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A Novel Antagonist of the Immune Checkpoint Protein Adenosine A2a Receptor Restores Tumor-Infiltrating Lymphocyte Activity in the Context of the Tumor Microenvironment

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Abstract

BACKGROUND: Therapeutic strategies targeting immune checkpoint proteins have led to significant responses in patients with various tumor types. The success of these studies has led to the development of various antibodies/inhibitors for the different checkpoint proteins involved in immune evasion of the tumor. Adenosine present in high concentrations in the tumor microenvironment activates the immune checkpoint adenosine A2a receptor (A2aR), leading to the suppression of antitumor responses. Inhibition of this checkpoint has the potential to enhance antitumor T-cell responsiveness. *METHODS:* We developed a novel A2aR antagonist (PBF-509) and tested its antitumor response *in vitro*, in a mouse model, and in non-small cell lung cancer patient samples. *RESULTS:* Our studies showed that PBF-509 is highly specific to the A2aR as well as inhibitory of A2aR function in an *in vitro* model. In a mouse model, we found that lung metastasis was decreased after treatment with PBF-509 compared with its control. Furthermore, freshly resected tumor-infiltrating lymphocytes from lung cancer patients showed increased A2aR expression in CD4+ cells and variable expression in CD8+ cells. *Ex vivo* studies showed an increased responsiveness of human tumor-infiltrating lymphocytes when PBF-509 was combined with anti-PD-1 or anti-PD-L1. *CONCLUSIONS:* Our studies demonstrate that inhibition of the A2aR using the novel inhibitor PBF-509 could lead to novel immunotherapeutic strategies in non-small cell lung cancer.

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Introduction

Metastatic lung cancer remains an incurable disease. The standard treatment is chemotherapy, with targeted agents for patients who have actionable driver mutations. This has produced a survival benefit; however, median survival is only 12.3 months for patients with non-small cell lung cancer (NSCLC) [1]. Recently, immunotherapy with agents preventing the binding of the immune checkpoint PD-1 to its ligand PD-L1 was found to be efficacious in lung cancer. PD-1 is one of several T-cell surface receptors called immune checkpoint proteins, which delivers a negative signal to T-cells when it engages its ligand PD-L1 expressed on antigen-presenting cells [2]. This is a feedback

Abbreviations: A2aR, adenosine A2a receptor; CAF, cancer-associated fibroblast; CHO, Chinese hamster ovary; IC_{50}, concentration of compound that displaces the binding of radioligand by 50%; Kb, binding constant; Ki, inhibition constant; NECA, 5″-N-ethylcarboxamidoadenosine; NSCLC, non-small cell lung cancer; TIL, tumor-infiltrating lymphocyte.

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mechanism whereby immune responses are dampened when no longer needed. This mechanism of control of T-cells can be co-opted by tumors to escape rejection by the immune system [3,4]. A marker of malignancy is an inflamed tumor microenvironment [5], and inflammatory cytokines such as γ-interferon can induce PD-L1 expression on tumor cells [6]. Therefore, tumor antigen-specific T-cells activated in tumor-draining lymph nodes are shut down when they enter into the tumor microenvironment. This discovery has led to the development of a therapeutic strategy that involves monoclonal antibodies designed to prevent PD-1 binding to PD-L1. There are a number of different monoclonal antibodies specific for PD-1 or PD-L1 that are in clinical development for lung cancer, all having similar tumor response rates of 15% to 20% [7].

The adenosine A2a receptor (A2aR) is another T-cell surface immune checkpoint protein operational within the tumor microenvironment. It is known that the extracellular tumor microenvironment contains high levels of adenosine as a consequence of anaerobic glycolysis in hypoxic regions and preferential utilization of aerobic glycolysis for energy metabolism in non-hypoxic regions (the Warburg effect), producing a relative excess of AMP and tumor cell expression of the ectonucleotidase CD73, which catabolizes AMP to produce adenosine [8,9]. Adenosine produced within the hypoxic microenvironment of inflamed tissue has been shown to limit the exuberance of inflammatory responses to reduce collateral damage of normal tissue by inflammatory cells and cytokines [10,11]. This is due to a direct inhibitory effect of adenosine on T-cells that express adenosine A2aR, a T-cell surface immune checkpoint protein [12], leading to discovery that adenosine in the tumor microenvironment interferes with antitumor immunity [13] and suggesting that antagonism of the A2aR could be an effective cancer immunotherapeutic [14].

It has been shown that the use of an adenosine A2aR antagonist leads to synergistic tumor suppression with anti-PD-1 in murine models. Beavis and colleagues reported that an adenosine A2aR antagonist could protect mice from developing B16F10-CD73^{hi} experimental metastases [15]. In a more recent paper, this group demonstrated that the combination of a blocking anti-PD-1 antibody and an A2aR antagonist were synergistic in the prevention of experimental and spontaneous metastases in two separate mouse models [16].

A2aR signaling in the brain contributes to neurologic conditions such as Parkinson disease, which has led to the development of A2aR antagonists that have been tested in randomized trials for these diseases [17]. As a class, these drugs are quite safe [18,19]. Here, we describe the characteristics of PBF-509, a novel and potent A2aR antagonist that has already undergone two clinical trials to assess tolerability and safety (data has not been disclosed yet) [20,21]. It enhances antitumor immune responses in murine and in human *ex vivo* models as a consequence of inhibiting the A2aR on T-cells where it functions as an immune checkpoint, contributing to immune evasion in the tumor microenvironment. Thus, PBF-509 may function as an anticancer immunotherapeutic agent in cancer patients.

Methods

Cell Lines

CHO-A1 and HEK-A2B cell lines were purchased from Euroscreen (now part of Perkin Elmer). Hela-A2A and Hela-A3 cells were obtained in house. These four cell lines were obtained more than 10 years ago and were characterized by means of radioligand binding saturation and competition with reference compound studies. This characterization was carried out each time a new batch of membranes was prepared for experiments. B16-CD73+ and MCA205 cells (used in the tumor model) were generously provided by Dr. Mark J. Smyth (Queensland University, Australia) and were not authenticated.

Radioligand Binding Competition Assay

A1, A2A, A2B, and A3 human receptors expressed in transfected Chinese hamster ovary (CHO; hA1), HeLa (hA2A and hA3), and HEK-293 (hA2B) cells were used. Concentration-response binding competition curves were carried out by assaying six different concentrations (range: between 10 nM and 100 μM). The inhibition constant (Ki) of each compound was calculated by the Cheng-Prusoff equation: $Ki = IC_{50}/(1 + [L]/Kd)$, where IC_{50} is the concentration of compound that displaces the binding of radioligand by 50%, [L] is the free concentration of radioligand, and Kd is the dissociation constant of each radioligand. IC_{50} values were obtained by fitting the data with nonlinear regression with the use of Prism 2.1 software.

cAMP Accumulation Inhibition Assay

These assays were performed with adenosine receptors transfected using a cAMP enzyme immunoassay kit (Amersham Biosciences). HEK-293 cells were seeded (10,000 cells/well) in 96-well culture plates and incubated at 37°C in an atmosphere with 5% CO_2 in Eagle medium nutrient mixture F-12, containing 10% fetal calf serum and 1% L-glutamine. Cells were washed 3 times with 200 μl of assay medium (Eagle medium nutrient mixture F-12 and 25 mM HEPES; pH 7.4) and preincubated with assay medium containing 30 μM rolipram and test compounds at 37°C for 15 minutes. A second incubation step with 10 μM 5″-N-ethylcarboxamidoadenosine (NECA) was performed for 15 minutes at 37°C (total incubation time of 30 minutes). 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). Data were fitted by non-linear regression using GraphPad Prism v2.01 (GraphPad Software). We calculated concentration-response curves by assaying six different concentrations (range from 10 nM to 100 μM). Data were expressed as binding constant (Kb) by following the formula reported by Leff and Dougall [22]: $Kb = IC_{50}/(2 + ([A]/[A50]^n)(1/n) - 1$, where IC_{50} is the concentration of compound that inhibits NECA by 50%; [A] is the concentration of NECA employed in the assay, [A50] is the NECA EC_{50} value, and n is the Hill slope of the curve.

Experimental Tumor Model

Wild-type C57Bl/6 female mice were purchased from Charles River Laboratories and maintained at the Centre de Recherche du Centre Hospitalier de l'Université de Montréal (Montreal, Quebec, Canada). All experiments were carried out in accordance with guidelines set by the Animal Experimental Ethics Committee. C57Bl/6 mice were injected intravenously with 3×10^5 B16F10 tumor cells retrovirally gene-modified to express CD73 (herein referred to as B16-CD73+) [15] or 10^5 MCA205 cells. Mice were then treated daily with vehicle control or PBF-509 by oral gavage from day 0. Vehicle consisted of 0.1% Tween 80 and 0.5% sodium carboxymethylcellulose in water. At day 15 post-injection of tumor cells, mice were killed, lungs were harvested, and tumor nodules were counted under dissecting microscope.

Cell Culture

Primary human fibroblasts were isolated from portions of lung tumors resected from patients for clinically indicated reasons. The tumors were mechanically and enzymatically (collagenase, DNase,

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