**A2B adenosine receptor antagonists rescue lymphocyte activity in adenosine-producing patient-derived cancer models**

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**ABSTRACT**

**Background** Adenosine is a metabolite that suppresses antitumor immune response of T and NK cells via extracellular binding to the two subtypes of adenosine-2 receptors, A2Rs. While blockade of the A2AAR subtype effectively rescues lymphocyte activity, with four A2BAR antagonists currently in anticancer clinical trials, less is known for the therapeutic potential of the other A2BAR blockade within cancer immunotherapy. Recent studies suggest the formation of A2AAR/A2BAR dimers in tissues that coexpress the two receptor subtypes, where the A2BAR plays a dominant role, suggesting it as a promising target for cancer immunotherapy.

**Methods** We report the synthesis and functional evaluation of five potent A2BAR antagonists and a dual A2AAR/A2BAR antagonist. The compounds were designed using previous pharmacological data assisted by modeling studies. Synthesis was developed using multicomponent approaches. Flow cytometry was used to evaluate the phenotype of T and NK cells on A2BAR antagonist treatment. Functional activity of T and NK cells was tested in patient-derived tumor spheroid models.

**Results** We provide data for six novel small molecules: five A2BAR selective antagonists and a dual A2AAR/A2BAR antagonist. The growth of patient-derived breast cancer spheroids is prevented when treated with A2BAR antagonists. To elucidate if this depends on increased lymphocyte activity, immune cells proliferation, and cytokine production, lymphocyte infiltration was evaluated and compared with the potent A2BAR antagonist AZD-4635. We find that A2BAR antagonists rescue T and NK cell proliferation, IFNγ and perforin production, and increase tumor infiltrating lymphocytes infiltration into tumor spheroids without altering the expression of adhesion molecules.

**Conclusions** Our results demonstrate that A2BAR is a promising target in immunotherapy, identifying ISAM-R56A as the most potent candidate for A2BAR blockade. Inhibition of A2BAR signaling restores T cell function and proliferation. Furthermore, A2BAR and dual A2AAR/A2BAR antagonists showed similar or better results than A2AAR antagonist AZD-4635 reinforcing the idea of dominant role of the A2BAR in the regulation of the immune system.

**WHAT IS ALREADY KNOWN ON THIS TOPIC**

⇒ A2AAR is a low affinity adenosine receptor that is activated by high adenosine concentration. With adenosine being an anti-inflammatory and immunosuppressive metabolite that accumulates at high concentrations in the tumor microenvironment, targeting A2AAR is a promising metabolic immune checkpoint.

**WHAT THIS STUDY ADDS**

⇒ We synthesized high affinity non-xanthine A2BAR antagonists with improved T and NK cell activities under exogenous and endogenous adenosine suppression. A2BAR inhibition furthermore improves infiltration of tumor-infiltrating lymphocytes into patient-derived 3D spheroids.

**HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE AND/OR POLICY**

⇒ These findings provide practical readouts to study adenosine-mediated suppression and identified putative A2 BAR antagonists to alleviate suppression of T and NK cells.

**BACKGROUND**

Accounting for its heterogeneity with more than 100 distinct types, cancer is a complex and dynamic disease. This complexity has been rationalized to 10 transforming hallmarks, of which avoiding immune destruction explains how the immune system plays an essential role during tumorigenesis. Therapies based on activating the immune system can result in beneficial responses in patients with metastatic cancer. Treatment with antibodies targeting the immunological checkpoint axis PD-1–PD-L1/2 can result in potent antitumor T cell activation and clinically meaningful long-lasting responses. Primary resistance to immune checkpoint
therapy can be attributed to the absence of intratumoral T cells. This has fueled the search for strategies to convert immune-excluded tumors to immune-infiltrated tumors. Since the tumor microenvironment (TME) often imposes metabolic stress and dysregulation on tumor infiltrating lymphocytes (TILs), targeting immune metabolism represents a promising direction.

Purinergic signaling involves extracellular purine nucleosides [Adenosine (ADO)] and nucleotides (ATP and AMP) as signaling molecules. ADO, an ubiquitous metabolite with critical anti-inflammatory and immunosuppressive roles, downregulates inflammatory cytokine secretion as well as decreases the effector function and proliferation of T and natural killer (NK) cells (online supplemental figure 1). Several tumors display transformed purine metabolism thereby facilitating the production of ADO and reducing its degradation. Oxygen deprivation reduces the availability of energy source and promotes the accumulation of extracellular ATP. Moreover, hypoxia is a strong inducer of the ectonucleotidases CD39 and CD73. The released ATP suffers the consecutive action of these ectonucleotidases resulting in increased ADO concentration from nanomolar (nM) in physiological conditions to micromolar (μM) range in the TME.

Extracellular ADO binds to purinergic type-1 G-protein coupled receptors (GPCRs), accordingly named adenosine receptors (AR)—A1AR, A2A AR, A2B AR and A3AR. A2A AR is highly expressed in most immune cells, with evidences supporting that its activation in the TME suppresses antitumor immune responses, enhancement of regulatory T cells immune suppressive activity and inhibition of antigen presentation by dendritic cells. These reports set the foundation for inhibiting A2A AR in hypoxic tumors to improve antitumor immune responses. Consequently, A2A AR antagonism has emerged as a prototypical approach of small molecule immunotherapeutic, with recent encouraging clinical outcomes in treatment-refractory cancer, along the four A2 AR antagonists already in clinical trials.

While the A1AR, A2A AR and A3AR receptor subtypes bind ADO with high affinity, the A2B AR exhibits a low affinity footprint among different A2AR antagonists, a dual A2B AR/A2A AR antagonist and an A2A AR antagonist were evaluated for their immunomodulatory effect. Non-xanthinic scaffold exhibits improved pharmacokinetic properties and bring structural novelty to adenosine antagonists reported to date. Using healthy donor in vitro and patient-derived ex vivo models, we demonstrated that antagonizing A2B AR signaling significantly alleviated adenosine-mediated suppression across different lymphocyte subsets. Notably, marked differences in the outcomes of A2B AR antagonist drug screening were observed when comparing exogenous and endogenously produced ADO. These results show a comparative immunological footprint among different A2A AR antagonists, a dual A2B AR/A2A AR antagonist and an A2A AR antagonist in clinical trials, strongly suggesting that the A2B AR is a promising target in cancer immunotherapy.

MATERIALS AND METHODS

Chemistry

The synthesis of the previously described antagonists, analytical procedure and spectroscopic and analytical data for all the compounds is detailed in online supplemental information. For the synthesis of ISAM–R56A, a mixture of isopropyl 4-(furan-2-yl)-2-methyl-1,4-dihydro benzo-1,2a-pyrimidine[1,2a]pyrimidine-3-carboxylate (ISAM–M89A, 5 mmol), 2-fluorobenzyl bromide (3 mmol) and potassium carbonate (4 mmol) in 4 mL of DMF was orbital stirred in a coated Kimble vial at 80°C for 5 hours. After completion of the reaction, as indicated by TLC, the solvent was removed in vacuum and the obtained oily residue was purified by column chromatography on silica gel to obtain two regioisomers (ISAM–M89A and ISAM–M89B). For the synthesis of ISAM–M89A, a mixture of 2-amino-5-chlorobenzimidazole (7.5 mmol), 3-furancarboxaldehyde (5 mmol), isopropyl acetate (5 mmol) and ZnCl2 (0.5 mmol) in 2.5 mL of THF was orbital stirred in a coated Kimble vial at 80°C for 12 hours. After completion of the reaction, as indicated by TLC, the solvent was removed and the obtained oily residue was purified by column chromatography on silica gel, to obtain two regioisomers (ISAM–M89A and ISAM–M89B).

Binding affinity of adenosine receptor subtypes

The affinity and selectivity profiles of the ligands obtained was studied in vitro, radioligand binding assays, at the four
human specific ARs subtypes, using experimental protocols previously described. All ligands were prepared and tested as racemic mixtures. Human ARs expressed in transfected CHO (A1AR), HeLa (A2AR and A1AR) and HEK-293 (A2AR) cells were employed. The following radioligands were used for binding experiments: [3H]DPCPX for A1AR and A2AR, at 2 and 25 nM, respectively; [3H]ZM241385 at 3 nM for A1AR; and [3H]NECA at 30 nM for A2AR. Non-specific binding was determined in the presence of R-PIA 10 μM for A1AR, NECA 50 μM for A2AR, NECA 400 μM for A2AR and R-PIA 100 μM for A2AR. The biological data are expressed as K, (nM, n=3). K values were obtained by fitting the data with non-linear regression using Prism 5.0 software (GraphPad, San Diego, CA, USA). Results are the mean of three experiments, each performed in duplicate.

Blockade of hCD73 assays
The selected AR antagonists were tested at 1 μM and 10 μM. Assays (human CD73) were carried out in a white 384-Optiplate (Perkin Elmer 6007290). Test compounds and the standard (αβ-methylene adenosine, Sigma M3763), 0.5 μg/mL enzyme (Cayman RYD-5795-EN-010), 300 μM AMP (Sigma A2252) and 100 μM ATP (A2383) were added in a final volume of 25 μL/well, using 25 mM Tris-HCl, 5 mM MgCl2·6H2O, pH=7.4 as assay buffer. The reaction mixture was incubated at 37°C for 15 min, after incubation 25 μL of Cell Titer-Glo Luminescence cell viability (Promega G7571) was added and shaken during 2 min before incubation at RT for 10 min. Luminescence at 100 ms was measured in the Perkin Elmer Enspire multimode plate reader.

cAMP assays
Assays were performed in transfected A2AR using a cyclic AMP (cAMP) enzyme immunoassay kit (Amersham Biosciences) following previously described protocols. HEK-293 cells were seeded (10,000 cells/well) in 96-well culture plates and incubated at 37°C in an atmosphere with 5% CO2 in Eagle’s Medium Nutrient Mixture F-12 (EMEM F-12), containing 10% fetal calf serum (FCS) and 1% L-Glutamine. Cells were washed three times with 200 μL assay medium (EMEM-F12 and 25 mM HEPES pH=7.4) and pre-incubated with assay medium containing 30 μM rolipram and test compounds at 37°C for 15 min. Ten μM NECA was incubated for 15 min at 37°C (total incubation time 30 min). Reaction was stopped with lysis buffer supplied in the kit and the enzyme immunoassay was carried out for detection of intracellular cAMP at 450 nm in an Ultra Evolution detector (Tecan). The dose-response curve of NECA-elicited cAMP formation was used to determine the initial choice of A2AR antagonist concentration (online supplemental figure 2).

Computational modeling
A previously reported model of the A2AR in complex with the reference antagonist ISAM–140 was the starting point to investigate the binding mode of the antagonists here reported. Briefly, the inactive hA2AR conformation was generated by homology modeling based on a curated alignment with the A2AR, of known structure, followed by some refinement steps as previously described. The initial binding orientation of ISAM–140, initially obtained by automated docking with GOLD, was herein refined with a round of MD simulations, consisting of: (i) insertion of the A2AR-ISAM–140 complex on an atomistic model of the membrane, solvation, and a 5 ns MD equilibration protocol as implemented in the PyMolModSim module of the GPCR-ModSim webserver; (ii) a short MD equilibration of the binding site, consisting on a 25 Å radius solvated sphere with the software Q as detailed in online supplemental information. The remaining ligands were modeled in the equivalent stereoisomer and aligned with the ‘Flexible Ligand Superposition’ in Schrödinger to this pose of ISAM–140. Each complex was subject to the same MD equilibration of the binding site outlined for ISAM–140, with representative snapshots shown in figure 1C.
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Cell viability assay (Cell-Titer Glo)

From breast cancer single cell suspension, 3×10^3 cells were seeded and cultured in an ultra-low attachment 384-well plate (Corning) with DMEM-F12 media (Thermo Fisher Scientific) containing 20% FBS and 1% PS. Treatment with A2B AR antagonist at various concentrations are stated in the Results section. After 4 days, Cell-Titer Glo reagent (Promega) was used to measure cell viability in accordance to the manufacturer’s instruction. EnSpire Multilabel Reader (PerkinElmer) was used to read the luminescence.

Proliferation assay with exogenous adenosine

Freshly isolated PBMCs and NK cells were stained with 5μM FITC-conjugated carboxyfluorescein succinimidyl ester (CFSE, BioLegend) in phosphate-buffered saline (PBS) (Life Technologies) at room temperature for 5 min. CFSE stained cells were washed with flow cytometer buffer three times. CFSE stained cells were cultured in 96-well plate with X-VIVO20% and 1% PS for 3 and 6 days, respectively. Healthy PBMCs were incubated with Human T-activator CD3/CD28 beads (Thermo Fisher Scientific) and IL-2 (Novartis) at 100 IU/mL. Healthy donor NK cells were incubated with 1000 IU/mL IL-2 only. 2×10^5 PBMCs and 5×10^4 NK cells were counted for treatment with ADO (Sigma-Aldrich) and 12 μM of A2B AR antagonist at the same day.

Proliferation assay with CD73-expressing patient-derived sarcoma spheroid

A malignant peripheral nerve sheath tumor (MPNST) patient-derived sarcoma cell line was used to grow (PBS) (Life Technologies) at room temperature for 5 min. CSFE stained cells were washed with flow cytometer buffer three times. CSFE stained cells were cultured in 96-well plate with X-VIVO20% and 1% PS for 3 and 6 days, respectively. Healthy PBMCs were incubated with Human T-activator CD3/CD28 beads at 1:4 ratio (Thermo Fisher Scientific) and IL-2 (Novartis) at 100 IU/mL. Healthy donor NK cells were incubated with 1000 IU/mL IL-2 only. 2×10^5 PBMCs and 5×10^4 NK cells were counted for treatment with ADO (Sigma-Aldrich) and 12 μM of A2B AR antagonist at the same day.

Figure 1  Pyrimidine derivatives as potent and highly selective A2BAR antagonists. (A) Synthesis of the novel A2B AR antagonist (ISAM–R56A) and the dual A2AAR/A2B AR antagonist (ISAM–M89A) and structure of four A2B AR antagonists previously published and the A2A AR clinical candidate (AZD–4635) employed in the study.17 26–28 (B) Adenosine receptors binding data, A2B AR functional data and hCD73 inhibitory data of the A2B AR antagonists employed in the study. aDisplacement of specific [3H]DPCPX binding in human CHO cells expressed as Kᵢ in nM (n=3). bDisplacement of specific [3H]4-(2-[7-amino-2-[2-furyl]1 2 4 triazolo[2,3-a] 5-triazin-5-ylamino)ethyl)phenol binding in human HeLa cells expressed as Kᵢ in nM (n=3). cDisplacement of specific [3H]DPCPX binding in human HeLa cells expressed as Kᵢ in nM (n=3). dDisplacement of specific [3H]NECA binding in human HeLa cells expressed as Kᵢ in nM (n=3). eInhibition of hCD73 in presence of α,β-methylene adenosine, ATP and AMP (expressed as Kᵢ in nM). fCAMP production in HEK-293 cells in presence of NECA and test compound at a concentration of 10μM expressed as Kᵢ in nM (n=3) (concentration-response curves shown in online supplemental figure 1). (C) Binding mode of the three most potent compounds disclosed in this study. All three compounds interact with N254 and F173, both of which are crucial in AR ligand recognition. Up: ISAM–140 (light blue), center: ISAM–R56A (purple) only forms one hydrogen bond with N254, but additionally explores a pocket between TM6 and TM7, down: ISAM–M89A (orange), where the Cl atom protrudes deep in the binding pocket.
spheroid by seeding 1×10⁴ cells per well in a 96-well ultra-low attachment plate (Thermo Fisher Scientific) with DMEM-F12 media containing 20% FBS and 1% PS for 5 days. 1×10⁴ CSFE-labeled healthy PBMCs, ratio of 10:1, were added to the CD73 expressing, adenosine-producing spheroid at day five. Similar to the proliferation assay with exogenous ADO, CD3/CD28 beads at 1:4 ratio and IL-2 at 100IU/mL were added. A₂B AR antagonism treatment at 12 μM was added at the same day.

**Spheroid infiltration by autologous TILs**

Spheroids were prepared using patient-derived sarcoma cell lines. 1×10³ cells were seeded per well in 96-well ultra-low attachment plate with DMEM-F12 media containing 20% FBS and 1% PS for 5 days. 3×10⁴ CSFE-labeled autologous expanded TILs, at Effector:target ratio of 3:1, were added to the spheroid at day five with A₂B AR antagonism treatment at 12 μM. After 3 days, spheroid was removed and split into two groups— IN and OUT. IN indicates TILs infiltrated into the sphere, while OUT indicates TILs that did not infiltrate into the sphere. Spheroids were washed with PBS at least two times. GentleMACS tumor dissociation kit (Miltenyi Biotec) was used to digest the spheroids for FC analysis.

**Real-time imaging**

Brightfield and phase contrast images under 4X objective were acquired every 6 hours on IncuCyte S3 system (Essen BioScience). For breast cancer patient-derived spheroid culture, 1×10⁴ cells per well were seeded with A₂B AR antagonist at 12 μM treatment for 6 days real time imaging. For spheroid infiltration, after the addition of CSFE-labeled autologous expanded TILs and treatment with A₂B AR antagonist at 12 μM, green fluorescence images were acquired for 3 days. All spheroid invasion analysis was performed using top hat segmentation with IncuCyte software.

**Extracellular ADO uptake assay via pAMPK staining**

Experimental setup was adopted from a previous study whereby AR antagonist was added to pretreat cells before exogenous adenosine treatment for intracellular and cytokine staining.²⁷ In brief, PBMCs were treated with 12 μM of A₂B AR antagonists for 90 min before the addition of 50 μM of adenosine for 2 hours. Cells were harvested after 3 hours of CD3/CD28 bead stimulation before intracellular staining with phospho-AMPK (Thr183, Thr172) rabbit primary antibody (Thermo Fisher Scientific) and antirabbit secondary antibody (BD biosciences).

**IFNγ and perforin cytokine production assay**

Similar experimental setup was performed as the extracellular ADO uptake assay. Cells were harvested after 2 days of CD3/CD28 bead stimulation before treatment with PMA/ionomycin (Sigma Aldrich) and golgi-inhibitors (BD biosciences). After 3 hours of subsequent incubation, intracellular staining was performed for IFNγ and perforin (online supplemental table 1).

**Flow cytometry analysis**

Cell surface was stained with mouse monoclonal anti-human antibodies against CD3, CD4, CD8, CD45RA, CD56 and CD19 listed in online supplemental table 1. Cell surface antibodies and live/dead (L/D) marker were incubated with samples at 4°C for 20 min after washing two times with flow cytometry buffer containing 5% FBS in PBS. For spheroid infiltrated TIL phenotyping, digested IN and OUT spheroids were stained with cell surface marker and analyzed on NovoCyte (ACEA Biosciences) with the use of FlowJo software (Tree Star) by gating single cell based on forward and side scatters. A representative gating strategy for CD8 naïve T cells is shown in online supplemental figure 3A. Compensated flow cytometry standard (FCS) files with only live cells were concatenated for downstream tSNE analysis using the ‘cytofkit’ R package (https://github.com/JinmiaoChenLab/cytokit).

**Statistical analysis**

Experimental replicates are presented as mean±SD and median in box plot stated in the figure legend of the result section. Statistical analysis was performed using Prism 8 (GraphPad Software) and stated in figure legends.

**RESULTS**

**Synthesis and pharmacological characterization of potent A₂B AR antagonists**

The pyrimidine derivatives studied here were obtained using a modified procedure of the reliable Biginelli reaction,³⁸ consisting of the catalyzed condensation of an aldehyde, a β-keto-ester and a 1,3-dinucleophile. The synthesis and binding data of SY1AF–30,²⁹ SY1AF–80,²⁹ SY1KO–24³⁸ and ISAM–140⁴⁰ (figure 1A) were recently described by our group in the context of a program to develop novel A₂B AR antagonists. In addition, we present two previously undisclosed derivatives (ISAM–R56A and ISAM–R89A, figure 1A). These compounds are structurally related to ISAM–140 and they were discovered in the context of the structure activity relationship (SAR) exploration and structural diversification of the tricyclic scaffold present in this prototypical A₂B AR antagonist. Besides providing distinctive and not-self-evident structural novelties, this pair of compounds offer interesting pharmacological data from quantitative (Kᵢ in the low nM range) and qualitative (dual profile) points of view.

The synthetic pathway employed to prepare the novel A₂B AR ligands is shown in figure 1A, where it can be observed that both transformations exploit the tautomericism present in the precursors. Briefly, treatment of ISAM–140 with 2-fluorobenzyl bromide under basic conditions produced a mixture of regioisomers (ISAM–R56A and ISAM–R56B) that was separated using column chromatography. The Biginelli-inspired ZnCl₂-catalyzed condensation of 2-amino-5-chlorobenzimidazole, 3-furanecarboxaldehyde and isopropyl acetoacetate gave a (1:1) mixture of two tricyclic regioisomers (ISAM–M89A and M89B) that was subsequently converted into the tautomeric forms ISAM–R56A and ISAM–R89A, respectively.
and ISAM–M89B) that differ in the position (7/8) of the halogen atom. The unequivocal assignment for each regioisomer was determined by NMR techniques.

The binding data obtained from the five A2B AR antagonists, the dual A2B AR/A2A AR ligand and the A2A AR clinical candidate (AZD-4635) are depicted in figure 1B. The previously reported A2B AR antagonists (SY1AF–30, SY1AF–80, SY1KO–24, ISAM–140) exhibit affinity values in the low nanomolar range (K_i 3.50–24.3 nM) and excellent subtype selectivity. Moreover, the diverse substitution pattern at the central pyrimidine scaffold provides chemical entities with different topologies, physicochemical features and distinctive binding modes, lately affecting its pharmacodynamic and pharmacokinetic profiles. ISAM–M89A and ISAM–R56A are novel non-xanthinic A2B AR ligands discovered during the detailed exploration of the SAR around the tricyclic scaffold of ISAM–140 by halogen introduction and N-alkylation, respectively.

The selected A2B AR and A2B AR/A2A AR ligands were tested in cAMP assays (figure 1B) to evaluate their ability to inhibit NECA-stimulated (100 nM) cAMP production. These experiments demonstrated that all of them inhibited cAMP accumulation, thus validating its A2B AR antagonistic behavior (figure 1B). A comparison of their K_i and K_B values revealed complete agreement (data within 1–7-fold) between the binding and functional assays. Ectonucleotidases, in particular CD73, play a key role in the context of adenosine-mediated tumor immune escape. Consequently, we evaluated the effect of A2B AR ligands on the blockade of hCD73 as part of the pharmacological characterization of the selected A2B AR ligands (figure 1B). These experiments revealed that none of the five A2B AR antagonists, nor the A2A /A2L dual antagonist, showed any noticeable inhibitory effect on hCD73 (1 μM or 10 μM). These data excluded a dual A2B AR-CD73 inhibition, allowing us to attribute the pharmacological effects described below to the specific A2B AR antagonistic effect (or the dual A2B AR antagonism in the case of ISAM–M89A) of these ligands.

**Binding mode of A2B AR antagonists**

The binding mode of the three most potent compounds (ISAM–140, ISAM–R56A and ISAM–M89A) was investigated using a previously reported A2B AR homology model in complex with ISAM–140. Such a model could successfully explain the stereospecific recognition of trifluorinated derivatives (figure 1C), and the new antagonists ISAM–R56A and ISAM–M89A could indeed adopt an analogous binding mode within the orthosteric A2B AR cavity (figure 1C). In all three cases, the tricyclic core is stabilized by interaction with the AR conserved residues N254, F173, a common denominator for AR ligands. Ligand ISAM–M89A specifically maintained a double hydrogen bond with N254, allowing the chlorine atom in position eight to bind deeper in the binding pocket, surrounded by H251, N186, and Q990. The N1 substituted ISAM–R56A, which could only retain one hydrogen bond with N254, presented, on the other hand, the extensive interactions of the 2-fluorobenzyl group in a A2B AR specific hydrophobic pocket located between TM6 and TM7, formed by residues V253(I), V250(L), A271(L), M272, A275, I276 and L278 (parenthesis indicating the corresponding A2A AR residues, if different). These specific interactions might explain the high affinity and the retained high selective profile for the A2B AR.

**Blockade of A2B AR reduces patient-derived breast cancer spheroid growth**

The expression of A2B AR gene (ADORA2B) was investigated using The Cancer Genome Atlas (TCGA)—Pan cancer publicly available dataset across 33 tumor types (online supplemental figure 4A–D). With high ADORA2B gene expression in breast cancer, the blockade of the adenosinergic pathway has been widely studied in breast cancer. To investigate if our novel A2B AR antagonists would affect breast cancer growth, we used a drug screening platform based on breast tumor resections. It is composed of majority tumor cells at 73.2%, with 11.2% immune cells based on flow cytometry analysis of EpCAM and CD45 expression, respectively (online supplemental figure 4E,F). On exposure to ISAM–140, ISAM–R56A and ISAM–M89A for 4 days, the relative cell viability of these breast cancer spheroids was significantly reduced with a more pronounced effect by ISAM–M89A (figure 2A). To confirm this, antagonist effects of the cell growth, the kinetics of the observed cytotoxic effect mediated by the antagonists was studied using real-time imaging within the same spheroid culture setting (figure 2B). With the low and middle antagonist concentrations being statistically equal in cell viability, the latter concentration of ISAM–140, ISAM–R56A and ISAM–M89A significantly affected the spheroid growth over time compared with untreated control (figure 2C). Despite the low CD3 T cell frequency in these tumor resections (online supplemental figure 4F), blockade of A2B AR still reduced cell viability and spheroid growth compared with untreated spheroids.

**Blockade of A2B AR rescues adenosine-mediated suppression of T and NK cell proliferation**

To elucidate if the antitumor effects observed by the A2B AR antagonists could alter lymphocyte activity, the different antagonists were added directly to T and NK cells in the presence of exogenous adenosine. While no statistical difference in viability at different adenosine concentrations was observed, a trend of decreasing viability with increasing adenosine concentration among CD8 T cell subsets was observed. Conversely, the viability of CD56 positive NK cells was not affected by adenosine (online supplemental figure 3B). However, a dose-dependent adenosine-mediated suppression of cell proliferation was observed across the different subtypes of T and NK cells with naïve CD8 T cells being the most affected (online supplemental figure 3C,D).
Due to its suppressive effect on cell proliferation without compromising viability, adenosine concentration at 0.1 mM was used to investigate the ability of the different A2B AR antagonists in rescuing adenosine-mediated suppression of lymphocyte proliferation. A subapoptotic concentration of 12 μM A2B AR antagonists was added to proliferating lymphocyte cultures (data not shown). ISAM–140 rescued the proliferation of CD45RA+ (naïve and effector) CD8 T cells, CD45RA- CD4 T cells (central and effector memory) and CD45RA+ CD4 T cells, and NK cells (figure 3). While ISAM–R56A did not restore the proliferation of T cell subsets, NK cell proliferation was significantly rescued. None of the other A2B AR antagonists (SY1AF–30, SY1AF–80 and SY1KO–24) had any measurable impact on the rescue of lymphocyte proliferation (figure 3). Similarly, the frequency of cells expressing A2A AR and A2B AR ranging between 10% and 20% did not differ between lymphocyte populations and was highly donor dependent (online supplemental figure 5E,F).

A2BAR antagonists inhibit extracellular ADO by downregulating pAMPK in T cells with upregulation of expression of CD69, IFNy, and perforin

To demonstrate the ability of A2B AR antagonists to inhibit extracellular ADO uptake in lymphocytes, one of the downstream cellular ADO signaling molecules—phosphorylated-AMP activated protein kinase (pAMPK) was analyzed in total CD4 and CD8 T cells (figure 4A,B). ISAM-R56A significantly downregulated pAMPK among both CD4 and CD8 T cells in the presence of exogenous ADO.
Figure 3  Rescue of lymphocyte proliferation by A₂B_AR antagonism. Relative proliferation of: (A) CD45RA− CD4 T cells, (B) CD45RA+ CD4 T cells, (C) CD45RA− CD8 T cells, (D) CD45RA+ CD8 T cells, and (E) NK cells, after incubation with exogenous adenosine 0.1 mM and A₂B_AR antagonist 12 µM for 3 (A–D) and 6 (E) days. (n=10 healthy donors) CD45RA expression differentiates naïve and effector T cells from central and effector memory T cells. Box plots with minimum, first quartile, median, third quartile, and maximum are presented. Unpaired and non-parametric statistical analysis—Mann-Whitney test was performed against untreated control with *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. (F–J) Representative CFSE histogram on the corresponding lymphocyte subset. CFSE, carboxyfluorescein succinimidyl ester.
Similarly, SY1AF-80 and ISAM-M89A significantly inhibited ADO uptake in CD4 and CD8 T cells, respectively.

With the rescue of adenosine-mediated suppression of lymphocyte proliferation, effector function through proinflammatory cytokines—IFNy and perforin, and the early activation marker—CD69 were successively examined. IFNy production by CD4 and CD8 T cells was successfully rescued by several antagonists (figure 4C,D). Similarly, perforin production by cytotoxic CD8 T cells was significantly upregulated on A2aAR antagonists (figure 4E), of which, SY1AF-30 and ISAM-R56A significantly improved both IFNy and perforin production on
adenosine suppression. CD69 expression, associated with early lymphocyte activation, was also increased (figure 4F,G) whereby ISAM-M89A had positive effect on both CD4 and CD8 T cells. Finally, both ISAM-R56A and ISAM-M89A rescued killing of breast cancer TILs against autologous tumor cells while ISAM-R56A also rescued killing in sarcoma TILs (online supplemental figure 6).

**A2AR antagonists rescue CD8 naïve T cell proliferation in adenosine-producing tumor spheroids**

Sarcomas had the highest alteration frequencies for ADORA2B gene amplification as well as highest median copy number at DNA level when compared against 33 TCGA tumor types (online supplemental figure 4C,D). To investigate the effect of A2AR antagonism on lymphocyte proliferation in a more physiologically relevant model, a sarcoma spheroid model that produce endogenous adenosine to better mimic the TME instead of using exogenous adenosine was developed. The relative production of adenosine was validated in spheroids including patient-derived tumors and the commercial U2OS cell line (online supplemental figure 7A). The expression of the ectonucleotidases CD39 and CD73 was highest in primary tumor spheroids from undifferentiated pleomorphic sarcoma, myxofibrosarcoma, and malignant peripheral nerve sheath tumors (MPNSTs, online supplemental figure 7B). Based on these results, lymphocyte proliferation was analyzed in MPNST-derived sarcoma spheroids. In contrast to the rescue of lymphocyte proliferation observed with exogenous adenosine, the A2AR antagonists SY1AF–30, SY1AF–80 and ISAM–R56A significantly rescued naïve and effector CD8 T cell proliferation based on CD45RA expression (figure 5).

**A2AR antagonism improves TIL infiltration into autologous patient-derived sarcoma spheroids**

To increase the translational impact of the immunomodulatory effects of the A2AR antagonists, their ability in rescuing adenosine-mediated suppression was tested in patient-derived ex vivo expanded tumor-infiltrating lymphocytes (TILs) and autologous sarcoma spheroids. These expanded TILs were mainly composed of CD4 and CD8 effector memory cells (online supplemental figure 7C–F). Real-time imaging showed the blockade of A2AR, especially by ISAM-R56A, resulted in an overall improved TIL infiltration in comparison with untreated tumor spheroids (figure 6A,B). Although not statistically significant, both ISAM–R89A and AZD-4635 treatments resulted in a median increased infiltration of 7% and 20%, respectively, whereas ISAM–140 did not have any effect on TIL infiltration (figure 6B). To confirm the frequency of TIL infiltration, the spheroids were collected and analyzed for CSFE positive and CD73 negative TILs by flow cytometry. Despite a high variability, ISAM–R56A treatment showed significantly enhanced TIL infiltration into the spheroids (figure 6C).

With the recruitment and retention of T cells within the tumor, expression of adhesion molecules is required. Thus, the expression of tissue resident T cells markers CD11a, CD49a, CD69 and CD103, was analyzed to gain insights into the phenotype of spheroid-infiltrated T cells. T-distributed stochastic neighboring embedding (tSNE) analysis revealed that the majority of infiltrating TILs are CD8 positive (cluster 1) and CD4 positive (cluster 9) T cells (figure 6D). Among them, the infiltrated CD8 T cells were enriched with 2.5-fold higher CD49a and CD103 coexpression, while no significant difference was observed, the infiltrated CD4 T cells showed an enrichment of CD69 (figure 6E–H). Furthermore, the total CD3 T cells were 20% greater inside the spheroid. To investigate the effect of A2AR antagonism in modulating the phenotype of infiltrated TILs, various antagonists were added to the TIL-tumor spheroids. All antagonists increased the CD8/CD4 T cell ratio though it was statistically significant only in the presence of ISAM–140 (figure 6I). In contrast, no significant change in the expression of the adhesion molecules CD11a, CD69, and CD49a/CD103 between untreated and A2AR antagonist-treated spheroid cultures was observed (figure 6J–L, online supplemental figure 8).

**DISCUSSION**

While blockade of A2AR is well documented to rescue T and NK cell proliferation, the effect of targeting the A2AR in lymphocytes remains almost unexplored.\(^8\)\(^\text{13}\)\(^\text{16}\) Only recently, it was reported that A2AR deficiency in tumor-bearing mice resulted in an increased infiltration of dendritic cells to promote cross-priming of adoptively transferred tumor antigen-specific T cells.\(^21\) A2AR activation plays critical roles during tumor development including but not limited to proliferation, angiogenesis, invasion and metastasis, as well as immune suppression, which make the antagonists of this receptor hold great promise for the development of new polyvalent cancer therapeutics.\(^1\)\(^6\) To investigate lymphocyte activity on blockade of A2AR, a set of five potent and selective A2AR antagonists and a dual A2AR/A2AR antagonist were synthesized and evaluated. Herein, documented ligands were conceived in the context of a hit to lead program based on the 1,4-dihydrobenzo\(^5\) imidazo[1,2-a] pyrimidine scaffold. The obtained data highlight the potential of this scaffold to provide potent AR antagonists while illustrating how subtle structural modifications can strongly affect the affinity and selectivity profile of the novel ligands. The main SAR and selectivity trends identified within the series were substantiated by a molecular modeling study based on a receptor-driven docking model of A2AR constructed based on the crystal structure specific of the human A2AR.

Given the short half-life of endogenous adenosine,\(^47\) exogenous adenosine is still commonly used to study adenosine-mediated immunosuppression in the TME. In general, lymphocyte assays are based on phenotypic analysis and often complemented with read-outs to analyze canonical functions such as cell viability, proliferation and the ability to produce inflammatory cytokines as well as...
Figure 5  Rescue of lymphocyte proliferation in sarcoma spheroids by A2B AR antagonism. Relative proliferation of: (A) CD45RA-CD4 T cells, (B) CD45RA+CD4 T cells, (C) CD45RA- CD8 T cells and (D) CD45RA+ CD8 T cells after antagonist 12 µM treatment for 3 days in MPNST patient-derived sarcoma spheroid. (n=5 healthy donors) Box plots with minimum, first quartile, median, third quartile, and maximum are presented. Unpaired and non-parametric statistical analysis—Mann-Whitney test was performed against untreated control with *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. (E) Representative CSFE dilution of CD45RA+ CD8 T cells. CD45RA expression differentiates naïve and effector T cells from central and effector memory T cells. MPNST, malignant peripheral nerve sheath tumors; UPS, undifferentiated pleomorphic sarcoma.

as to recognize and kill target cells. Throughout these experiments, the A2B AR antagonists were evaluated in lymphocyte viability and proliferation assays. Since prognosis is often associated with the frequency of TILs,51–53 we developed two models to study the blockage of A2B AR in recusing antitumor immune responses. An exogenous adenosine in vitro healthy donor model and endogenously produced adenosine ex vivo patient-derived model were used to better study the dynamic crosstalk of adenosine in the TME as well as being clinically relevance. As a result, A2B AR antagonists successfully rescued antitumor immune response through cytotoxicity of patient-derived
spheroid cultures, proliferation of lymphocytes and tumor spheroid immune cell infiltration accompanied with differences in phenotype.

The six ligands examined exhibited high A2B AR affinity and excellent selectivity profiles. Two of them (ISAM–R56A and ISAM–M89A) were herein originally reported, by introduction of a 2-fluorobenzyl group or a chlorine atom at positions 1 and 8 of the tricyclic core, respectively. Indeed, ISAM–R56A (Ki=1.50 nM) is confirmed as one of the most potent A2B AR antagonist published to date, while ISAM–M89A exhibited a highly promising dual A2B AR/A2A AR antagonistic profile (while devoid of affinity for the remaining AR subtypes). The selected ligands revealed negligible CD73 inhibitory action, thus allowing to attribute the herein observed effects to their specific A2B AR antagonistic effect. The high affinity of these compounds could be explained by a computational model of the A2B AR in complex with these ligand chemotype, which had been used in the design of these series of antagonists.

By exposure of these A2B AR antagonists to breast cancer patient-derived cells, their antitumor activity was first revealed through a reduced tumor spheroids growth rate. Despite the highly variable donor-dependent expression of A2ARs, the immunomodulatory effect of A2B AR antagonism was further demonstrated with the successful rescue of T and NK cell proliferation under exogenous adenosine-mediated suppression, with CD8 naïve T cell being the most responsive of the T cell types examined. These findings correspond to an adenosine-producing human melanoma cell line, showing higher suppression of CD8 T cell proliferation than CD4 T cells.54 A2B AR antagonists were subsequently shown to inhibit extracellular ADO uptake via downstream pAMPK in total CD4 and CD8 T cells. Even with varying adenosine

Figure 6 Infiltration of TILs into autologous patient-derived sarcoma spheroids after A2B AR antagonist treatment. (A) Representative real-time imaging of spheroid TIL infiltration over 3 days under 4× objective. Green areas demarcate CFSE-labeled TILs infiltrated into the red boundary tumor spheroid. (B) Relative TIL infiltration into autologous spheroids (n=8 donors) measured by flow cytometry. Antagonist 12 µM was added with CSFE-labeled TILs after spheroid formation on day five. (C) Representative flow cytometry plot of CFSE positive TILs in CD73+ tumor spheroid. (D) tSNE analysis and (E) annotated pie chart of TILs within (IN) and outside of (OUT) the spheroids without treatment (n=8 donors, indicated within the pie chart). Wilcoxon Rank Sum test was performed to compare the various IN vs OUT adhesion markers. Expression of (F) CD3 T cells, (G) CD49a+ CD103+ among CD8 T cells and (H) CD69 MFI among CD4 T cells IN and OUT of the spheroids without treatment (n=8 donors). (J) Relative CD8/CD4 TIL ratio IN the spheroids. Differential expression of relative (J–L). CD49a+ CD103+ cells IN the spheroids (n=8 donors) of CD3, CD8, and CD4 TILs. Antagonist 12 µM was added for J–L. Outlier is presented as diamond shape and defined by Tukey’s rule, which is not included in statistical analysis. Box plots with minimum, first quartile, median, third quartile, and maximum are presented. Unpaired and non-parametric statistical analysis—Mann-Whitney test was performed against untreated control with *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. CFSE, carboxyfluorescein succinimidyl ester; TIL, tumor infiltrating lymphocyte; tSNE, T-distributed stochastic neighboring embedding.
susceptibility between donors, improved production of proinflammatory cytokines IFNγ and perforin along with the expression of the activation marker CD69 was observed. In addition, the obtained discrepancies could be related with drug availability, binding kinetics and physicochemical properties, influencing a different cell response for drugs with similar affinity.

Patient outcome in various sarcoma types is shown to correlate with the presence of TILs. Patient-derived spheroids can retain the unique characteristic of the original tumor compared with 2D monolucr or patient-derived xenograft by enabling cell-cell and cell-extracellular interactions. Using a patient-derived sarcoma spheroids model, we observed that treatment with A2B AR antagonists improved autologous TIL infiltration into sarcoma spheroids. Tumor spheroid-infiltrating lymphocytes were enriched for the expression of the tissue resident markers CD49a and CD103 in CD8 T cells and CD69 in CD4 T cells. A recent study identified a unique population of CD8 TILs coexpressing CD39 and CD105 that were reactive against both primary and metastatic tumors. Another study defined CD8 tissue resident T cells in human epithelia with cytotoxic function to express CD49a and correlate with inflammatory skin diseases. In vivo A2AR antagonism has also been reported to upregulate the expression of CD69 on TILs, while A2B AR antagonism enhanced CXCR3-dependent TIL responses. Notably, a recent study demonstrated that adenosine can mediate functional and metabolic suppression of tumor-infiltrating CD8+ T cells. Thereby, A2B AR antagonism not only potentially increase TIL infiltration into solid tumors through modulation of adhesion molecules but may also improve the overall metabolic fitness of tumor-infiltrating T cells.

ISAM–140, a potent and highly selective A2B AR antagonist (K_{i}=3.49 nM), was shown as an optimal A2B AR binder by computational modeling and proved to be an efficient antagonist in functional cAMP assay. We here demonstrate that this compound exerts biologically improved immune cell proliferation on T and NK cells in an A2B AR expression independent manner in healthy donor PBMCs. This response was dose-dependent with a higher concentration further restoring proliferation (data not shown). This lymphocyte proliferation rescue was accompanied with upregulated IFNγ production in both CD4 and CD8 T cells. ISAM–140 was also nontoxic and even improved cell viability (data not shown). In addition, a novel A2B AR antagonist—ISAM–R56A, with K_{i} to the A2B AR similar to the corresponding A2B AR value of the preclinical A3 AR antagonist—AZD–4635 (1.50 nM vs 1.70 nM, respectively), improved TILs infiltration into autologous patient-derived sarcoma spheroids. CD8-naïve T-cell proliferation response was also observed in the adenosine-producing spheroid model, while a specific NK cell proliferation response was noted in the exogenous adenosine assay. CD8 T cell IFNγ and perforin production was also significantly rescued under exogenous adenosine suppression. Thereby, the potential of ISAM–R56A to induce tumorous TILs infiltration is highly promising. This induction of tumorous CD8 T cell infiltration was already observed from the clinical trial of a dual A3 AR/A2B AR antagonist—AB928 combined treatment with anti-PD1.

CONCLUSION
With only one A2B AR antagonist currently registered in a clinical trial, our findings close the gap of lacking alternatives and provide insights to practical readouts related to adenosine-mediated immune suppression. We established a systematic workflow to screen novel small molecule antagonists that enabled the identification of ISAM–R56A as the most potent candidate for A2B AR blockade. With ISAM–R56A, cytotoxic immune cells can be relieved from adenosine-mediated suppression to proliferate and infiltrate into adenosine-producing solid tumors expressing CD73. Besides using appropriate immunocompetent or xenograft in vivo models to evaluate the pharmacology and preclinical safety of these novel small molecule antagonists as potential complements to existing immunotherapies, future directions on downstream hypoxia-HIF-1α of A2B AR-cAMP signaling axis shall be mimicked in vitro and ex vivo assays.
cells are under influence of the adenosine-2A adenosine receptor pathway. Front Immunol 2012;3:1–12.
15 Panther E, Coriati S, Idzko M, et al. Adenosine affects expression of membrane molecules, cytokine and chemokine release, and the T-cell stimulatory capacity of human dendritic cells. Blood 2003;101:3985–90.
16 Sitkovsky MV. Lessons from the A2A adenosine receptor antagonist–enabled tumor regression and survival in patients with treatment-refractory renal cell cancer. Cancer Discov 2020;10:16–19.
17 Borodovsky A, Barbon CM, Wang Y, et al. Small molecule AZD4635 inhibitor of A2A R signaling rescues immune cell function including CD103+ dendritic cells enhancing anti-tumor immunity. J Immunother Cancer 2020;8:1–17.
18 Congreve M, Brown GA, Borodovsky A, et al. Targeting adenosine A2B receptor antagonism for treatment of cancer. Expert Opin Drug Discov 2018;13:997–1003.
19 Hinz S, Navarro G, Borroto-Escuela D, et al. Adenosine A2A receptor ligand recognition and signaling is blocked by A2A receptors. Oncotarget 2018;9:24423 doi:10.18632/oncotarget.24423
20 Maj T, Wang W, Crespo J, et al. Oxidative stress controls regulatory T cell apoptosis and suppressor activity and PD-L1-blockade resistance in tumor. Nat Immunol 2017;18:1332.
21 Chen S, Akdemir I, Fan J, et al. The expression of adenosine A2B receptor on antigen-presenting cells suppresses CD8+ T-cell responses and promotes tumor growth. Cancer Immunol Res 2020;8:canim.0833.2019.
22 Corona SP, Sobhani N, Generali D. Adenosine A2B receptor: novel anti-cancer therapeutic implications. J Cancer Metastasis Treat 2017;3:206.
23 Antonioli L, Blandizzi C, Pacher P, et al. Immune, inflammation and cancer: a leading role for adenosine. Nat Rev Cancer 2013;13:842–57.
24 Leone RD, Emens LA. Targeting adenosine for cancer immunotherapy. J Immunother Cancer 2018;6:1–9.
25 Moriyama K, Sitkovsky MV. Adenosine A2A receptor is involved in cell surface expression of A2B receptor. J Biol Chem 2010;285:39271–88.
26 Mallo-Abreu A, Prieto-Díaz R, Jespers W, et al. Nitrogen-Walk approach to explore biosimilar replacements in a series of potent G-protein coupled receptor antagonists. J Med Chem 2020;63:7721–39.
27 Jiang J, Seel CJ, Temirak A, et al. A3a adenosine receptor antagonists with picomolar potency. J Med Chem 2019;62:4032–55.
28 Carabajales C, Azuaje J, Oliveira A, et al. Enantiospecific Recognition at the A3a Adenosine Receptor by Alkyl 2-Cyanoimino-4-substituted-6-methyl-1,2,3,4-tetrahydropyrimidine-5-carboxylates. J Med Chem 2017;60:3372–82.
29 Crespo A, El Maatougui A, Biagini P. Discovery of 3,4-diarylpyrimidine-2(1H)-ones as a novel class of potent and selective A2B2 adenosine receptor antagonists. ACS Med Chem Lett 2013;4:103–6.
30 El Maatougui A, Azuaje J, González-Gómez M, et al. Discovery of potent and highly selective A2B2 adenosine receptor antagonist chemotypes. J Med Chem 2016;59:1967–83.
31 Mallo-Abreu A, Majellaro M, Jespers W, et al. Trifluorinated pyrimidine-based A3a antagonists: optimization and evidence of stereospecific recognition. J Med Chem 2019;62:9315–30.
32 Jakola V-P, Griffith MT, Hanson MA, et al. The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. Science 2008;322:1211–7.
33 Esquerra M, Siretskiy A, Bello X, et al. GPCR-ModSim: a comprehensive web based solution for modeling G-protein coupled receptors. Nucleic Acids Res 2016;44:W455–62.
34 Bauer P, Barrozo A, Purg M, et al. Q6: a comprehensive toolkit for empirical valence bond and related free energy calculations. SoftwareX 2016;7:389–95.
35 Schrödinger, Schrödinger, LLC New York, 2009.
36 Yang Y, Neo SY, Chen Z, et al. Thioredoxin activity confers resistance against oxidative stress in tumor-infiltrating NK cells. J Clin Invest 2012;130:5508–22.
37 Mastic-Gavillet B, Navarro Rodrigo D, Décombaz L, et al. Adenosine mediates functional and metabolic suppression of peripheral and tumor-infiltrating CD8+ T cells. J Immunother Cancer 2019;7:1–16.
38 Konje CO. Biochemical active dihydropyrimidines of the Bigenillic-type—a literature survey. Eur J Med Chem 2000;35:1043–52.
39 Jespers W, Schiedel AC, Heitman LH, et al. Structural mapping of adenosine receptor mutations: ligand binding and signaling mechanisms. Trends Pharmacol Sci 2018;39:75–89.
40 Gill D, Alcazar CR, Jinni SJ, Ekram MB, et al. Immune escape in breast cancer during In Situ to invasive carcinoma transition. Cancer Discov 2017;7:1098–115.
41 Gourdin N, Bossee M, Rodriguez C, et al. Autocrine adenosine regulates tumor polyfunctional CD73’CD4’ effector T cells devoid of immune checkpoints. Cancer Res 2018;78:3604–18.
42 Loi S, Pommey S, Haibe-Kains B, et al. CD73 promotes anthracycline resistance and poor prognosis in triple negative breast cancer. Proc Natl Acad Sci U S A 2013;110:11091–8.
43 Mittal D, Sinha D, Barkauskas D, et al. Adenosine 2B receptor expression on cancer cells promotes metastasis. Cancer Res 2016;76:4372–82.
44 Stagg J, Divisekera U, McLaughlin N, et al. Anti-CD73 antibody therapy inhibits human breast tumor growth and metastasis. Proc Natl Acad Sci U S A 2010;107:1547–52.
45 Terp MG, Olesen KA, Arnspsang EC, et al. Anti-human CD73 monoclonal antibody inhibits metastasis formation in human breast cancer by inducing clustering and internalization of CD73 expressed on the surface of cancer cells. J Immunol 2013;191:4165–73.
46 Turcotte M, Allard D, Mittal D, et al. CD73 promotes resistance to HER2/ErbB2 antibody therapy. Cancer Res 2017;77:5652–63.
47 Zhi X, Chen S, Zhou P, et al. RNA interference of ecto-5’-nucleotidase (CD73) inhibits human breast cancer cell growth and invasion. Clin Exp Metastasis 2007;24:439–48.
48 Zhou X, Zhi X, Zhou P, et al. Effects of ecto-5’-nucleotidase on human breast cancer cell growth in vitro and in vivo. Oncol Rep 2007;17:1341–6.
49 Sepulveda C, Palomo I, Fuentes E. Role of adenosine A2B receptor overexpression in tumor progression. Life Sci 2016;166:32–9.
50 Sekhon BK, Roubin RH, Tan A, et al. High-throughput screening platform for anticancer therapeutic drug cytotoxicity. Assay Drug Dev Technol 2008;6:711–21.
51 Rusakiewicz S, Semeraro M, Sarabi M, et al. Immune infiltrates are prognostic factors in localized gastrointestinal stromal tumors. Cancer Res 2013;73:3499–510.
52 Fuji H, Arakawa A, Utsumi D, et al. CD8(+) tumor-infiltrating lymphocytes at primary sites as a possible prognostic factor of cutaneous angiosarcoma. Int J Cancer 2014;134:2393–402.
53 Balch CM, Riley LB, Bae YJ, et al. Patterns of human tumor-infiltrating lymphocytes in 120 human cancers. Arch Surg 1990;125:200–5.
54 Morandi F, Morandi B, Horenstein AL, et al. A non-canonical adenosinergic pathway led by CD38 in human melanoma cells induces suppression of T cell proliferation. Oncotarget 2015;6:25602–18.
55 Rusakiewicz S, Semeraro M, Sarabi M, et al. Immune infiltrates are prognostic factors in localized gastrointestinal stromal tumors. Cancer Res 2013;73:3499–510.
56 Bray LJ, Hutmacher DW, Bock N. Addressing patient specificity in the engineering of tumor models. Front Bioeng Biotechnol 2019;7:217.
57 Liao W, Wang J, Xu J, et al. High-throughput three-dimensional spheroid tumor model using a novel stamp-like tool. J Tissue Eng 2019;10:2041731419889184.
58 Amsen D, van Gisbergen KPJM, Hombrink P, et al. Tissue-resident memory T cells at the center of immunity to solid tumors. Nat Immunol 2018;19:538–46.
59 Duhem T, Duhem R, Montler R, et al. Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors. Nat Commun 2018;9:2724.
60 Cheuk S, Schlums H, Gallais Sérézal I, et al. CD49a Expression Defines Tissue-Resident CD8+ T Cells Poised for Cytotoxic Function in Human Skin. Immunity 2017;46:287–300.
61 Leone RD, Sun I-M, Oh M-H, et al. Inhibition of the adenosine A2A receptor modulates expression of T cell coinhibitory receptors and improves effector function for enhanced checkpoint blockade and act in murine cancer models. Cancer Immunol Immunother 2018;67:1271–84.
62 Young A, Ngiow SF, Madore J, et al. Targeting adenosine in BRAF-mutant melanoma reduces tumor growth and metastasis. Cancer Res 2017;77:4684–96.
63 Arndtler JD, de Souza PL, Gutierrez R, et al. AB928, a novel dual adenosine receptor antagonist, combined with chemotherapy or AB122 (anti-PD-1) in patients (PTS) with advanced tumors: preliminary results from ongoing phase I studies. JCO 2019;37:2604.
Correction: A2B adenosine receptor antagonists rescue lymphocyte activity in adenosine-producing patient-derived cancer models

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In this article the x-axis on figures 4B, D, F and G have been updated to include the correct values.

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