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Discovery of CT3021, a Novel Potent Adenosine A2a/A2b/A1 Receptor Triple Antagonist

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ABSTRACT

In a CD73-positive tumor microenvironment (TME), ATP released from apoptotic tumor cells converts to adenosine, generating an adenosine-rich immunosuppressive environment. Adenosine activates G-protein coupled receptors expressed on immune cells to achieve immune suppression, with a mechanism of action involving (1) dampening antitumor effector cells (T_{eff} and NK cells) via adenosine engagement on A2a receptor, (2) reducing dendritic cell antigen presentation and promoting myeloid-derived suppressor cell (MDSC) differentiation by activating A2b and A2a receptors, and (3) inducing chemotactic migration of immature plasmacytoid dendritic cells (pDC) to infiltrate tumor tissue via A1 receptor. Furthermore, A1 receptor expression in certain tumors mediates an adenosine-driven tumor cell proliferation, likely through A1 receptor Gi coupling to the Hippo-YAP signal transduction pathway. Thus, pharmacologic inhibition of A2a, A2b, and A1 receptors by a triple antagonist in the context of adenosine-rich TME may represent an attractive strategy to achieve a better outcome in treating solid tumors.

We discovered and characterized a novel adenosine receptor antagonist CT3021. CT3021 exhibits high binding affinity to A2a, A2b, and A1, with a K_i value of 0.37 nM, 1.76 nM, and 1.26 nM, respectively, and is selective against the A3 receptor and other 83 pharmacologically and/or toxicologically relevant targets. Schild analysis demonstrated that CT3021 is a competitive antagonist with a calculated K_B value of 0.18 nM on A2a receptor in a whole cell cAMP assay in transfected HEK293 cells. In functional antagonist assays, CT3021 maintains its high potency full antagonism under an adenosine-rich TME-like condition (10-100 μM adenosine) both in transfected HEK293 cells and in isolated human T lymphocytes. CT3021 has a high oral bioavailability, a limited CNS exposure, and a favorable ADME profile. CT3021 is well tolerated in rats after repeat dosing. Further toxicology and efficacy studies are underway to explore the potential of CT3021 as a best-in-class adenosine receptor antagonist immunotherapy for the treatment of CD73 positive and A1 receptor positive solid tumors.

Introduction

Extracellular adenosine in a tumor TME engages G-protein coupled receptors A2a, A2b, and A1 subtypes at multiple cellular junctures to promote tumor immune escape and tumor growth (**Figure 1**). Several candidates in clinical development either are subtype-selective, or lose potency in a high adenosine environment. We hypothesize that a triple antagonist across A2a, A2b, and A1 that can counter the action of high adenosine concentration seen in TME might offer a better clinical outcome. This type of antagonist would be expected to block adenosine negative action on effector cells, reduce the immune suppressive MDSC differentiation, and block pDC chemotaxis toward high adenosine cue in the tumor. Furthermore, for A1 expressing tumors, blocking A1 is also expected to inhibit tumor growth, because Gi-coupled A1 receptor can modulate Hippo-YAP pathway within tumor cells and blocking A1 can suppress YAP-directed tumor growth. CT3021 was discovered that met the selection cretieria through a medicinal chemistry design and structure vs. activity relationship study. Results of our profiling studies (**Table 1**) indicate that CT3021 is a potential "*Best-in-Class*" molecule addressing high TME adenosine-mediated tumor immune escape and tumor growth in patients with high CD73 and A1 expressing solid tumors.

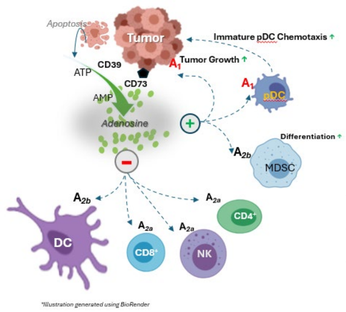

*Illustration generated using BioRender

Figure 1. Adenosine receptor subtypes and their role in tumor growth and immune escape in an adenosine-rich tumor microenvironment.

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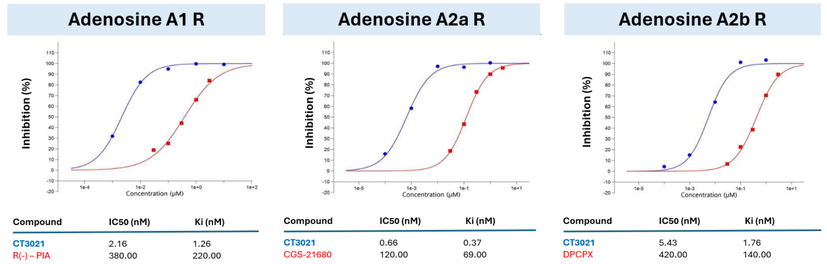
Table 1 CT3021 Profile Summary

Parameters	CT3021 Profile
Adenosine Receptor Binding Affinity (Ki)	0.37 nM (A2a), 1.76 nM (A2b), 1.26 nM (A1), *NR* (A3)
Functional Antagonism in TME	Full antagonist to A2a/A2b under TME-like high Adenosine (10-100 μM)
Orthologue Activity	Active
LM stability, $t_{1/2}$, min (species)	158 (Hu); 50 (Mk), 72 (R), 47 (M), 80 (D)
Metabolite ID (hepatocyte, 240 min)	Rat: Parent (47.44%),Oxidation (30.24%), De-alkylation (7.66%), others: minor Human: Parent (87.56%),Oxidation (6.55%), De-alkylation (1.05%), others minor
CYP Inhibition, P450 Isotype (IC_{50}, μM)	> 50 μM (2C8; 2C9; 2C19; 1A2; 2D6; 2B6; 3A4/5)
PK (rat, mouse)	Orally bioavailable (%*F* = 100), peripherally restricted (B/P ratio = 0.04-0.06)
Eurofins SafetyScreen 87-panel @ 10 μM	NR *(except Adenosine receptors*)
hERG (Patch-Clamp)	IC_{50} = 4.0 μM
14-day repeat oral dosing in male rats	Well tolerated with minor clinical signs/macro-microscopic findings at 300 mg/kg/d. NOEAL = 100 mg/kg/d (Cmax = 14852 ng/mL, AUC = 85030 h*ng/mL)

Results and Discussion

Figure 2 showed that CT3021 has high binding affinity to three adenosine receptor subtypes A1, A2a, A2b, with *Ki* values of 1.26 nM, 0.37 nM, and 1.76 nM, respectively in Eurofins radioligand binding assays (antagonist mode). CT3021 does not bind to adenosine A3 receptor, nor does it exhibit activities toward other 83 targets in the Eurofins 87-panel safety screen (data not shown). Schild analysis of CT3201 on A2a receptor expressed in HEK293 cells with NECA (**Figure 3A**) or adenosine (**Figure 3B**) as an agonist in cAMP assays indicated that CT3021 is a potent competitive antagonist on A2a, with a calculated K_B value of 0.18 nM. A comparison of binding affinity of major adenosine receptor blockers in development is given in **Table 2,** showing CT3021 superiority over competitors. Next, CT3021 potency in a functional assay under high adenosine concentrations that mimic a high-adenosine TME condition was determined for A2a and A2b, and side-by-side directly compared with competitor compounds (**Figure 4**). This study revealed that CT3021 is the only compound having potent dual antagonism toward A2a and A2b under high TME adenosine up to 100 μM. CT3021 also inhibited adenosine-induced cAMP in anti-CD3/anti-CD28 activated human Pan-T lymphocytes isolated from healthy donor whole blood (**Figure 5**). CT3021 is orally bioavailable when dosed in mouse and rat, with %*F* = 100. In rat, a dose-proportional exposure (AUC) was observed from 10 mg/kg to 300 mg/kg after single oral dosing, with plasma Cmax reaching 61.5 μg/mL at 300mg/kg dose and limited brain exposure (**Figure 6**). To demonstrate that CT3021 can lead to an immune-dependent tumor growth inhibition, CT26 tumor cells were inoculated in syngeneic wild-type Balb/*c* mice and immune-deficient NCG mice (GemPharmatech). After dosing CT3021 in these mice, CT26 tumor growth inhibition was only observed in syngeneic wild-type Balb/c mice but not in immune-deficient NCG mice (**Figure 7**). CT26 tumors collected from mice had low levels of expression for A1 receptor and CD73. Further studies with a CD73 and A1 positive tumor model should shed more light on the potential efficacy benefit of a triple antagonist such as CT3021 in treating CD73/A1 positive tumors.

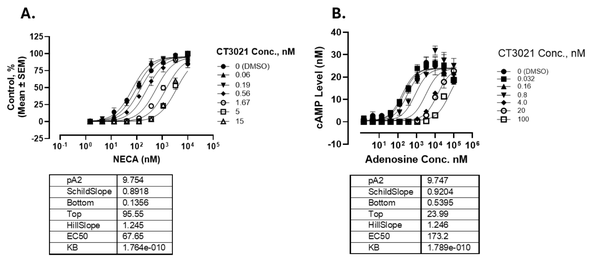


Compound	IC50 (nM)	Ki (nM)
CT3021	2.16	1.26
R(-) – PIA	380.00	220.00

Compound	IC50 (nM)	Ki (nM)
CT3021	0.66	0.37
CGS-21680	120.00	69.00

Compound	IC50 (nM)	Ki (nM)
CT3021	5.43	1.76
DPCPX	420.00	140.00

Figure 2. Radioligand binding assays of CT3021 in antagonist mode for adenosine receptors A1, A2a and A2b.



pA2	9.754
SchildSlope	0.8918
Bottom	0.1366
Top	95.55
HillSlope	1.245
EC50	67.65
KB	1.764e-010

pA2	9.747
SchildSlope	0.9204
Bottom	0.5395
Top	23.99
HillSlope	1.246
EC50	173.2
KB	1.789e-010

Figure 3. Schild Analysis of CT3021 on A2a receptor stably expressed in HEK 293 cells, using NECA (A) or Adenosine (B) as agonist and cAMP production as receptor activation readout.

Table 2. Comparison of Binding Affinity of Major Adenosine Receptor Blockers in Development

Compound	A_{2a}, nM	A_{2b}, nM	A_1, nM	A_3, nM	Brain Penetration
AB928 [1, 7]	K_B =1.4	K_B =2.4	K_B =64	K_B = 489	~1%
AZD 4635 [2,5]	K_i = 1.5; K_d = 2.6	K_i = 64			NA
CPI-444 [3,5, 8]	K_i = 3.54; K_d = 0.83	K_i = 1528	K_i = 192	K_i = 2455	NA
EOS100850 [5]	K_d = 0.30	1200-fold	270-fold	>40000-fold	~1%
INCB106385 [4]	K_B = 0.35; K_i = 0.24	K_i = 9.5			<1%
HM87277 [6]	IC_{50} = 4.4	IC_{50} = 10.5	IC_{50} = 0.3	IC_{50} > 1000	NA
SEL330-639 [9]	K_i = 2.2; K_d = 2.6	K_d = 11.5			No CNS
M1069	IC50 = 0.13 nM	IC50 = 9.0 nM			
CT3021 [10]	**K_B = 0.18; K_i = 0.37**	**K_i = 1.76**	**K_i = 1.26**	NR	~4%**

** NR, no response; NA, not available; N, no CNS penetration, ** male rat, % CNS level at T_{max}*

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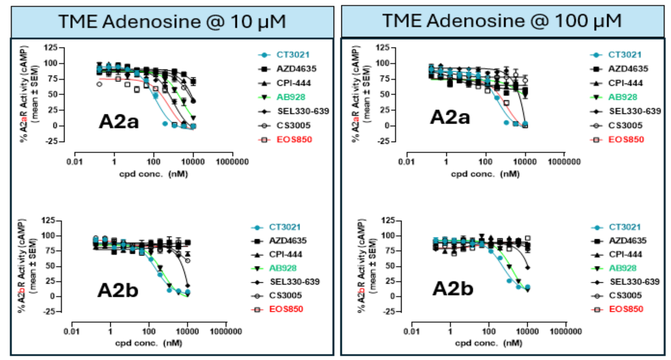
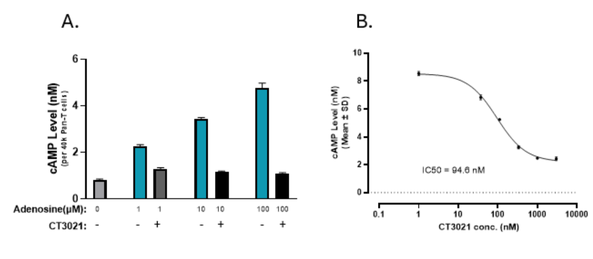


Figure 4. A direct comparison of CT3021 with competitors in functional assays under high adenosine assay conditions. *Note: 1. EOS850 is A2a-selective, and maintain potency under high adenosine level as reported; 2. AB928 is a dual A2a/2b antagonist, but lose potency on A2a at high adenosine level, retain potency on A2b. 3. A2a is the major receptor expressed on tumor killing CTL cells; 4. CT3021 is potent on both A2a and A2b receptors under high adenosine level.*

Figure 5. Inhibition of cAMP production in activated human T lymphocytes. (A). Activated Pan-T lymphocytes were incubated with adenosine (1 μM – 100 μM) and with or without CT3021 for 1 hr. cAMP levels were determined. (B). CT3021 dose response inhibition of NECA (10 μM) induced cAMP production in activated human primary T cells.



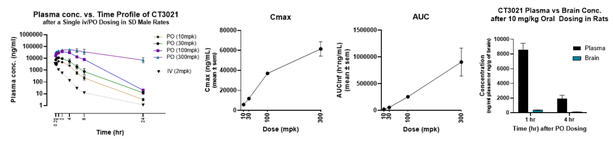
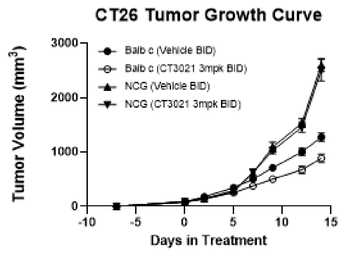
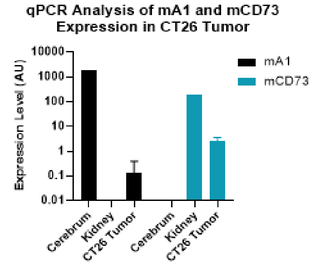


Figure 6. CT3021 PK profile in male Sprague Dawley rats.

PK parameters	Unit	iv	PO			
	mg/kg	2	10	30	100	300
Cl_obs	mL/min/kg	7.52				
$T_{1/2}$	h	2.06	2.51	2.62	1.89	6.94
C0	ng/mL	4218				
T_{max}	h		1.3	1.17	2	4
C_{max}	ng/mL		5786	11783	37002	61522
AUC_{last}	h*ng/mL	4449	22323	56177	255849	806840
AUC_{inf}	h*ng/mL	4464	22336	56228	255908	906039
$AUC_{%Extrap\_obs}$	%	0.354	0.0569	0.114	0.0223	9.5
MRT_{tlf_obs}	h	1.22	2.91	3.33	4.50	9.8
AUC_{last}/D	h*ng/mL	2225	2232	1873	2558	2689
Vss_obs	L/kg	0.544				
F	**%**		***100***	***84***	***114***	***135***
CNS Exposure			***B/P @1hr: 367/8550 (ng) = 0.04 B/P @4hr: 118/1936 (ng) = 0.06***			

Figure 7. (A) CT26 tumor growth inhibition by CT3021 in immune-competent Balb/c mice and immune-deficient NCG mice. (B). Analysis of A1 and CD73 expression in CT26 tumors collected from vehicle dosed Balb/c mice.





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