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# Targeting Adenosine in BRAF-Mutant Melanoma Reduces Tumor Growth and Metastasis

Arabella Young[1,2], Shin Foong Ngiow[1,2,3], Jason Madore[4,5], Julia Reinhardt[6], Jennifer Landsberg[7,8], Arash Chitsazan[9,10], Jai Rautela[11,12], Tobias Bald[1], Deborah S. Barkauskas[1], Elizabeth Ahern[1,2,13], Nicholas D. Huntington[11,12], Dirk Schadendorf[8], Georgina V. Long[4,5], Glen M. Boyle[14], Michael Hölzel[6], Richard A. Scolyer[4,5], and Mark J. Smyth[1,2]

## Abstract

Increasing evidence exists for the role of immunosuppressive adenosine in promoting tumor growth and spread in a number of cancer types, resulting in poor clinical outcomes. In this study, we assessed whether the CD73-adenosinergic pathway is active in melanoma patients and whether adenosine restricts the efficacy of clinically approved targeted therapies for commonly mutated $BRAF^{V600E}$ melanoma. In AJCC stage III melanoma patients, CD73 expression (the enzyme that generates adenosine) correlated significantly with patients presenting nodal metastatic melanoma, suggesting that targeting this pathway may be effective in advanced stage disease. In addition, dabrafenib and trametinib treatment of $CD73^{+}$ $BRAF^{V600E}$-mutant melanomas caused profound CD73 downregulation in tumor cells. Inhibition of BRAF and MEK in combination with the A2A adenosine receptor provided significant protection against tumor initiation and metastasis formation in mice. Our results suggest that targeting adenosine may enhance therapeutic responses for melanoma patients receiving targeted or immune-based therapies. *Cancer Res; 77(17); 4684–96. ©2017 AACR.*

## Introduction

The recent clinical success of immunotherapies, such as FDA-approved antibodies directed toward cytotoxic T lymphocyte–associated antigen 4 (CTLA4) and programmed death 1 (PD1), has provided renewed hope for identifying curative therapeutic strategies for the treatment of metastatic melanoma (1, 2). In fact, targeting these complementary immunosuppressive pathways in combination potentiates durable responses in advanced melanoma (3–5). Similarly, advances in targeted therapies toward activating *BRAF* mutations, with specific FDA-approved BRAF inhibitors, such as vemurafenib and dabrafenib, provide improved progression-free and overall survival in comparison with chemotherapy (6, 7). However, a proportion of patients remain nonresponsive to these therapeutic strategies, indicating the presence of alternate immune or tumor-derived adaptive resistance mechanisms impeding an active antitumor response.

BRAF mutations are frequently identified in cutaneous melanoma (8, 9), and while the majority of tumors initially respond to BRAF-directed therapies, patients commonly develop therapeutic resistance. This is often associated with reactivation of the MAPK pathway, particularly by downstream MEK, revealing that cotargeting BRAF and MEK delays acquired resistance, improving therapeutic efficacy (10–12). Combining dabrafenib and the MEK inhibitor trametinib improved overall survival compared with monotherapeutic BRAF inhibition in previously untreated *BRAF*-mutated melanoma patients (13, 14). Interestingly, combining dabrafenib and trametinib also modulates the tumor microenvironment (TME) by increasing the presence of tumor-specific antigens, altering the expression of immune-related molecules, and increasing intratumor $CD8^{+}$ T cells early during treatment (EDT; refs. 15–17). Preclinical studies combining BRAF and MEK inhibition have identified further therapeutic benefit when used alongside immunotherapies such as adoptive T-cell therapy or immune checkpoint blockade (15). However, a phase I clinical trial with ipilimumab and vemurafenib in combination was discontinued due to severe hepatotoxicity (18). Alternative combination approaches that target both oncogenic signatures specific to the tumor and immune-activating strategies, with limited toxicity, need to be identified.

One such pathway that may impede the active antitumor immune response in melanoma is the adenosinergic pathway.

[1]Immunology in Cancer and Infection Laboratory, QIMR Berghofer Medical Research Institute, Herston, Queensland, Australia. [2]School of Medicine, University of Queensland, Herston, Queensland, Australia. [3]Department of Microbiology and Institute for Immunology, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania. [4]Melanoma Institute Australia, Sydney, New South Wales, Australia. [5]Sydney Medical School, The University of Sydney, Sydney, New South Wales, Australia. [6]Unit for RNA Biology, Department of Clinical Chemistry and Clinical Pharmacology, University of Bonn, Bonn, Germany. [7]Laboratory of Experimental Dermatology, Department of Dermatology and Allergy, University of Bonn, Bonn, Germany. [8]Department of Dermatology, University Hospital Essen, West German Cancer Center, University Duisburg-Essen, Essen, Germany. [9]QIMR Berghofer Medical Research Institute, Herston, Queensland, Australia. [10]The University of Queensland Diamantina Institute, Translational Research Institute, The University of Queensland, Brisbane, Queensland, Australia. [11]The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia. [12]Department of Medical Biology, The University of Melbourne, Victoria, Australia. [13]Cancer Care Services, Royal Brisbane and Women's Hospital, Queensland, Australia. [14]Cancer Drug Mechanisms Group, QIMR Berghofer Medical Research Institute, Herston, Queensland, Australia.

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A. Young and S.F. Ngiow contributed equally to this article.

**Corresponding Author:** Mark J. Smyth, QIMR Berghofer Medical Research Institute, 300 Herston Road, Herston 4006, Queensland, Australia. Phone: 617-3845-3957; Fax: 617-3362-0111; E-mail: mark.smyth@qimrberghofer.edu.au

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Adenosine signaling, particularly via the A2A adenosine receptor (A2AR), potently reduces effector functions of cytotoxic lymphocytes ($CD8^+$ T cells and NK cells) while also promoting recruitment and polarization of immunosuppressive cell types, including myeloid-derived suppressor cells (MDSC) and T regulatory cells (Tregs; reviewed in ref. 19). Previously, others and we have identified that targeting adenosine generation by blockade of the ectonucleotidase CD73 or downstream A2AR inhibition enhances tumor control and antimetastatic activity (20–25). Similarly, inhibiting conditions that initiate adenosine production, such as hypoxia, also provide protection against tumor formation (26, 27). In addition, therapeutic approaches targeting the adenosinergic pathway alongside immune checkpoint blockade and chemotherapies display enhanced antitumor efficacy in combination (28–32). Importantly, targeting adenosine in solid tumors, by using anti-CD73 (NCT02503774) or small-molecule A2AR antagonism (NCT02403193 and NCT02655822), has entered clinical trials.

In this study, we identified that more advanced clinical stage disease, as well as *TP53* mutation, are associated with increased CD73 expression in melanoma. In addition, inhibition of adenosine signaling with an A2AR antagonist in combination with BRAF and MEK inhibition provided improved tumor control in mouse models of BRAF-mutated melanoma. These findings suggest that targeting adenosine may provide therapeutic benefit for melanoma patients by alleviating immunosuppression.

## Materials and Methods

### Patient and specimens

This study was undertaken in accordance with the Declaration of Helsinki and with Human Ethics Review Committee approval and patient's informed consent from the Melanoma Institute Australia (MIA; Protocol No. X10-0305 and HREC/10/RPAH/539) and Institutional Ethics Committee boards in Germany.

### The Cancer Genome Atlas tissue microarray

Archival tissue pathology specimens were identified from the MIA Research Database, for which matched fresh frozen tissue specimens had been banked by the MIA Biobank and included in The Cancer Genome Atlas (TCGA) project ($n = 95$, from which 77 patients had sufficient tumor tissue for evaluation of CD73 staining of one core per patient). Criteria for inclusion in the TCGA project were AJCC stage III lesions from patients without distant metastases at the time of tumor banking on the basis of clinical examination and CT scans. All patients had a history of primary cutaneous melanoma and no patient received immunotherapy or BRAF/MEK inhibitor treatment. Patient demographics, primary tumor characteristics, and follow-up data were retrieved from the MIA Research Database. Patient specimens were annotated using morphologically stained hematoxylin and eosin tissue sections to identify tumor areas from which tissue microarrays were constructed using a tissue microarrayer at 1.0-mm diameter tissue core size.

### Combination targeted therapy–treated patients and metastatic melanoma biopsy specimens

Slides of BRAF-mutant melanoma patients treated with BRAF inhibition (dabrafenib or vemurafenib) were provided from the biopsy collection of Dirk Schadendorf (Essen, Germany). Pretreatment samples of 13 patients with BRAF-mutant metastatic melanoma were taken 39–558 days (median 214 days) before treatment. Four of 13 patients were biopsied during treatment with BRAFi (dabrafenib or vemurafenib), 3 of 13 patients were biopsied within one week, and 6 of 13 patients were biopsied greater than one week (15–167 days) after withdrawal of BRAFi due to progressive disease. Best clinical response was defined as partial response (PR), stable disease (SD), and progressive disease (PD) based on RECIST 1.1 criteria.

From the MIA, a cohort of 13 patients with BRAF-mutant metastatic melanoma who had at least two lesions amenable to excision biopsy before and during systemic therapy with combination BRAFi (dabrafenib) and MEK inhibitor (MEKi; trametinib) were included in this study. Excision biopsies were taken within 7 days prior to commencing the BRAFi and MEKi (PRE), early during treatment at day 3–15 (EDT), and at week 12. Biopsies of new or growing melanoma metastases (PROG) were performed if possible. The BRAFi and MEKi were ceased no more than 24 hours prior to surgery for those requiring a general anesthetic for therapeutic surgical resection of progressing lesions.

### Mice

C57BL/6 wild-type (WT) mice were purchased from the Walter and Eliza Hall Institute for Medical Research or bred in-house at the QIMR Berghofer Medical Research Institute. C57BL/6 A2AR-deficient (B6.A2AR.ko) mice were described previously (24). C57BL/6 *Tyr*::$CreER^{T2}$; $Braf^{CA}$; $Pten^{lox/lox}$ (TBP) mice were further backcrossed ten times to the C57BL/6 background since first described (33). WT and all gene-targeted strains were used between the ages of 6 to 30 weeks. Groups of 5 to 12 mice per experiment were used for experimental tumor assays, to ensure adequate power to detect biological differences. All experiments were approved by the QIMR Berghofer Medical Research Institute Animal Ethics Committee.

### Tumor cell lines

The LWT1 melanoma was derived from SM1WT1 cells at the QIMR Berghofer in 2013. The C57BL/6 mouse melanoma cell line LWT1 was maintained as described previously (34). LWT1 cells were grown in RPMI1640 (Gibco) supplemented with 10% FCS, 1% Glutamax (Gibco), and 1% penicillin/streptomycin (Gibco) and maintained at 5% $CO_2$. Human melanoma cell lines SK-MEL28 and MZ7 were obtained from MH in 2015 and cultured in RPMI1640 containing 10% FCS, 2 mmol/L L-Glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all media components from Life Technologies) and maintained at 5% $CO_2$, as previously described (35). All cells tested negative for mycoplasma using the Lonza MycoAlert mycoplasma detection kit and only the human cell lines were STR profiled.

### Reagents

A2AR antagonist SCH58621 was purchased from Sigma and used at 1 or 10 mg/kg i.p. per dose as specified. Adenosine analogue 5′-(N-Ethylcarboxamido)adenosine (NECA) purchased from Sigma was given at 0.05 mg/kg. BRAF inhibitor (PLX4720) supplied by Plexxikon Inc. was dissolved in DMSO injected daily intraperitoneally at 10 mg/kg. MEK inhibitor GSK1120212 (trametinib, supplied by RBWH Oncology Pharmacy) was dissolved in DMSO, and then further diluted in an aqueous mixture of 0.5% hydroxypropyl methyl cellulose (HPMC; Sigma) and 1% polysorbate 80 (PS80; Fluka) to be delivered by oral gavage at 0.6 mg/kg.

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### *In vivo* treatments

For experimental metastasis, LWT1 cells were injected intravenously in a 200 μL volume and treatment commenced immediately after (day 0) and day 3, as indicated. Lungs were harvested on day 14 and normal tissue counterstained by intratracheal injection of 10% India ink prior to fixation in Fekete solution. For tamoxifen-inducible tumors, 4-HT solution was prepared in DMSO as described previously (33). 4-HT was applied topically on the back skin or ear of TBP mice to induce localized melanoma. Mice harboring established melanoma (from day 20 to 26) were treated daily with intraperitoneal injections either alone or in combination as specified with 10 mg/kg PLX4720, 10 mg/kg or 1 mg/kg A2ARi (SCH58261) and 0.05 mg/kg NECA, or an equivalent volume of vehicle DMSO or oral administration of vehicle or trametinib 0.6 mg/kg throughout the treatment period, as specified. Mice were monitored for melanoma development, and tumor growth determined using digital calipers, and tumor sizes represented as mean ± SEM or the growth curves of individual mice with tumors in each group. At indicated time points, tumors were excised and mass measured (mg) and inguinal lymph nodes harvested to investigate metastasis for individual mice in each group.

### *In vitro* treatments

Human melanoma cell lines SK-Mel28 and MZ7 were treated 24 hours postseeding with vehicle (DMSO), 100 nmol/L dabrafenib, or 50 nmol/L trametinib (GSK1120212; both Selleckchem) for 96 hours. For flow cytometry analysis, $2 \times 10^5$ cells were stained with Brilliant Violet 421 anti-human CD73 (AD2; Biolegend) and propidium iodide (PI). Cells were acquired using a FACSCanto II flow cytometer (BD Biosciences) and gated for the PI-negative cell population for further analysis of CD73 expression.

### Bone marrow chimeras

TBP mice received two doses of 550 cGy total body irradiation, separated by 3 hours to minimize gastrointestinal toxicity. Following this, C57BL/6 wild-type (WT) or B6.A2AR.ko donor bone marrow was injected intravenously on day 0. After 8 weeks of bone marrow reconstitution, tumor induction was initiated by tamoxifen application and the subsequent tumor incidence and survival monitored.

### Immunohistochemistry

For human CD73 staining, tissue microarray sections were cut at 3 μm onto superfrost+ glass slides and stored at 4°C until IHC was performed. IHC was performed on a Dako autostainer/PT-Link system using a high pH target retrieval buffer (Dako, K8005) as per the manufacturer's instructions. The primary antibody against CD73 (clone D7F9A Rabbit mAb; Cell Signaling Technology #13160) was incubated for 45 minutes at room temperature at a 1:200 dilution and visualized using the MACH3 Rabbit HRP polymer detection system (Biocare; M3R531) and DAB Chromogen Kit (Biocare; BDB2004) as per the manufacturer's instructions. IHC was evaluated on tissue microarray cores by assigning a score based on tumor cell CD73 immunostaining as follows; negative (0), weakly cytoplasmic no evidence of membrane staining (1), moderate CD73 expression with incomplete membrane staining (2), strong complete membrane staining (3). For whole tissue section CD73 immunostaining, a score for the intensity of tumor CD73 staining was assigned as above and then multiplied by the percentage area of CD73-positive tumor cells across the entire specimen. CD73 IHC for TMA and BRAF/MEK inhibitor-treated patients was evaluated by J. Madore and reviewed by R.A. Scolyer.

Immunohistopathology for BRAF inhibitor alone treated melanoma patients was performed with rabbit-anti-human NT5E/CD73 pAB (Sigma HPA017357, 1:600, antigen-retrieval pH 6, 10 minutes) followed by enzyme-conjugated secondary antibodies and the LSAB-2 color development system (DAKO). Stained sections were examined with a Leica DMLB immunofluorescence microscope. Images were acquired with a JVC digital camera KY-75FU and processed with Adobe Photoshop. A semiquantitative scoring system (0 = no expression, 1 = low, 2 = intermediate, 3 = high) has been applied for CD73 expression intensity on melanoma cells and pathologic analysis was performed by J. Landsberg.

For SOX10 staining, lymph nodes and tumor from TBP tumor-bearing mice or skin were formalin-fixed prior to paraffin-embedding. Sections from paraffin-embedded lesions were dewaxed and treated with Dako low pH antigen retrieval solution at 100°C for 20 minutes. Endogenous peroxidase activity was quenched in $H_2O_2$, and sections blocked with 1% BSA. Primary SOX10 antibody (clone N-20 goat polyclonal IgG, Santa Cruz Biotechnology) was applied, followed by appropriate secondary antibodies. After washing, color was developed with Vector red chromagen.

### Flow cytometry analysis

Tumor-bearing ears were harvested from tamoxifen-treated TBP mice, as indicated. Ears were minced and digested with 1 mg/mL collagenase IV (Worthington Biochemical Corporation) and 0.02 mg/mL DNase I (Roche) and homogenized to prepare single-cell suspensions. For surface staining, cell suspensions were stained with eFluor780 anti-CD45.2 (104; eBioscience), Brilliant Violet 605 anti-CD4 (RM4-5; Biolegend), PE anti-TCRβ (H57-597; Biolegend), PE-Cy7 anti-NK1.1 (PK136; eBioscience), Brilliant Violet 421 anti-CD8a (53-6.7; Biolegend), and Alexa Fluor 647 anti-FR4 (12A5; Biolegend) in the presence of anti-CD16/32 (2.4G2). 7AAD or Zombie Aqua (Biolegend) were used to exclude dead cells. Cells were acquired on the BD LSR II Fortessa or BD FACSCanto (BD Biosciences) and analysis was carried out using FlowJo (Tree Star).

### Statistics

Statistical analyses were carried out using GraphPad Prism. Significant differences were determined by log-rank test, ANOVA, and Holm-Sidak multiple comparison test of all pairwise combinations were determined unless indicated, paired *t*-test and unpaired *t*-test as specified. Significance by $\chi^2$ test and Fisher exact test with Freeman–Halton extension was determined using Spotfire from TIBCO. Values of $P < 0.05$ were considered significant.

## Results

### CD73 expression is heterogeneous in melanoma

CD73, the enzyme that generates adenosine, is associated with poorer prognosis in triple-negative breast cancer and high-grade serous ovarian cancer (31, 36). While it has been suggested that cell lines derived from metastatic melanoma predominantly express CD73, the overall CD73 expression in an intact TME and its prognostic value remains unclear (37).

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Therefore, we sought to further characterize CD73 expression in melanoma, as this marker is most closely linked with extracellular adenosine production, to determine whether CD73 expression and therefore changes to adenosine levels were associated with survival or disease status.

To assess the prognostic value of CD73 in melanoma, we first evaluated CD73 expression in tissue microarrays (TMAs) from advanced stage metastatic melanoma patients (AJCC stages III and IV). Interestingly, CD73 displayed heterogeneity in its expression pattern in melanomas ranging from negative to strong expression (Fig. 1A–D). Due to the variation in CD73 expression, we determined whether positive CD73 protein or high *NT5E* (CD73) gene expression were associated with survival. No significant survival association was identified for *NT5E* gene or positive CD73 protein expression, respectively (Supplementary Fig. S1A and S1B).

We next determined whether CD73 expression correlated with disease stage. Interestingly, in AJCC stage III patients with an N stage greater than 0, CD73 score (defined by level of CD73 intensity) was significantly increased ($^{*}$ $P < 0.05$; Fig. 1E). Therefore, although CD73 expression is not an independent prognostic factor in melanoma, it was associated with increasing expression in later stage disease. This indicates that the CD73-adenosinergic pathway may be an immunosuppressive mechanism employed by the tumor in late stages of disease, suggestive of its value as a possible therapeutic target for advanced disease.

### Increased CD73 expression associates with driver mutations in melanoma patients

As melanoma has distinct and well-characterized driver mutations, we next determined whether CD73 expression was

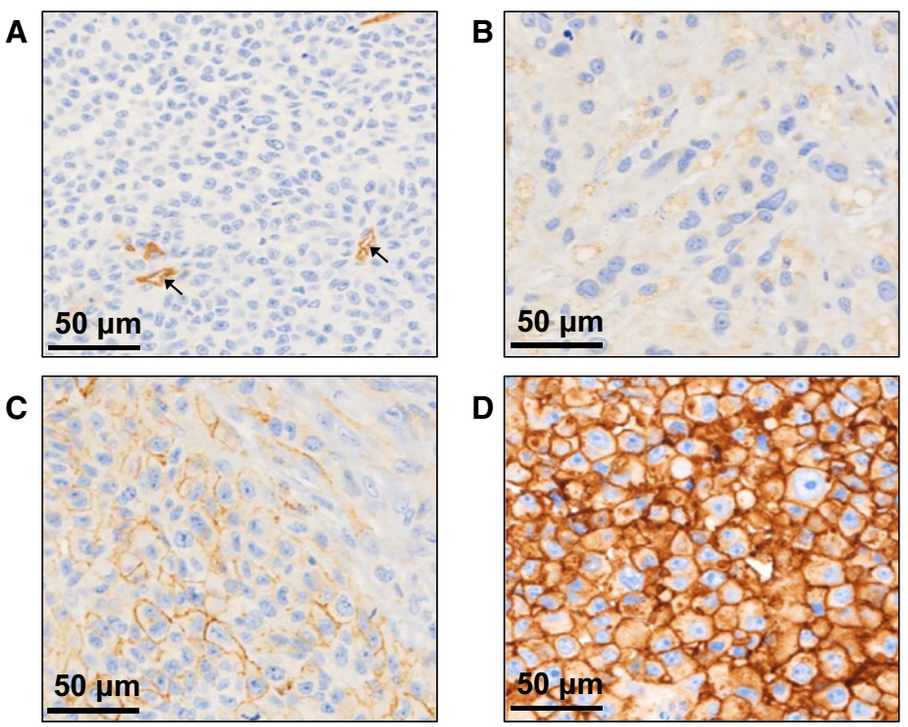


**Figure 1.**
Heterogeneous CD73 expression across human melanomas. Sample IHC (tissue array) for CD73 staining in various metastatic melanoma tumor specimens. **A,** Representative IHC images of CD73-negative tumor; arrows, CD73-positive tumor-associated endothelial cells. **B,** Weakly positive CD73 tumor signal. **C,** Moderate positive CD73 tumor signal with incomplete membrane staining. **D,** Strong positive tumor cell CD73 signal with complete membranous staining. **E,** Higher levels of CD73 protein expression associated with N stage greater than 1 at diagnosis ($^{*}$, $P < 0.05$; Fisher exact test Freeman–Halton extension). Scale bars, 50 μm.

**E**

| CD73 Score | N stage at diagnosis | |
|---|---|---|
| | N0 | N1–3 |
| Negative | 12 | 4 |
| Weak | 27 | 3 |
| Moderate | 6 | 6 |
| Strong | 3 | 2 |
| | 48 | 15 |

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**Table 1.** TP53 mutation status is associated with increased CD73 expression

| CD73 Score | *BRAF* Mutation status | *TP53* Mutation status | Total[a] |
|---|---|---|---|
| Negative | 6 (32%) | 1 (5%) | 19 |
| Weak | 18 (53%) | 8 (24%) | 35 (34) |
| Moderate | 7 (50%) | 5 (36%) | 15 (14) |
| Strong | 7 (88%) | 6 (75%) | 8 |
| *P* value | 0.066 | 0.002[b] | 77 (75) |
| $\chi^2$ | 7.18 | 14.76 | |
| df | 3 | 3 | |

NOTE: Tissue microarray analysis of CD73 protein expression identified an association between *TP53* mutation status and increasing CD73 protein expression. A similar though not statistically significant CD73 pattern was also observed for *BRAF* mutation status ($P = 0.066$; $\chi^2$ test). Results shown represent number and the (% of total).

[a]Mutation data available for these patients.

[b]$P < 0.01$; $\chi^2$ test.

associated with these mutational changes. In particular, increased CD73 expression was identified as significantly associated with *TP53* mutation in melanoma patients (Table 1). Since *BRAF* is the most common mutation in cutaneous melanoma (8, 9) we also assessed whether intensity of CD73 expression was altered. Whilst not significant within our cohort of patients, a trend of increasing CD73 score with *BRAF* mutation status was also apparent ($P = 0.066$; Table 1).

### Loss of A2AR adenosine signaling inhibits BRAF$^{V600E}$-mutant melanoma tumor growth

Given the tendency for increased CD73 expression and therefore potentially adenosine production in BRAF-mutated human melanoma, we assessed experimentally whether inhibition of adenosine signaling by the high-affinity A2AR improved anti-tumor immunity and impaired tumor development. We used the well-established BRAF$^{V600E}$-mutant, PTEN-deficient, tamoxifen-inducible melanoma model (*Tyr*::CreER$^{T2}$:*Braf*$^{CA}$:P*ten*$^{lox/lox}$; herein referred to as TBP; ref. 33) and generated wild-type or A2AR-deficient bone marrow chimeras. Following 8 weeks of immune cell reconstitution, we induced melanoma formation by tamoxifen application. Notably, mice with A2AR-deficient hematopoietic cells displayed significantly reduced tumor initiation compared with wild-type reconstituted mice (Fig. 2A and B; Supplementary Fig. S2A).

Next, we assessed whether prophylactic antagonism of the A2AR provided protection against tumor development. Prior to tumor induction, TBP mice were treated with A2AR inhibitor (A2ARi) and this was continued until day 35. Interestingly, during the treatment period, significantly reduced tumor growth was observed (Fig. 2C and D). However, at completion of A2ARi administration tumors rebounded to equivalent size compared with the vehicle-treated control group (Fig. 2E). While these changes in tumor growth were significant but minor, it is expected that higher therapeutic doses may enable improved tumor control.

Following, we aimed to identify whether the A2ARi altered immune infiltration into the TME. We initiated melanoma development on the ear whereby, 8 weeks posttamoxifen, significant tumor burden was observed (Supplementary Fig. S3A). We utilized this localized TME in the ear, alternatively to the back skin, to capture changes to immune infiltration during the early phases of tumor initiation within a defined, localized region as the TBP tumor model is poorly infiltrated. Therefore, following 6 weeks of tumor development, we administered either the A2ARi or vehicle via intradermal injection into the tamoxifen-treated ear. Tamoxifen-treated ears displayed significantly increased mass compared with the contralateral ear from the same mouse that did not receive tamoxifen (Supplementary Fig. S3B). The A2ARi further increased ear mass compared with vehicle in the tumor-bearing ear, which we attribute to a potential increase in inflammatory infiltration in the local TME (Supplementary Fig. S3B). Overall, tamoxifen-treated ears, assessed by flow cytometry, displayed a significant increase in overall leukocyte number and increased proportion of immune cell infiltrate compared with the nontamoxifen-treated ear (Supplementary Fig. S3C and S3D). Of particular note was the increased infiltration of CD8$^+$ T cells and NK cells following administration of the A2ARi compared with the vehicle-treated tumors (Fig. 2F–I). CD8$^+$ T cells, but not NK cells, also displayed increased frequency of expression of the activation marker CD69 (Fig. 2J and K). While CD4$^+$ T cells were not significantly altered in their proportion or number between vehicle and A2ARi-treated tumor-bearing ears, the proportion of FR4$^+$CD4$^+$ T cells was reduced (Fig. 2L; Supplementary Fig. S3E and S3F). A large proportion of CD4$^+$ T cells expressing FR4 are Tregs (38). Using this marker, we determined the CD8$^+$ T-cell/Treg ratio was significantly enhanced in tumor-bearing ears treated with A2ARi (Fig. 2M). This indicates that A2AR inhibition enhances infiltration of cytotoxic NK cells and CD8$^+$ T lymphocytes while also decreasing a proportion of immunosuppressive CD4$^+$ Tregs, both potentially improving tumor control.

### Adenosine inhibits metastatic control of BRAF-mutant melanoma

We next aimed to identify whether heightened *in vivo* adenosine levels modulated tumor growth and progression. TBP mice were treated with tamoxifen and following tumor development at day 23, we commenced daily administration of an adenosine analogue, NECA. Notably, NECA did not significantly alter primary tumor growth (Fig. 3A). In contrast, NECA enhanced macroscopic lymph node pigmentation and significantly promoted metastatic formation within the lymph nodes (by histologic investigation; Fig. 3B and C). Melanoma metastases were identified as melanocytic cell clusters located within the subcapsular sinus region of the lymph node, which reflects the positioning of metastases seen in the tumor draining lymph nodes of melanoma patients (39). Lymph node metastases were confirmed by the presence of nuclear expression of SOX10, a neural crest transcription factor highly expressed by melanoma cells (Fig. 3D and E; ref. 40). Expression of SOX10 within skin and tumor were also determined as positive controls (Supplementary Fig. S4A and S4B).

### Combining BRAF and A2AR inhibition reduces tumor growth and metastasis in an inducible and experimental BRAF-mutant melanoma

Previously, BRAF and MEK inhibition have been shown to downregulate *NT5E* gene expression in A375 human melanoma cells and we further confirmed reduction of *NT5E* mRNA and CD73 protein levels in BRAF-mutant melanoma cell lines (Fig. 4A; Supplementary Fig. S5A and S5B; ref. 16). Next, we assessed whether BRAF-targeted therapy with either dabrafenib or vemurafenib alters CD73 expression in 13 melanoma patients. Consistent with changes identified *in vitro*, 4 of 4 patients biopsied while receiving BRAF inhibitor (BRAFi) treatment showed decreased

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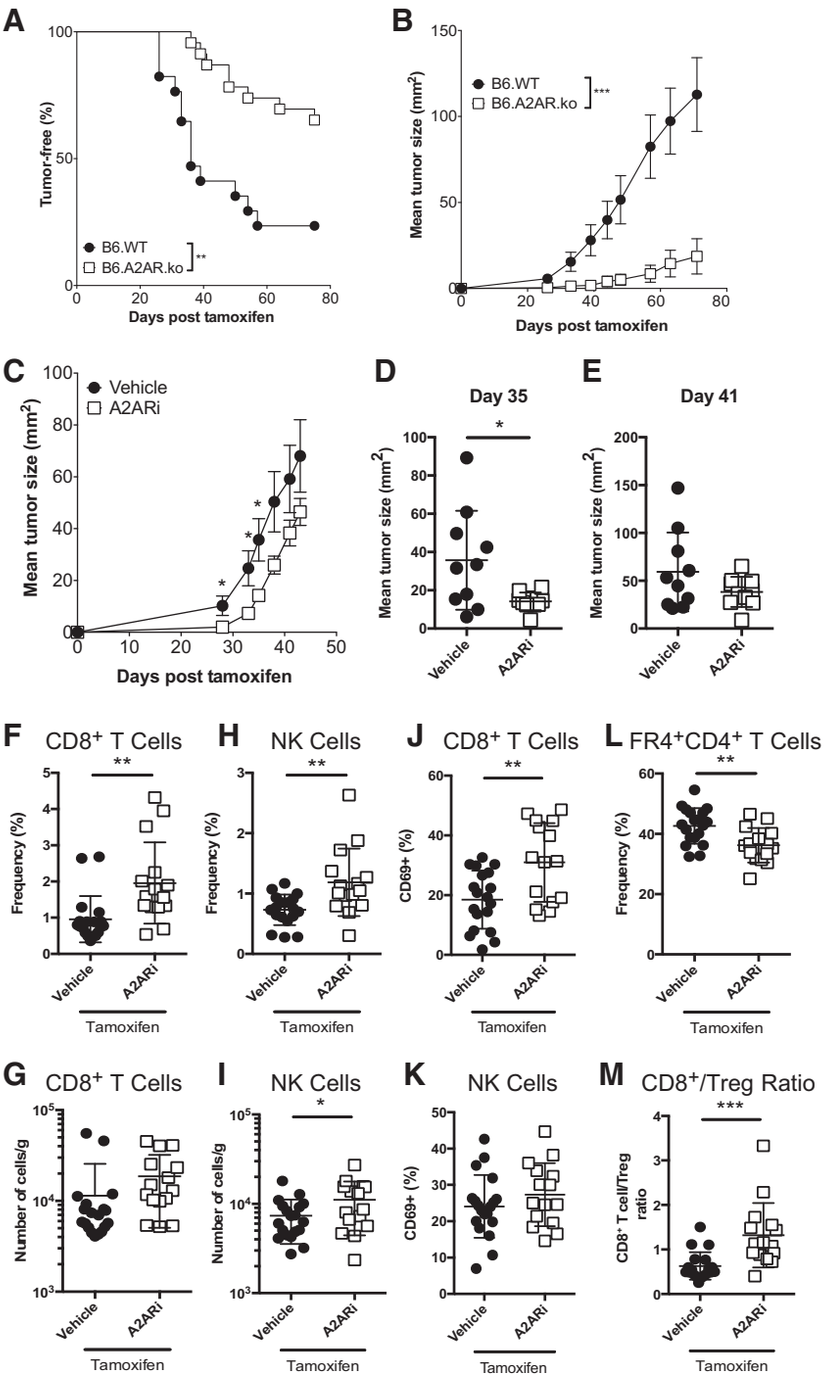


**Figure 2.**

Inhibition of A2AR signaling limits BRAF$^{CA/+}$PTEN-deficient tumor growth. TBP mice were sublethally irradiated and reconstituted with bone marrow from WT or B6.A2AR.ko mice. After 8 weeks, tumor induction by tamoxifen was performed and subsequently monitored for tumor development over 75 days. **A,** Survival curves defined as the percentage of tumor-free mice (following two increasing growth measurements > 9 mm$^2$) at each time point. Results shown are from two pooled experiments and 17–23 male mice per group are shown. **B,** Tumor growth was measured using digital calipers and tumor sizes from one representative experiment of 8–10 male mice are presented as mean ± SEM. Statistical significance for tumor incidence and tumor growth was determined by log-rank (Mantel–Cox) test and unpaired *t* test at day 71, respectively (**, $P < 0.01$; ***, $P < 0.001$). **C–E,** TBP mice were treated with vehicle or A2AR inhibitor (A2ARi; SCH58261, 1 mg/kg) from day -2 prior to tamoxifen induction until day 35. **C,** Tumor growth was measured using digital calipers and tumor sizes are presented as mean ± SEM. Tumor sizes for individual mice at day 35 (**D**) and day 41 (**E**) are presented as mean ± SD. Results shown are from one experiment of 10 mice per treatment group as indicated. Statistical significance was determined by unpaired *t* test at the specified time points (*, $P < 0.05$). **F–M,** Tamoxifen induction of tumors was performed on the left ear of TBP mice. After 6 weeks, vehicle or A2ARi (SCH58261, 1 mg/kg) was intradermally injected into the tumor-bearing ear for 10 consecutive days. Flow cytometry was performed and proportions (**F** and **H**) or number normalized (**G** and **I**) to ear mass of CD8$^+$ T cells and NK cells was determined. Frequency of CD69-expressing CD8$^+$ T cells (**J**) and NK cells (**K**) in the TME was identified. Proportions of FR4$^+$ T cells within the CD4$^+$ T-cell population (**L**) and CD8$^+$/Treg ratio was identified (**M**). Results shown are presented as the means ± SD from two pooled experiments of 15–19 mice per treatment group as indicated. Statistical significance was determined by unpaired *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

CD73 expression (Fig. 4B; Supplementary Table S1). Three progressing patients, biopsied less than a week posttreatment, showed no change in CD73 expression (Supplementary Fig. S5C; Supplementary Table S1). Of the melanoma patients that were biopsied more than a week following BRAFi withdrawal, 3 of 6 patients showed decreased CD73 expression and 3 of 6 increased CD73 expression compared with their pretreatment tumor biopsy (Fig. 4C; Supplementary Table S1). Notably, all patients that demonstrated decreased CD73 expression displayed a partial response as their best therapeutic response, defined by RECIST 1.1 (Supplementary Table S1). In contrast, 4 of 6 patients that either showed increased or no change in CD73 expression only managed to achieve stable or progressive disease (Supplementary Table S1). Therefore, CD73 downregulation driven by BRAF inhibition may be beneficial for therapeutic efficacy. Alternatively, incomplete loss of CD73 expression may assist tumor escape from MAPK-targeted therapies.

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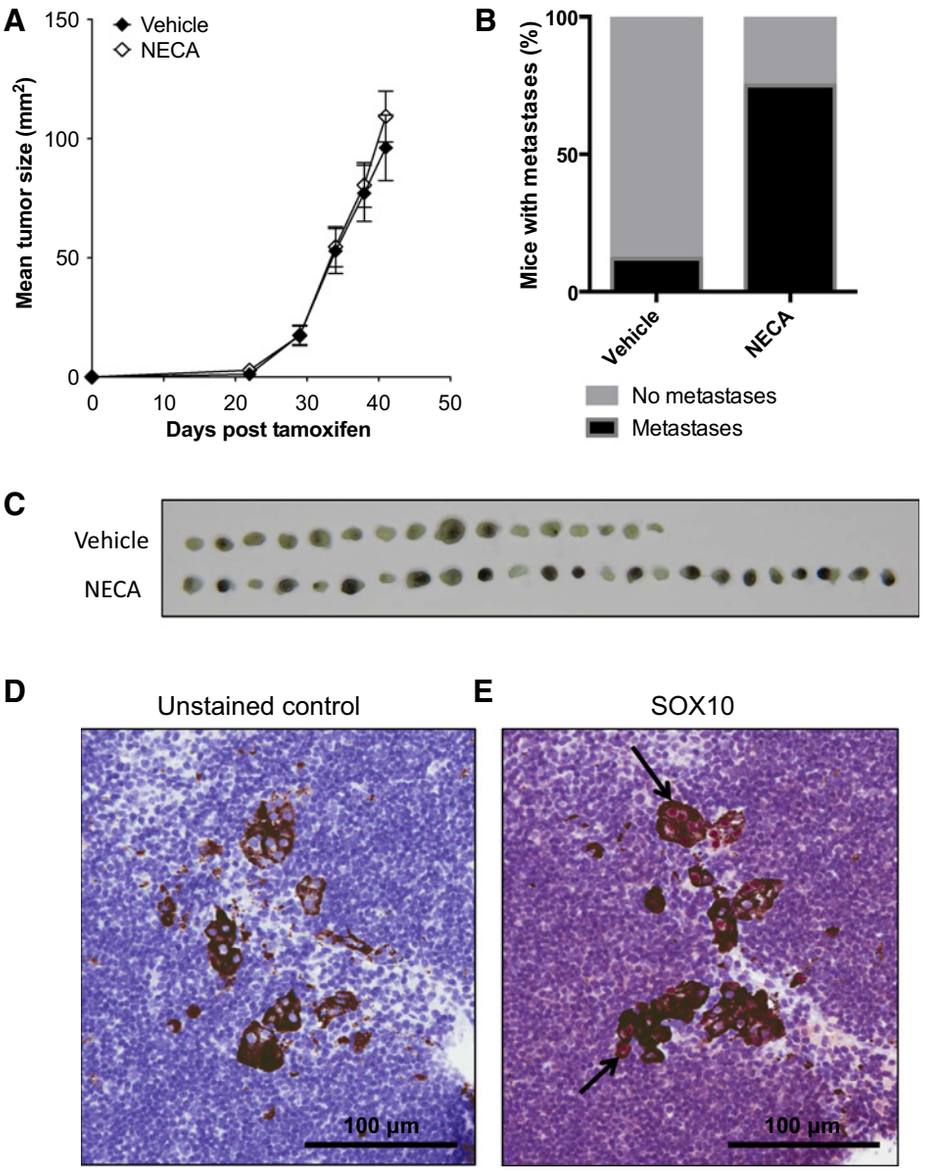


**Figure 3.**

Adenosine enhances metastatic spread, but not $BRAF^{CA/+}PTEN$-deficient primary tumor growth. Tumor induction by tamoxifen was performed in TBP mice. Following the development of tumor, mice were treated daily with the adenosine analogue NECA (0.05 mg/kg) from day 23 to day 42. **A,** Tumor growth was measured using digital calipers and tumor sizes presented as mean ± SEM. **B,** At day 42, inguinal lymph nodes were excised and percentage of mice with metastases was assessed by histologic sections. **C,** Macroscopic changes to lymph node pigmentation following NECA treatment are shown. **D** and **E,** Representative histologic images of subcapsular lymph node metastases (**D**) and nuclear SOX10 staining (red) within the lymph node metastases (**E**).

Recently, we have shown that targeting both CD73 and the A2AR in combination enhances antitumor immunity (25). Therefore, as BRAF inhibition is able to decrease CD73 expression and improve therapeutic response in melanoma patients, we next experimentally assessed whether A2AR inhibition further increased the efficiency of BRAFi therapy. Interestingly, combined A2AR and BRAF inhibition provided significantly reduced tumor growth in TBP mice compared with vehicle or single-agent treatment alone (Fig. 5A and B; Supplementary Fig. S5D). As we previously observed that increased systemic adenosine augmented metastatic burden in lymph nodes, we determined whether combining A2AR and BRAF inhibition therapeutically limited metastatic spread. Notably, the combination reduced lymph node pigmentation, which previously correlated with lower metastatic burden (Supplementary Fig. S5E). Following this, we wanted to determine whether this combination approach provided greater efficacy in a transplantable metastatic melanoma model. Using the LWT1 BRAF-mutant experimental metastatic melanoma (34), we identified significant antimetastatic activity when BRAF and A2AR inhibitors were administered concurrently (Fig. 5C and D).

This indicates that combining BRAF-targeted therapies and A2AR inhibition may provide improved protection against tumor growth and distant metastasis formation, qualities requisite of an effective melanoma therapy.

### Triple combination therapy targeting BRAF, MEK, and A2AR provides improved antimetastatic activity

BRAF and MEK inhibition are currently used in combination as standard of care for the treatment of $BRAF^{V600E}$-mutant melanoma patients receiving targeted therapies. Therefore, we assessed whether 13 BRAF-mutant melanoma patients treated with a combination of dabrafenib and trametinib displayed modulated CD73 expression during treatment. Within this

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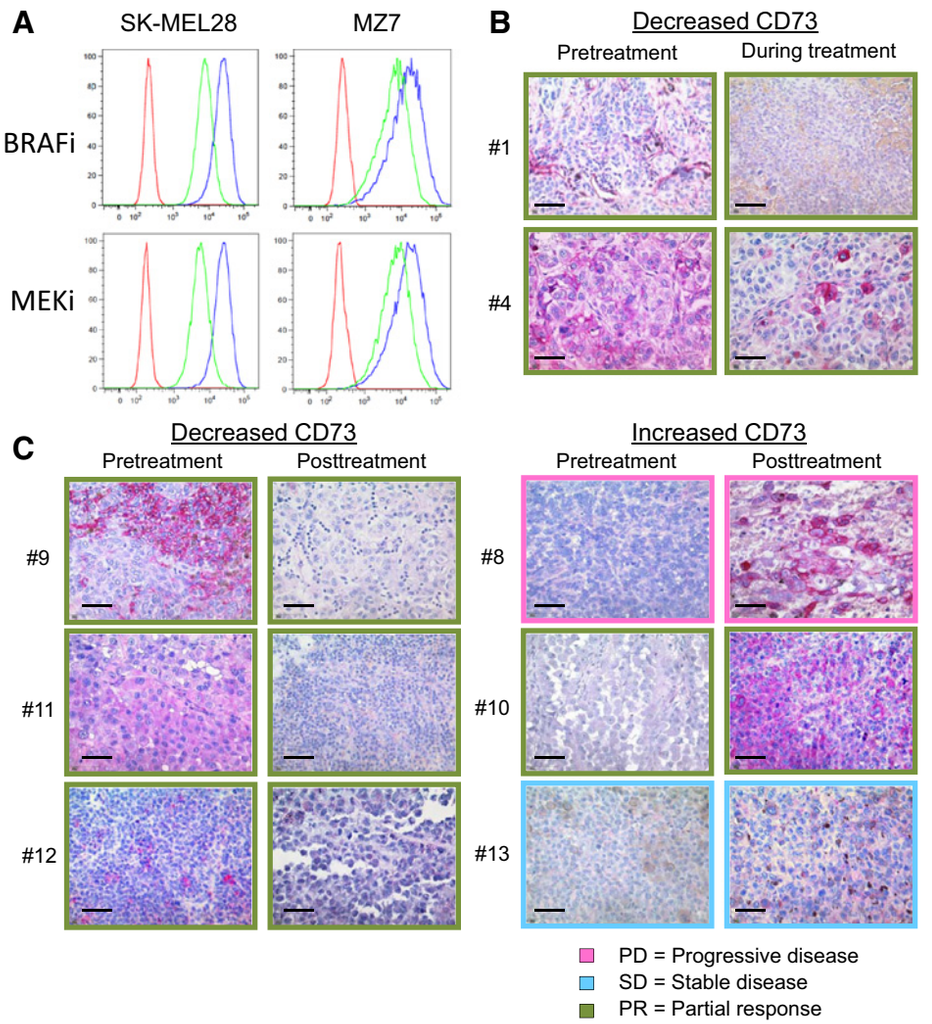


**Figure 4.**
BRAF inhibition reduces CD73 expression from melanoma patients. **A,** Human BRAF-mutant melanoma cell lines SK-MEL28 and MZ7 were treated with BRAFi (dabrafenib, 100 nmol/L) or MEK inhibitor (trametinib, 50 nmol/L) for 96 hours prior to assessing CD73 expression by flow cytometry. Representative histograms from biological triplicates of vehicle-treated (blue), BRAF or MEK inhibitor-treated (green), or unstained cells (red) are shown. **B** and **C,** CD73 expression from melanoma biopsies taken pretreatment compared with during (**B**) and more than one week (**C**) after BRAF inhibitor–targeted therapy. Best therapeutic response was assessed according to RECIST 1.1 criteria; patient (#) details are shown in Supplementary Table S1. Scale bars, 50 μm.

cohort, 7 of 13 patients were CD73 negative prior to treatment and remained negative at week 12 or progression. Notably, 6 of 13 patients with CD73 positivity at baseline or EDT significantly decreased tumor-derived CD73 expression in response to dabrafenib and trametinib combination therapy (Fig. 6A and B). Together, in two separate cohorts of melanoma patients, tumor-derived CD73 expression was identified to be downregulated in response to either BRAFi alone or in combination with MEK-targeted therapies.

Therefore, as BRAF and MEK inhibition potently decreases CD73 expression, we wanted to establish whether A2AR inhibition could provide protection additional to this therapeutic combination. Using the LWT1 BRAF-mutated metastatic melanoma model, the triple combination of BRAF, MEK, and A2AR inhibition provided significantly greater antimetastatic activity compared with the clinically approved BRAF and MEK combination therapy (Fig. 6C; Supplementary Fig. S6A). Next, we assessed the triple combination in the TBP primary tumor model. While the MEK inhibitor (trametinib) potently impacted on tumor growth alone, this was further enhanced in the triple combination with BRAF and A2AR inhibition providing significantly improved tumor control (Fig. 6D).

Together, this indicates that BRAF and MEK inhibition negatively regulates CD73 expression and A2AR inhibition may be a useful therapeutic addition to both BRAF and MEK inhibition, for melanoma patients.

## Discussion

Adenosine is gaining increasing prominence as an immunosuppressive metabolite utilized by the tumor to facilitate immune evasion and tumor growth (19). Similarly, the ability of CD73 to identify patients with poorer prognosis and reduced response to therapeutic modalities is receiving attention (19, 31, 36). In melanoma, CD73 increases with more advanced clinical staging, indicative that the adenosinergic pathway may be increasingly active in later-stage disease. We also identified that exogenous adenosine increased metastatic dissemination, but not primary tumor growth, suggesting a role for adenosine in promoting cancer spread. Previously, a similar relationship between elevated CD73 levels in human metastatic melanoma cell lines, but not those derived from melanocytes or primary tumor, was identified *in vitro* (37). Furthermore, CD73 was coexpressed with molecules (such as N-cadherin, MMP-2, caveolin-1, CD44 and several

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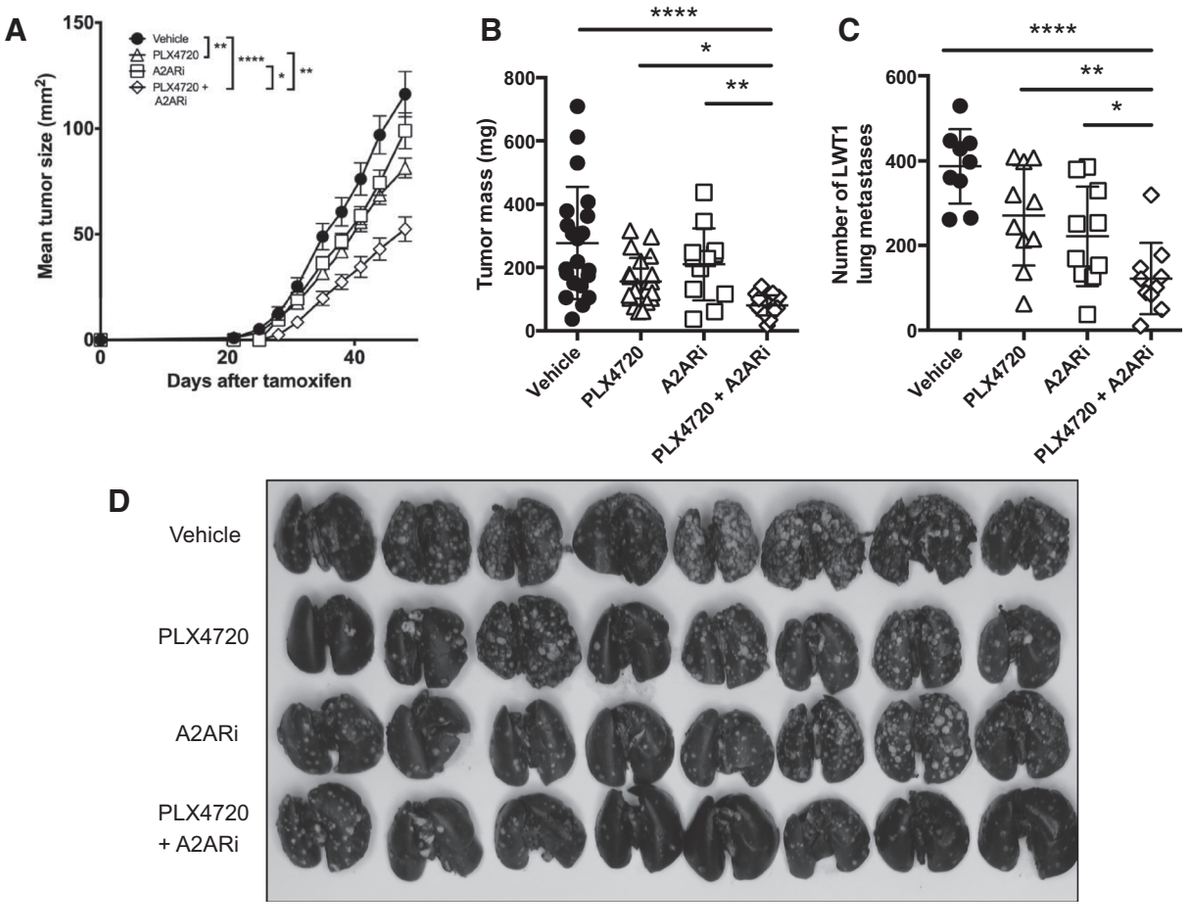


**Figure 5.**

BRAF inhibition and A2AR inhibition limits tumor growth and metastasis in BRAF-mutant melanoma. Tumor induction by tamoxifen was performed in TBP mice. Following the development of tumor, mice were treated daily with vehicle, BRAFi (PLX4720, 10 mg/kg), and A2ARi (SCH58261, 10 mg/kg) from day 23 to day 48. **A,** Tumor growth was measured using digital calipers and tumor sizes are presented as mean ± SEM. Results shown are from one representative experiment of groups of 5–12 mice. Statistical significance was determined by one-way ANOVA and Holm–Sidak multiple comparison test between tumor sizes at day 48 (*, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$). **B,** Tumors were excised at day 48 or 49 and tumor mass measured and are presented as mean ± SD. Results are pooled from two experiments and 12–21 mice per group are shown. Improved tumor control for the combination therapy–treated mice was statistically significant (*, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; one-way ANOVA and Holm–Sidak multiple comparison test comparing PLX4720 + A2ARi–treated mice pairwise to single-agent and vehicle treatment). C57BL/6 WT mice were injected intravenously with the LWT1 BRAF-mutated melanoma ($7.5 \times 10^5$ cells). On day 0 and 3, mice were treated intraperitoneally with vehicle, A2ARi (SCH58261, 10 mg/kg), PLX4720 (10 mg/kg), or combinations as specified. **C,** Lungs were harvested on day 14 and normal tissue was counterstained with India ink and amelanotic macrometastases counted. Results are from one representative experiment and 9–10 mice per group ± SD are shown. Improved metastatic control was statistically significant (*, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; one-way ANOVA and Holm–Sidak multiple comparison test comparing PLX4720 + A2ARi–treated mice pairwise to single-agent and vehicle treatment). **D,** Metastatic burden of lungs at day 14 following treatment from one representative experiment are shown.

integrins) related to adhesion and epithelial-to-mesenchymal transition (EMT), resulting in increased metastatic potential (37). Indeed, the accompanying work by Reinhardt and colleagues (41) mechanistically links CD73 expression in melanoma to nascent activation of an EMT-like program. Evidence that *NT5E* gene expression is epigenetically regulated by DNA methylation in melanoma has also identified an alternate risk factor for metastatic progression (42). In melanoma patient samples, deregulated methylation of the *NT5E* CpG island, which leads to higher CD73 expression, correlated with increased risk of developing distant visceral metastasis (42). Therefore, while not prognostic of survival, identifying patients with high CD73 expression, which potentially facilitates greater extracellular adenosine production, may represent a subset of patients that present with more aggressive melanoma.

In comparison with other cancer types, mutation of the tumor suppressor gene *TP53* is relatively low (between 10% and 20%) in melanoma (43). Within our dataset, a distinct increase in CD73 expression was identified in patients harboring a *TP53* mutation. Cancer types such as triple-negative breast cancer and high-grade squamous ovarian cancer display high levels of *TP53* mutation, as well as elevated CD73 expression, resulting in poor prognosis (31, 36). Dissecting whether *TP53*-mutated/CD73-expressing melanoma patients have worse prognosis in a larger cohort of patients will be of interest to further delineate the interaction between these molecules.

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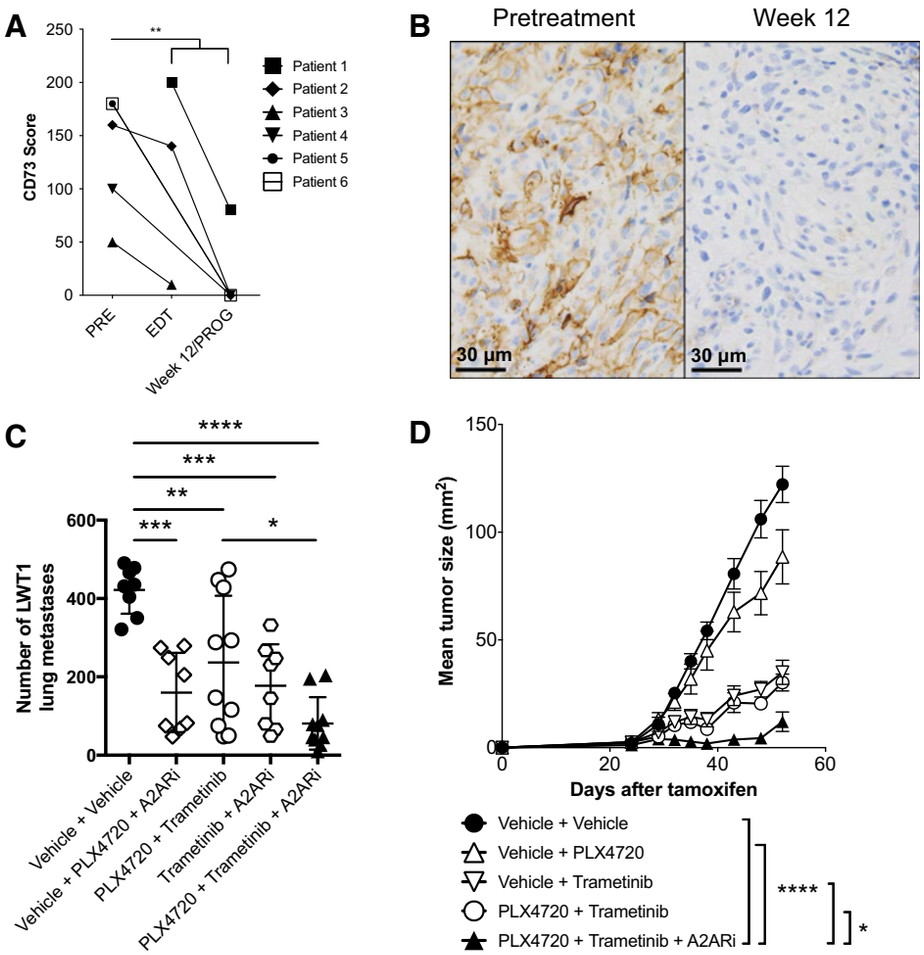


**Figure 6.**

Triple combination therapy targeting BRAF, A2AR, and MEK improves tumor control. CD73 expression of BRAF-mutant melanoma patients treated with dabrafenib and trametinib was assessed by IHC. Six patients were CD73$^+$ either pretreatment (PRE) or early during treatment (EDT). **A,** Kinetics of CD73 expression for CD73$^+$ BRAF-mutant melanoma patients following dabrafenib or trametinib. Decreased CD73 expression in CD73$^+$ patients comparing pretreatment (PRE) and posttreatment (EDT or week 12/PROG) was statistically significant (** $P < 0.01$; paired $t$ test). **B,** Representative image of changes to CD73 expression following dabrafenib and trametinib therapy in a CD73$^+$ tumor pretreatment. C57BL/6 WT mice were injected intravenously with the LWT1 BRAF-mutated melanoma ($7.5 \times 10^5$ cells). On day 0 and 3, mice were treated intraperitoneally with vehicle, A2ARi (SCH58261, 10 mg/kg), PLX4720 (10 mg/kg), or oral gavage of vehicle diluent or trametinib (0.6 mg/kg) combinations as specified. **C,** Lungs were harvested on day 14 and macrometastases counted. Results shown are from one representative experiment and 8–10 mice per group ± SD are shown. Improved metastatic control was statistically significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; one-way ANOVA and Holm–Sidak multiple comparison test comparing all pairwise combinations). **D,** Tumor induction by tamoxifen was performed in TBP mice. Following tumor development, mice were treated daily intraperitoneally with vehicle, BRAFi (PLX4720, 10 mg/kg), and A2ARi (SCH58261, 10 mg/kg) in combination with oral administration of vehicle or MEK inhibitor (trametinib, 0.6 mg/kg) from day 26 to day 48. Tumor growth was measured using digital calipers and tumor sizes presented as mean ± SEM. Results shown are from one experiment of groups of 6–10 mice. Statistical significance was determined by one-way ANOVA and Holm–Sidak multiple comparison test between triple therapy–treated mice pairwise to all other treatment groups shown at day 52 (*, $P < 0.05$; ****, $P < 0.0001$). Scale bars in **B**, 30 µm.

Nonetheless, for patients with advanced unresectable melanoma harboring a *TP53* mutation, immunotherapy is a standard of care. In preclinical models, combining adenosine-related therapies alongside immune checkpoint blockade has been shown to improve therapeutic efficacy, particularly with abundant tumor-derived CD73 (28–30, 32). Of note, combining anti-CD73 or A2AR antagonism with anti-PDL1 is currently being clinically tested in solid tumors, including malignant melanoma (NCT02503774 and NCT02655822).

A large proportion of BRAF-mutant melanoma patients display high levels of CD73 within their TME. Therapeutically, BRAF-mutant melanoma patients gain survival benefit through a combination of targeted therapies directed toward mutant BRAF and aberrant MEK reactivation (13, 14). In this study, A2AR antagonism potentiated the therapeutic efficacy of the clinically approved combination of BRAF and MEK inhibition. Therapeutic antagonism of A2AR signaling increased infiltration of cytotoxic immune cell populations, particularly CD8$^+$ T and NK cells, into the TME. Identifying strategies that enhance infiltration of immune cells into melanomas with limited TILs is a priority, as it is a significant prognostic factor that correlates with improved survival for melanoma patients (44). BRAF

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inhibition has also been shown to enhance activation and proinflammatory cytokine production of $CD8^+$ T cells and NK cells, which may facilitate the improved antitumor efficacy apparent from combinatorial A2AR and BRAF inhibitor treatment (34, 45). While some reports suggest MEK inhibition may suppress immune function, recent preclinical studies using *in vivo* models revealed that MEK inhibitors bolstered the efficacy of immunotherapies (15, 16, 46). Interestingly, combinatorial BRAF and MEK inhibition enhances tumor antigen and MHC expression while decreasing immunosuppressive molecules (15, 16). Similarly to Liu and colleagues, we have confirmed that BRAF and MEK inhibition decreased *NT5E* mRNA and CD73 protein expression in the BRAF-mutant melanoma cell line as well as patients receiving BRAF inhibition alone or in combination with trametinib (16). In fact, Reinhardt and colleagues elucidated a mechanism by which proinflammatory mediators (such as TNFα) promote CD73 expression in a MAPK-dependent manner by the transcription factor complex AP-1 (41). Recently, we demonstrated that cotargeting CD73 and A2AR adenosine signaling concurrently enables improved tumor control (25). Therefore, by downregulating CD73 within the TME, via BRAF and MEK inhibition, the potency of A2AR antagonism may be improved, highlighting a rationale for the improved therapeutic response following the triple combination.

Understanding whether therapeutic modalities regulate adenosine-related molecules in the TME and how this relates to therapeutic response will be important to elucidate the most effective combination treatment strategies. We identified profound CD73 downregulation in mutant BRAF patients receiving targeted therapies, leading to better therapeutic response in our small cohort of patients. However, larger cohorts are required with long-term assessment of tumor CD73 expression following tumor escape. Nonetheless, the correlation between CD73 downregulation and a favorable prognosis argues for an important role of the adenosinergic pathway in melanoma progression. In contrast to BRAF and MEK inhibition, a proportion of patients receiving cancer immunotherapies (either adoptive cell therapy directed toward MART-1 or anti-PD1 treatment) have been shown to increase CD73 expression (41). Many patients that exhibited CD73 upregulation were initially responsive to immunotherapies, but developed acquired resistance (41). This indicates that CD73 may be induced to dampen an active inflammatory response generated by these immunotherapies, highlighting a possible escape mechanism. Importantly, due to the dynamic regulation of CD73 in the TME, patients with $CD73^-$ tumors prior to receiving immunotherapies should not be excluded from receiving anti-CD73 therapies. Indeed, preclinical studies highlighting anti-CD73 treatment in combination with immune checkpoint blockade (30, 32) have led to clinical testing of this combination strategy (NCT02503774).

While the therapeutic effect of A2AR inhibition alone in primary melanomas is modest, its antimetastatic activity is comparable with mice treated with BRAF or MEK inhibitor alone or in combination. We speculate that this difference may be driven by adenosine accumulation in fast-growing primary tumors, in which hypoxia tends to occur, impeding the pharmacodynamics of the A2AR inhibitor (SCH58261) used within these studies. In contrast, A2AR antagonism is more effective in suppressing metastasis formation, where tumor density is relatively small and adenosine levels are less concentrated than in comparison with established primary tumors. This is further supported by the preferential effect of NECA in promoting metastasis of the TBP tumor, perhaps due to abundant adenosine production within the primary tumor leading to no difference in growth. However, we cannot discount that the increased metastatic potential observed in NECA-treated mice is due to reduced immune surveillance with greater levels of systemic adenosine dampening the antitumor immune response. Nonetheless, this may also partly explain the effect of prophylactic A2AR inhibition in significantly delaying tumor formation, as a monotherapy, but not sustaining long-term tumor suppression. Therefore, the tumor debulking effect of BRAF and MEK inhibitors may also enhance the therapeutic activity of A2AR antagonism.

The therapeutic benefit received by melanoma patients from either targeted or immune-based therapies is unprecedented. Much interest surrounds the most appropriate combinations and scheduling of therapies to maximize response. However, while preclinical models targeting BRAF inhibition and immune checkpoint blockade in combination display improved tumor control, there are clear toxicity concerns for their use in patients (15, 45, 47, 48). Concurrent administration of vemurafenib and ipilimumab (anti-CTLA4) induced severe hepatotoxicity (18). Similarly, anti-PD1 treatment preceding vemurafenib heightened an immune reaction, presenting as severe cutaneous and neurologic toxicities, resulting in patient hospitalization (49). In addition, melanoma patients receiving ipilimumab following dabrafenib and trametinib in a phase I/II clinical trial exhibited higher risk of intestinal perforations, resulting in closure of the triple combination study (50). Targeting adenosine offers an attractive combination partner as A2AR antagonists displayed excellent therapeutic safety profiles in the treatment of Parkinson disease. In addition, due to the short half-life of A2AR inhibitors, should adverse events occur treatment could be rapidly discontinued and thus reducing long-term toxicity.

Adenosine-related therapies appear to be formidable combination partners for relieving tumor-induced immunosuppression. In this study, we demonstrate that A2AR antagonism in combination with targeted BRAF and MEK inhibition enhanced immune responses to metastatic melanoma. As melanoma patients display increased CD73 expression with more advanced disease, targeting adenosine may limit immune evasion and tumor escape. With A2AR antagonists undergoing clinical trials for the treatment of solid tumors, their clinical use alongside targeted therapies in advanced melanoma should be investigated as a priority for improving survival outcomes for melanoma patients.

## Disclosure of Potential Conflicts of Interest

A. Young received speakers bureau honoraria from Arcus Biosciences. G.V. Long is a consultant/advisory board member for Bristol-Myers Squibb, Novartis, Roche, Amgen, Pierre Fabre, Array, and Merck. G.M. Boyle has ownership interest (including patents) from QBiotics. M.J. Smyth reports receiving a commercial research grant from Bristol Myers Squibb, Corvus Pharmaceuticals, and Aduro Biotech, is a consultant/advisory board member for Arcus Biosciences, and has provided expert testimony for Corvus Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

## Authors' Contributions

**Conception and design:** A. Young, S.F. Ngiow, E. Ahern, R.A. Scolyer, M.J. Smyth

**Development of methodology:** A. Young, S.F. Ngiow, A. Chitsazan, N.D. Huntington, R.A. Scolyer

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** A. Young, S.F. Ngiow, J. Madore, J. Reinhardt, J. Landsberg, J. Rautela, T. Bald, D.S. Barkauskas, G.V. Long, R.A. Scolyer, M.J. Smyth
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** A. Young, S.F. Ngiow, J. Madore, J. Reinhardt, J. Landsberg, J. Rautela, T. Bald, D.S. Barkauskas, E. Ahern, N.D. Huntington, D. Schadendorf, G.M. Boyle, M. Hölzel, R.A. Scolyer, M.J. Smyth
**Writing, review, and/or revision of the manuscript:** A. Young, S.F. Ngiow, J. Reinhardt, J. Landsberg, T. Bald, D.S. Barkauskas, E. Ahern, D. Schadendorf, G.V. Long, G.M. Boyle, M. Hölzel, R.A. Scolyer, M.J. Smyth
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** A. Young, S.F. Ngiow, E. Ahern, G.V. Long
**Study supervision:** A. Young, S.F. Ngiow, M.J. Smyth

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