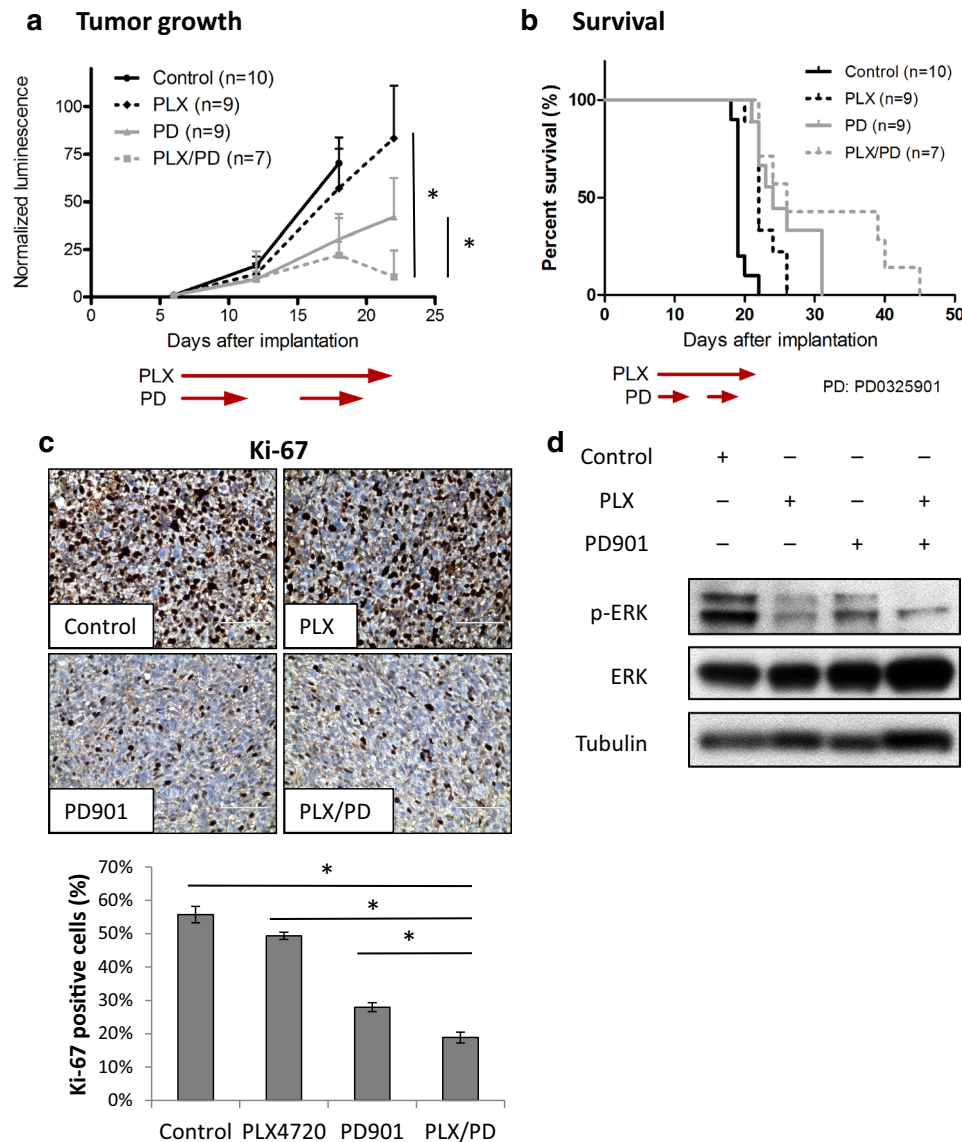


Fig. 3 Combined treatment with BRAF^{V600E} and MEK inhibitors suppressed tumor growth in intracranial BRAF^{V600E} AM-38 xenografts model. Mice intracranially injected with modified AM-38 cells were treated with vehicle (control), PLX4720 and PD0325901 alone or in combination. Tumor tissues were harvested for subsequent analysis. **a** All three therapies suppressed the growth of intracranial tumor, but combined therapy led to the most suppression (22 days post treatment, *p* value of combo vs. PLX4720=0.0317, combo vs. PD0325901=0.0265). **b** All three therapies prolonged the survival of mice, and combined treatment showed more benefit (statistically significant compared to PLX4720; modest but discernable com-

pared to PD0325901) (*p* value of control vs. PLX4720=0.0003, control vs. PD0325901<0.0001, control vs. combo=0.0002, combo vs. PLX4720=0.0388, combo vs. PD0325901=0.1612). **c** Ki-67 expression was suppressed by all three therapies (*p* value of control vs. PLX4720=0.0151, control vs. PD0325901=0.0001, control vs. combo=2.6741E-05). Combined treatment suppressed Ki-67 the most significantly (*p* value of PLX4720 vs. combo=1.1237E-05, PD0325901 vs. combo=0.0017). **d** Western blot analysis revealed that phosphorylation of ERK was inhibited by all three types of treatments, among which the combined treatment was the most effective to down-regulate ERK activity

Similarly, we found that the combination of PLX4720 and PD0325901 resulted in a more substantial blockade of MAPK signaling than either drug alone (Fig. 3d, Supplementary Fig. 3).

In an additional experiment with compounds currently in being used to treat cancer patients (BRAF^{V600E} inhibitor PLX4032 and MEK inhibitor GDC0973: currently being tested in patients with BRAF^{V600E} melanoma), we administered mono and combination therapy to mice with subcutaneous BRAF^{V600E} BT40 xenografts. As shown in Fig. 4a, PLX4032 therapy cause tumor growth stasis, whereas GDC0973 therapy promoted tumor regression that was made even more substantial by including PLX4032. Consistently, PLX4032 and GDC0973 alone or in combination significantly increased mice survival compared to control, while combined treatment has even better efficacy (statistically significant compared to PLX4032; modest but discernable compared to GDC0973) (Fig. 4b). Immunohistochemical (IHC) analysis showed that, compared to PLX4032 and GDC0973 monotherapies, combined treatment resulted in the largest decrease in tumor Ki-67 staining (Fig. 4c). Similarly, our data showed combination treatment as being most effective in suppressing ERK activation (Fig. 4d, Supplementary Fig. 4).

Combined BRAF^{V600E} and MEK inhibition decreases cutaneous squamous-cell carcinoma growth

cSCC can be an alarming consequence of vemurafenib monotherapy for melanoma patients [4, 24]. Both preclinical and clinical studies have shown that BRAF^{V600E} inhibition has the potential to accelerate the progression of cSCC, and that the addition of MEK inhibitors can inhibit the development of cSCC in patients [17, 25, 26].

To investigate whether the combination of BRAF^{V600E} and MEK inhibitors could reduce cSCC in our experiments, we used the RAS-mutant B9 cSCC cell line [17] to model secondary skin cancer. PLX4720 and PD0325901 alone or in combination were used to treat mice bearing B9 cSCC xenografts on one flank and BRAF^{V600E} BT40 xenografts on the other. Our results show that combination treatment caused the most substantial regression of BT40 xenografts (Fig. 5a, c), and that PD0325901, alone or in combination with PLX4720, was effective in preventing the growth of B9 cSCC xenografts (Fig. 5b, c).

Discussion

BRAF^{V600E} tumors targeted therapy using small molecule kinase inhibitors produces varying extent of responses in diverse BRAF^{V600E} tumors, from pronounced response in melanoma to limited response in colon and thyroid cancers

[4, 6, 7]. Our previously published results show only modest pre-clinical effect of PLX4720 against BRAF^{V600E} gliomas [10], which contrast with the robust preclinical efficacy observed when used in treating melanoma [27]. This disparity in anti-tumor activity prompted the current study, where we illustrate transient MEK-ERK blockade by BRAF^{V600E} inhibitor that results in only modest cell viability loss and cell cycle impairment in BRAF^{V600E} glioma (Fig. 1).

While BRAF^{V600E} is a potent oncogene and promotes increased MAPK pathway signal transduction [28], its expression also results in a significant activation of proteins whose role is to attenuate pathway signaling [29, 30]. These negative regulators act at various points along the RTK/RAS/MEK/MAPK axis [29, 31]. In BRAF^{V600E} tumors where RTK signaling is prominent, a recovery of RTK signaling is promoted by BRAF^{V600E} inhibition because of the inhibitor's repressive effect on negative regulators of RTK activity. As demonstrated in BRAF^{V600E} colon cancer and thyroid cancer, activation of HER family members was induced by BRAF^{V600E} inhibition [6, 7]. RTK activation is known to promote RAS activation, which recruits and activates wild-type RAF promoters. We believe this series of inhibitor responses is responsible for the recovery of the MAPK pathway in BRAF^{V600E} glioma cells treated with BRAF^{V600E} inhibitor. As demonstrated in our previous work [16], BRAF^{V600E} inhibition leads to MEK/ERK signaling suppression in BRAF^{V600E} glioma, which in turn down-regulates the EGFR phosphatase PTPN9. Inhibition of PTPN9 in turn results in sustained EGFR phosphorylation and enhanced EGFR activity, which promote the activation of RAS, then further activates c-RAF/MEK/ERK signaling. However, targeting single RTKs in gliomas can result in tumor cells adjusting by activating alternative RTKs and leave downstream signaling little affected [32, 33]. Our Phospho-RTK Array data also showed that besides EGFR, another RTK, Axl was activated in the presence of PLX4720 (Supplementary Fig. 5). Thus, we combined a downstream MEK inhibitor with BRAF^{V600E} inhibitor to treat BRAF^{V600E} gliomas.

Early melanoma studies demonstrated that near-complete target inhibition of the MAPK pathway was crucial to have a clinical effect, which reveals the prominent role of MAPK signaling in BRAF^{V600E} tumors [34]. In our experiments, we observed that, compared with BRAF^{V600E} inhibition only, combination of BRAF^{V600E} and MEK inhibition provides more substantial and durable anti-tumor effect in BRAF^{V600E} glioma (Figs. 2, 3, 4). This means that the MAPK signaling can also be a main contributor to BRAF^{V600E} glioma resistance when it is reactivated by BRAF^{V600E} inhibition. Therefore, combined treatment in our experiments is more effective because of additional MEK inhibition preventing MAPK reactivation. This

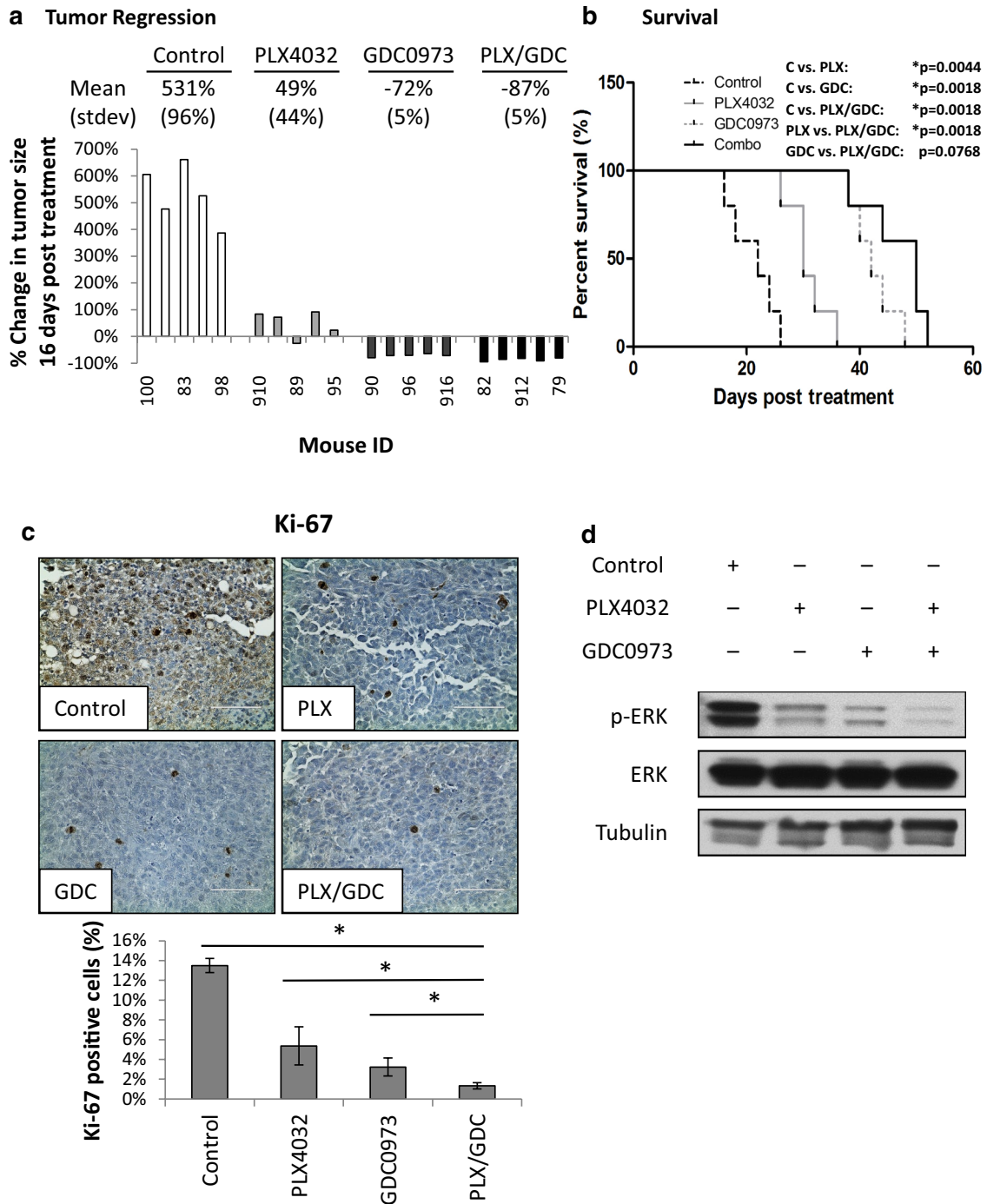
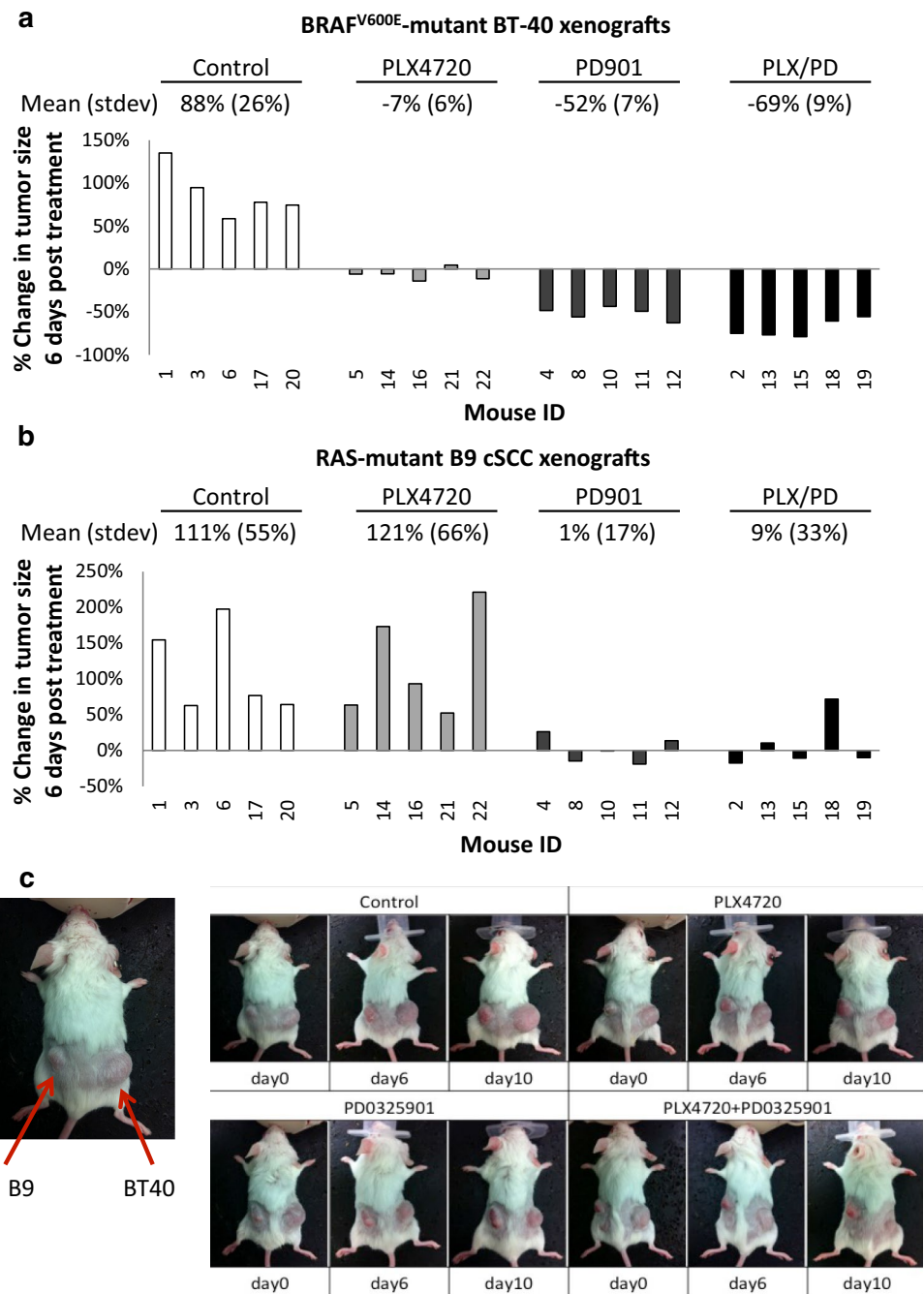


Fig. 4 PLX4032 and GDC0973 combined treatment reduced the growth of BRAF^{V600E} BT-40 astrocytoma xenografts. BT40 chunks were subcutaneously implanted into flank of scid mice. Mice were treated with vehicle (control), PLX4032 and GDC0973 alone or in combination. Tumor tissues were harvested for subsequent analysis. **a** All three therapies suppressed the growth of BT-40 tumors (p value of control vs. PLX4032=0.0001, control vs. GDC0973=0.0002, control vs. combo=0.0002). Mice treated with PLX4032 alone showed slow-down tumor growth, but mice treated with GDC0973 alone and combined with PLX4032 showed tumor shrink, in which, combined treatment led to the most significant tumor regressions (p value of GDC0973 vs. PLX4032=0.0053, combo vs. PLX4032=0.0034, combo vs. GDC0973=0.0028). **b**

All three therapies prolonged the survival of mice, and combined treatment showed more benefit (statistically significant compared to PLX4720; modest but discernable compared to PD0325901) (p value of control vs. PLX4032=0.0044, control vs. GDC0973=0.0018, control vs. combo=0.0018, combo vs. PLX4032=0.0018, combo vs. GDC0973=0.0768). **c** Compared to control, all three therapies inhibited Ki-67 expression significantly (p value of control vs. PLX4032=0.0024, control vs. GDC0973=0.0001, control vs. combo=1.1711E-05), among which, combined treatment induced the most inhibition (p value of combo vs. PLX4032=0.0235, combo vs. GDC0973=0.0268). **d** Phosphorylation of ERK was suppressed by all three therapies, while combined therapy inhibited the most ERK activity

Fig. 5 Combined BRAF^{V600E} and MEK inhibition reduced the risk of secondary skin malignancies. BT40 chunks were subcutaneously implanted into the right flank of scid mice. After 10 days, 1 × 10⁶ B9 cutaneous squamous cells were subcutaneously injected to the left flank of scid mice. Mice were treated with vehicle (control), PLX4720 and PD0325901 alone or in combination. **a** All three therapies suppressed the growth of BT40 tumors compared to control (p value of control vs. PLX4720=0.0014, control vs. PD0325901=0.0003, control vs. combo=9.1724E-05), among which combined treatment showed the most suppression (p value of combo vs. PLX4720=1.0885E-05, combo vs. PD0325901=0.0189). **b** Compared to control and PLX4720 alone, PD0325901 alone or combined with PLX4720 repressed the growth of B9 tumors significantly (p value of PD0325901 vs. control=0.0134, PD0325901 vs. PLX4720=0.0197; p value of combo vs. control=0.0165, combo vs. PLX4720=0.0231). (c) Images of mice carrying BT40 and B9 xenografts. In each group, images of one mouse (day 0, 6, and 10) were shown



is also confirmed by a variety of reports. In BRAF^{V600E} resistant glioma cells, our previous work did not find any obvious MAPK inhibition induced by BRAF^{V600E} inhibitor, which is related to the insensitivity of cell response to BRAF^{V600E} inhibitor [35]. In BRAF^{V600E} melanoma, Basile et al. found that the MAPK reactivation, as results of HRAS mutation or BRAF^{V600E} variants induced by long-term BRAF^{V600E} inhibition, is the main reason for tumor resistance to BRAF^{V600E} inhibitors [36]. Co-therapy using BRAF^{V600E} and MEK inhibitors has also been studied in BRAF^{V600E} melanoma patients, and early results show

improved efficacy in treating BRAF-mutant melanoma [25, 26, 37]. In BRAF^{V600E} colon cancer, Corcoran et al. confirmed that the inefficiency of BRAF^{V600E} inhibition is caused by EGFR-mediated reactivation of MAPK signaling, which can be overcome by combining with EGFR or MEK inhibitors [38].

Based on our in vitro and in vivo results, we believe that therapy with BRAF^{V600E} and MEK co-inhibition is a promising strategy for clinical trials against BRAF^{V600E} glioma treatment. In addition, BRAF^{V600E} inhibitors are known to promote the development of RAS-driven secondary

malignancies, which is a particular concern when treating pediatric patients. Reports have shown that 20–30% of melanoma patients treated with BRAF^{V600E} inhibitors develop keratoacanthomas/cutaneous squamous-cell carcinoma (cSCC) [4, 24, 37]. Preclinical results have shown that BRAF^{V600E} inhibition accelerates the progression of cSCC, whereas combined BRAF/MEK inhibition can prevent this [17]. Furthermore, clinical trial results have shown that combined treatment with BRAF and MEK inhibitors improves progression-free survival of patients with melanoma, and, as well, decreases the risk of cutaneous squamous-cell carcinoma [25, 26, 37]. Our in vivo results show that while BRAF^{V600E} inhibitor decreases the growth of BRAF^{V600E} gliomas, the combination of BRAF^{V600E} and MEK inhibitors not only suppresses BRAF^{V600E} glioma growth, but also inhibits the development of secondary skin malignancies (Fig. 5).

Initial clinical trials with MEK inhibitors showed significant toxicity, with rash and ocular reactions being dose-limiting [39]. For this reason, we used an intermittent dosing strategy of MEK inhibitors in this study, which has proven to be better tolerated by patients in contemporary clinical trials. It will be interesting to follow clinical trial progress for pediatric BRAF^{V600E} glioma, and observe what approaches are used for extending benefit to patients from inhibiting this activated oncogene.

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Compliance with ethical standards

Conflict of interest None declared.

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