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Oncological immunodiagnostics Curriculum

Targeting the CD73-dependent nucleotide-metabolizing
pathway in the tumor microenvironment

PhD Dissertation

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Abstract

Tumors thrive on a network of interactions with their heterogenous microenvironment. A molecule of notable relevance in most tumor microenvironment (TME) is adenosine. Because adenosine is both oncoprotective and immunosuppressive, both its production and signaling have been observed and validated as potential therapeutic targets. Despite the tremendous efforts over the years in exploiting this pathway for cancer immunotherapy, few to none of the known small molecule and monoclonal antibody inhibitors of components of the adenosine signaling pathway have made it to the FDA's approved drug list. In this study, we sought to identify novel inhibitors of CD73, the rate-limiting ectoenzyme in the production of adenosine from ATP and NAD, using computer-aided structure-based drug design approaches. Moreover, we aimed to elucidate the tumor and immune cell modulation properties of EOS100850, a novel small molecule inhibitor of A2A receptor, in chronic lymphocytic leukemia (CLL) mice models and in vitro. Though none of the identified inhibitors significantly attenuated the enzymatic activity of CD73 compared to the positive control, our study uncovered BG20, a potent activator of CD73. In a combination treatment of EOS100850 with venetoclax, both spleen weight and volume were significantly reduced with a trending reduction in leukemia burden compared to single treatment arms. Quite strikingly, we identified a classical switch of M2 to M1 macrophage subpopulation in combination treated mice, suggesting that A2AR targeting could augment macrophage-mediated antitumor immunity. Our study lends a solidified voice to the potential of A2AR targeting in CLL and sets the rationale to aggressively consider A2AR targeting as the missing piece to a more effective CLL treatment option.

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1. Introduction
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CHAPTER ONE

Literature Review, Research Question and Objectives

1.0 Literature Review

Tumors are master survivors; – by employing molecular arsenals and tricks of defense and deception, they have evolved over the years to strategically suppress targeted therapy, evade immune cell clearance, sustain proliferation, and metastasize. One of such molecular pathways of defense exploited by most tumors like chronic lymphocytic leukemia (CLL) is the adenosine signaling pathway [1-4]. Acute adenosine signaling is crucial to maintaining cellular homeostasis under conditions of stress such as ischemia or hypoxia and is arguably the most evolutionarily conserved anti-inflammatory mechanism safe-guarding tissue integrity against excessive or prolonged host immune responses [5]. Tumors have however hijacked this pathway to their advantage as prolonged local accumulation and signaling of adenosine is detrimental to health and facilitates the initiation and progression of chronic diseases like cancers.

a. Components of the CD73-dependent nucleotide-metabolizing pathway

The canonical adenosinergic axis is a well-defined network of ecto-nucleotidases (CD39 & CD73), receptors (A1, A2A, A2B & A3) and transporters (ENTs & CNTs) primarily regulating the production, signaling and uptake of adenosine respectively [6, 7]. Transforming growth factor-β (TGFβ) and hypoxia are two main drivers modulating molecules of this pathway [8-13]. Though at varying levels, adenosinergic molecules are expressed by both tumors and infiltrating immune cells, as well as stromal and endothelial cells. The highly hypoxic, increased cellular stress and elevated rate of apoptosis in the tumor microenvironment (TME) induce the accumulation of immunostimulatory ATP in the extracellular space (Figure 1.1) [14, 15]. Subsequently, ATP is quickly hydrolyzed by the sequential enzymatic activity of CD39 and CD73 resulting in the accumulation of immunosuppressive adenosine [16, 17]. Other non-canonical pathways for extracellular adenosine (eADO) accumulation involve the direct active expulsion of adenosine from the cytosol and the conversion of CD38/CD203a-mediated NAD+ to AMP [18], and subsequent hydrolysis to adenosine by CD73 or alkaline phosphatases [19]. eADO is tightly regulated, and as such has a half-life of few seconds [20]. It could either be

internalized through ENTs and CNTs [21], deaminated to inosine by adenosine deaminase [22, 23], or act as a signaling molecule via one of four G protein-coupled adenosine receptors [24, 25]. The latter is the dominant local action of adenosine in the TME [7, 11, 26]. Because of its immunosuppressive nature, molecules of both the production and signaling of adenosine have been considered and, to a greater extent, validated as promising therapeutic targets for CLL [27-29].
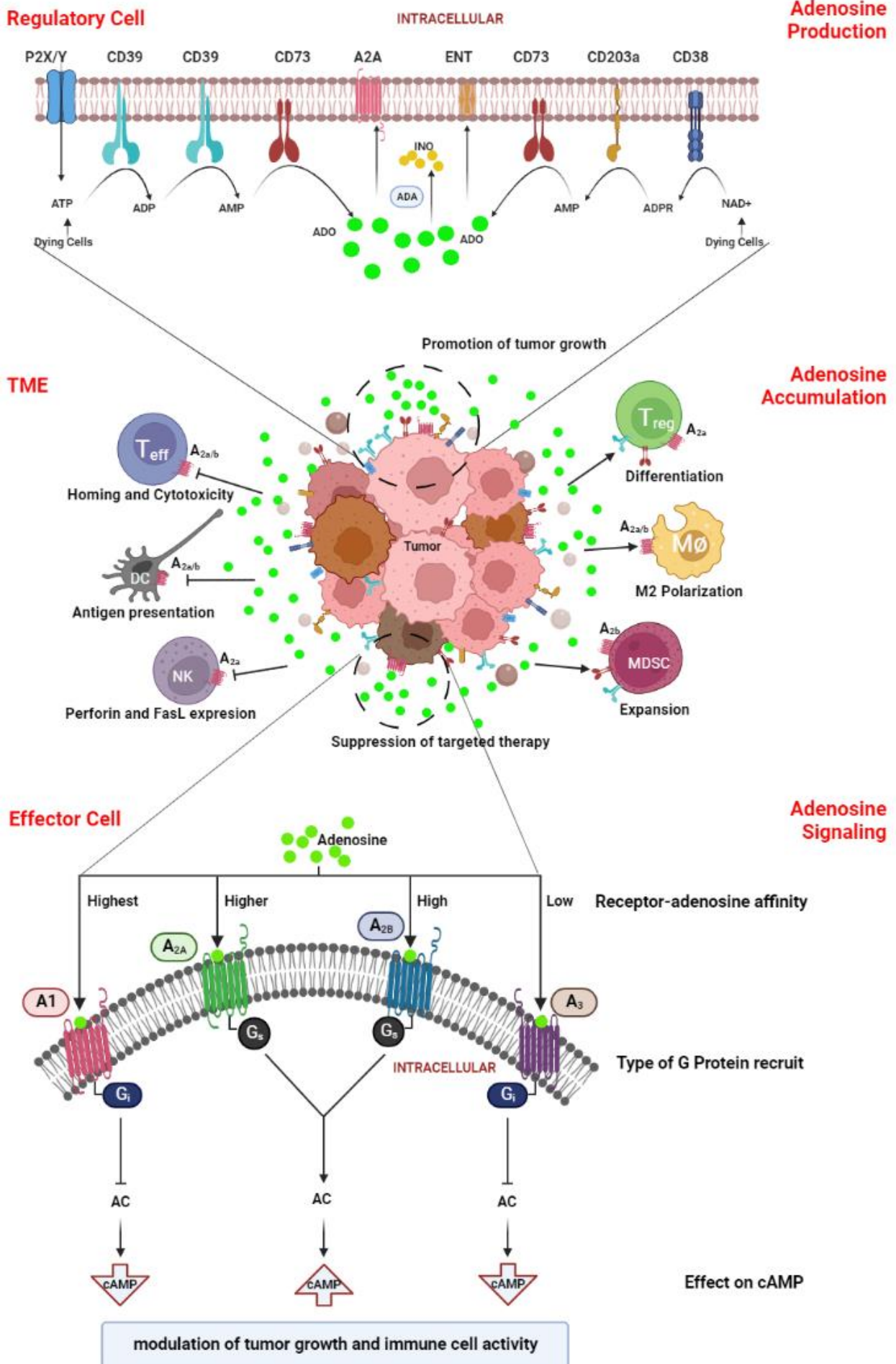
Fig. 1.1: CD73-generated adenosine suppresses immune cell activity mainly via A2A receptors in CLL tumor models. CD73 is the rate-determining ecto-enzyme in the build-up of adenosine in the tumor microenvironment (TME). Accumulation of ADO in the TME and its resultant signaling via A2AR, the most abundant receptor on CLL and immune cells, profoundly suppress antitumor immunity while promoting tumor growth and metastasis.

b. **CD73 is indispensable in the accumulation of adenosine**

Cluster of Differentiation 73 (CD73) is the rate determining ectoenzyme in the ultimate build-up of adenosine in the tumor microenvironment [16, 17, 30]. CD73 is found in almost all human tissues and highly expressed in most cancers. It is involved in purine salvage by mediating the siphoning of membrane-impermeable extracellular AMP, a common product of the catabolism of ATP and NAD, into membrane permeable adenosine. Because of this activity, it modulates cancer adenosinergic signaling and derived immunosuppression [6, 28, 31]. In addition, it co-stimulates T-cell activation mediated through the CD3/TCR complex [32, 33] and interestingly serves as tumor adhesion molecule to extracellular matrix proteins [5, 34, 35].

Mounting evidence has demonstrated the significant influence of CD73 on cancer proliferation, survival, and adaption to hypoxia, as well as on immune cell tumor-infiltration and activity [2, 36-39]. Consequently, it has been identified as a potential therapeutic target for the treatment of both solid and liquid malignancies [40]. Blocking CD73 with small molecule and monoclonal antibody inhibitors have been shown to suppress tumor growth independent of its effect on immune cells, inhibit angiogenesis and reduce metastasis [28, 38, 41-43]. CD73 gene-silencing or pharmacological targeting improves antitumor immune responses of inhibitors of immune checkpoint molecules such as PD-1, CTLA-4 and A2AR in colon, prostrate, breast, carcinoma, and melanoma tumor mice models [28, 44, 45]. Rationally, inhibiting CD73's enzymatic activity reduces adenosine accumulation in the tumor microenvironment which invariably releases the breaks on immunosuppression and impedes tumor growth and survival [40].

c. **A2AR is the most highly expressed adenosine receptor in most tumors**

CLL like most tumors thrives on a network of interactions with its heterogeneous microenvironment [46-49]. Tumor-mediated cell-cell interactions, as well as their chemokine, cytokine and soluble factor secretions create a highly inflammatory microenvironment that fosters the accumulation of immunosuppressive soluble factors such as CSF1, IL10 and VEGF, and cell types like myeloid-derived suppressor cells (MDSC). Compounding evidence have shown such chronic accumulations are markedly sufficient to resist therapy-induced apoptosis, dampen host immune attacks and promote cancer pathogenesis. A soluble

mediator of notable relevance of the CLL proliferation niche is adenosine [7, 39]. The degree of adenosine build-up and dependence on A2A receptor signaling within the TME is tumor/tissue/cell type specific. Under physiological conditions, A2AR protein expression or signaling is enriched in the basal ganglia and caudate of the brain, small and large intestines of the gastrointestinal tract, and in the bone marrow and lymphoid tissues [50]. Single cell cluster RNA data shows enhanced specificity in gastric mucus-secreting cells, plasmacytes, B and T-lymphocytes and a moderate enrichment in macrophages, dendritic and NK cells [51]. During a cancerous growth, however, A2AR expression and signaling levels are significantly upregulated, especially under hypoxia, mostly as a survival and growth promoting mechanism. The stimulatory-GPCR is markedly expressed in human lymphoma and melanoma cell lines with a moderate RNA expression profile in neuroblastoma, leukemia, breast, and liver cancer human cell lines [52]. Comparative RNA dataset of patient samples show enrichment in liver, renal, breast, testis, stomach, and lung cancer tissues [53], suggesting that these solid tumor types may benefit the most from A2AR signaling inhibition therapeutic options.

As A2AR is the most dominant type 1 purinergic G protein-coupled adenosine receptor in CLL [2], the purine nucleoside affects its immunosuppressive and anti-inflammatory responses mainly via A2AR on tumors and infiltrating immune cells [4, 54, 55]. Adenosine-A2AR signaling increases intracellular cAMP levels leading to downstream activation of PKA, phosphorylation of CREB and ultimate inhibition of the NF-κB pathway [25, 56, 57]. Moreover, elevated PKA activation positively modulates JNK, ERK, p38 and PI3K/AKT survival mechanistic pathways aiding tumors to thrive and grow despite the unfavorable harsh conditions of the TME to host immune cells [58]. In addition, A2AR signaling has been shown to facilitate tumor growth by elevating VEGF-mediated angiogenesis [43], proposing that blocking A2AR signaling may not only restore effective antitumor immune responses (discussed later), but also abrogate tumor growth by inhibiting adenosine-induced neovascularization.

i. A2AR signaling as a tumor growth promoting pathway

Tumor adenosine-A2AR signaling (TAAS) is oncoprotective [3, 40]. Mechanistically, TAAS, in part, reinforces survival pathway stimuli such as ERK and PI3K/AKT and upregulates molecules of the epithelial-mesenchymal transition (EMT) machinery, promoting tumor migration and invasiveness. A2AR signaling in hepatocellular carcinoma (HCC) led to Rap1-mediated P110β

plasma membrane localization, a trigger in PIP3 production and a resultant activation of the PI3K/AKT signaling pathway, fostering HCC progression and metastasis [32]. Moreover, upregulated A2AR expression and signaling in gastric cancer (GC) cell lines and human tumor xenografts were shown to positively correlate with cancer stemness and promoted GC migration and invasion via a PI3K-AKT-mTOR signaling pathway [33]. Stanniocalcin 2 (STC2) is a well-known glycosylated hormone involved in most cancer pathogenesis and progression. STC2 overexpression by Chen and Colleagues promoted ERK/MEK and PI3K/AKT-dependent colorectal cancer growth and migration *in vitro* and in a mouse xenograft model. Interestingly, inhibiting AKT-ERK pathways attenuated STC2-mediated EMT signatures [35], suggesting that A2AR signaling may crosstalk with STC2 production and/or activation.

We previously demonstrated how addition of adenosine to a culture of primary CLL cells significantly dampened both spontaneous and drug-induced apoptosis, and that by inhibiting A2AR signaling with SCH58261, adenosine's cytoprotection was markedly blunted [2]. This, in part, highlighted the survival advantage elevated adenosine levels offer to CLL via A2AR signaling. Chemokines modulate the homing and trafficking of lymphocytes. Like the CLL drug-induced apoptosis assay, Serra *et al* further showed that adenosine-A2AR signaling inhibits CLL chemokine-driven motility away from growth-favorable centers in a dose-dependent manner. Intriguingly, CGS21680-mediated A2AR activation and resultant increase in intracellular cAMP hampered CLL chemotaxis in a similar fashion, further strengthening the role of A2AR signaling in the growth and survival of hematological malignancies like CLL [2].

ii.   A2AR signaling as a major immunomodulatory axis

Several tumor-induced immunomodulatory pathways exist in the TME. These diverse signaling molecules interacting mostly through ligand or soluble factor-receptor cell-cell communications allow tumors to inactivate T cells (e.g., CD80/86 vs CTLA-4), escape macrophage phagocytosis (such as CD47 vs SIRPα), inactivate NK cells (e.g., MHC I vs iKIR) and upregulate Tregs by the binding of tumor-derived adenosine to A2A receptors on Tregs [59-61]. While such diversity presents a multi-faceted challenge to antitumor immunity strategies, a comprehensive profiling study and analysis of the major immunomodulatory axes in any given tumor type would continuously give us the edge and bring us a step closer to hitting the Achilles heel. For example, profiling of glioblastoma patients revealed that the dominant immunomodulatory targets on glioma-infiltrating lymphocytes (GILs) and myeloid-derived

cells (GIMs) was the CD39/CD73/A2AR pathway, followed by the PD-1 signaling pathway [62]. A2AR inhibition showed a considerable therapeutic response through the recovery of effector T functions in a mouse glioma model although immune recovery was hampered in the presence of gliomas, suggesting that a combinational targeting of potential exhaustion markers may potentiate the effectiveness of A2AR inhibitors in gliomas.

An effective immune-tumor attack relies on the coordinated effort of primed immune cells such as effector cytotoxic CD8+ T lymphocytes, M1-like inflammatory monocytes, matured NK & dendritic cells and limited expansion of regulatory T cells [63, 64]. Accumulating evidence has shown that adenosine-A2AR signaling potently mitigates antitumor immune responses by inhibiting the proliferation, differentiation and cytokine production such as interleukin-2, interferon (INF)-γ and tumor necrosis factor (TNF)-α of effector CD8+ T lymphocytes, consequently suppressing their cytotoxicity [29, 54, 65, 66]. Concurrently, A2AR receptor signaling modulates the CD3+ T cell population by promoting the generation of adaptive Treg cells and long term T cell anergy by increasing Foxp3 and LAG-3 expression [67], ultimately leading to a compromised environment where immune cells and tumors coexist tolerably. NK cells are antigen-independent, fast-acting effector lymphocytes of the innate immune system essential for speedy recognition and killing of infected or abnormally transformed cells. In these cells, A2AR signaling can impede their maturation, and release of pro-inflammatory cytokines and cytotoxic granules [68, 69]. In addition, adenosine-A2AR signaling has been shown to suppress DCs' ability to present antigens and induce T cell activation and differentiation [70-72], maintain an anti-inflammatory macrophage subset [73-75] and inhibits neutrophil migration and chemoattractant secretion [76, 77], as well as modulating the overall tumor-infiltration of the immune cell repertoire [66, 78].

iii.   A2AR signaling as an emerging resistant mechanism to immune checkpoint therapies

Immune checkpoint inhibitors (ICIs) such as PD-(L)1 and CTLA-4 antagonists as single agents or in combination with therapies such as CAR-T cell therapy, tyrosine kinase and VEGF inhibitors have emerged as the most promising cancer treatment modality for both solid and hematological malignancies [79, 80]. Despite the revolutionary improved survival outcomes offered to diverse cancer types like non-small-cell lung cancer (NCLC), metastatic melanoma and carcinoma patients, the majority of patients treated with ICIs do not experience complete

remission, and some do not respond at all. Relapse and disease progression do occur in those that do, at least in 20-30% of disease-free ICI-treated patients [81, 82]. Development of primary and acquired resistances, and immune-related adverse events have been known as major drawbacks to the clinical success of this class of checkpoint therapy. Expectedly, mechanisms fueling these shortcomings are intensely being studied. Among the molecular processes so far unraveled, (a) intensity of PD-L1 expression on tumors, (b) disruption of IFN-γ signaling pathway, (c) mutational tumor burden and (d) disrupted MHC1 antigen presentation via frameshift β$_2$-microglobulin (B2M) deletion have been observed as major players impeding ICI clinical outcomes [80, 83].

Intriguingly, A2AR signaling has been demonstrated to dampen IFN-γ production by effector CD8+ T cells, suppress antigen presentation by dendritic cells and may act as a master checkpoint pathway as it has been shown to enhance CRE-mediated expression of LAG-3, CTLA-4 and PD-1 on both regulatory and effector T lymphocytes [7, 84]. The success of immune checkpoint blockade (ICB) correlates with the intensity of receptor expression and/or its cognate ligand [80]. Interestingly, a number of inflammatory cytokines such as TNF-α and INF-γ in tumor regions and aberrant oncogenic pathways like NF-κB regulating the expression levels of PD-L1 on tumor cells for example are in part modulated by A2AR signaling, suggesting a possible dual advantage in combination studies involving these axes. A recent study by Takao and Colleagues involving 60 metastatic renal cell carcinoma (RCC) patients highlighted the inverse correlation between A2AR receptor expression and patients' response to inhibitors of vascular endothelial growth factor (VEGF), PD-1 and CTLA-4 [85]. Briefly, the authors noted that patients showed better response and longer survival when treated with PD-1 or VEGF monotherapy blocking agents or a combination of PD-1 and CTLA4 inhibitors only when the primary tumor had low A2AR receptor and high PD-L1 expression profiles. Thus, implicating adenosine-A2AR signaling as a key immunosuppressive arsenal invented by tumors to repress ICB efficacy.

d. **PoC preliminary data**

We previously demonstrated how addition of adenosine to a culture of primary CLL cells significantly dampened both spontaneous and drug-induced apoptosis, and that by inhibiting A2AR signaling with SCH58261, a small molecule A2AR antagonist, adenosine's cytoprotection was markedly blunted [2]. This, in part, highlighted the survival advantage elevated adenosine

levels in the TME offers to CLL via A2AR signaling. Chemokines modulate the homing and trafficking of lymphocytes. Like the CLL drug-induced apoptosis assay, Serra *et al* further showed that adenosine inhibits CLL chemokine-driven motility away from growth-favorable centers in a dose-dependent manner. Intriguingly, CGS21680-mediated A2AR activation and resultant increase in intracellular cAMP hampered CLL chemotaxis in a similar fashion, further strengthening the role of A2AR signaling in the growth and survival of CLL [2].
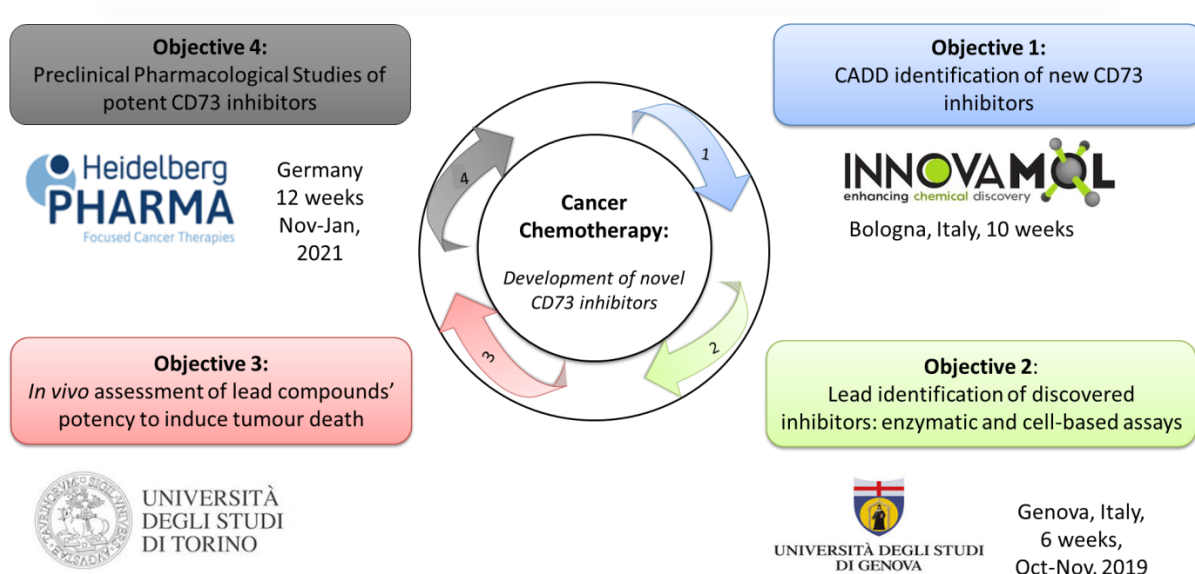
1.1 PROJECT AIM AND SPECIFIC OBJECTIVES

Though several inhibitors of CD73 and A2AR have been developed and few are in clinical trials, highly selective, potent and FDA approved drugs targeting the CD73-dependent nucleotide-metabolizing pathway in the tumor microenvironment are still limited.
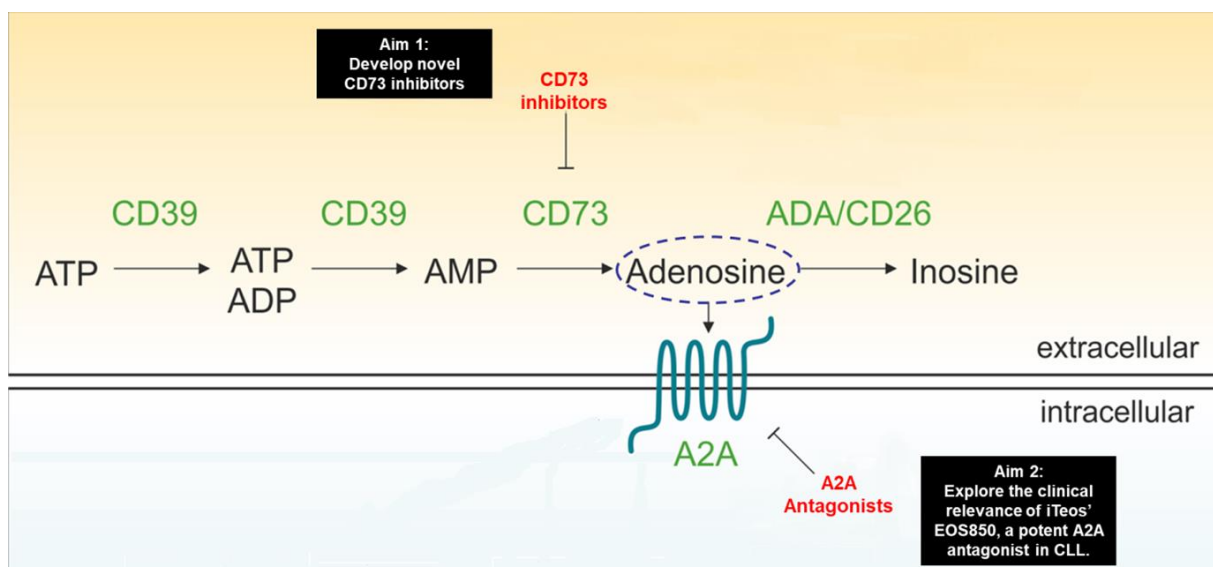
Fig 1.2: Targeting the CD73-dependent nucleotide-metabolizing pathway in the TME. The initial focus of the project was to develop novel CD73 inhibitors. Project objective was however revised during the PhD duration to explore the pharmacological properties and antitumor activity of EOS850, a potent A2AR inhibitor under development by iTeos therapeutics.

Initial Project Aim 1: The primary focus of the research project was to identify and develop novel CD73 inhibitors for the treatment of CD73-dependent malignancies using chronic lymphocytic leukemia (CLL) cancer models as illustrated in figure 1.2 above. In achieving this goal, the following specific objectives, coordinated by four of the partner institutions of the INTEGRATA ITN programme, were aimed at, graphically illustrated in figure 1.3 below:

1. Identify 20-30 CD73 inhibitors using computer-aided structure-based approach.
2. Test inhibitors' potency to significantly reduce the activity of CD73 in leukemic cell lines.
3. Optimize successful lead compounds.
4. Assess 3-5 lead compounds' potency to induce leukemic cell death in vitro.
5. Assess lead compounds' efficacy (2-3 compounds) in both immunocompetent and immunodeficient CLL mouse models.
6. Characterize the main pharmacological properties of selected 1-2 inhibitors

Fig. 1.3: INTEGRATA partners involved in the project and the period spent or hope to spend in achieving respective objectives. Objective 3 was amended to the revised project aim 2 below and has no specific duration within the PhD period because UNITO is where I am based and activities at the 3 partners are deemed as secondments. Objective 4 was not realized. Instead, I spent 10 weeks at iTeos Therapeutics for industrial experience.

Revised Project Aim 2: After elaborate work in fulfilling deliverables 1 and 2 above, it was observed that none of the identified inhibitors demonstrated superior inhibitory profiles compared to the positive control AMPCP. As a result, we decided to explore the antitumor immunity relevance of an already optimized A2AR antagonist, EOS850, under development by iTeos Therapeutics, a US-based pharmaceutical company with its R&D center in Belgium. iTeos has been in a long-standing collaboration with Prof. Silvia Deaglio's lab, and prior to the start of the PhD, our lab had already demonstrated to some extend the on-targetability of the small molecule and its potency to significantly suppress CGS21680-induced CREB phosphorylation. Project aim 2 also involved the combinational treatment with venetoclax. The rational and the working hypothesis are discussed in chapter three.

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CHAPTER TWO

IDENTIFICATION AND CELL-BASED TESTING OF NOVEL CD73 INHIBITORS

2.1 INTRODUCTION

The solving of the X-ray crystal structure of human CD73 bound to adenosine (α, β)-methylene diphosphate (AMPCP or APCP), a non-hydrolysable analog of ADP, paved an unprecedented avenue for the identification and design of effective nucleotide & nucleoside-derived CD73 inhibitors [1-5]. By modifying either the ribose sugar or the nucleobase, and sometimes the zinc-binding groups, several low micro- to nanomolar-ranged inhibitors have been reported. Notwithstanding, AB680, a typical derivative of AMPCP modification, is the first small molecule CD73 inhibitor to enter clinical trials recently [6, 7]. Beside these, other non-nucleotide-based potent antagonists such as sulfonamides [8], flavonoid-based compounds like quercetin [9], anthraquinone derivatives [10], phelligridin compounds [11] and monoclonal antibodies have been identified, exhibiting a mixture of competitive to non-competitive mode of inhibitions.

As an ecto-enzyme, CD73 predominantly functions as a dimer anchored by a glycosyl phosphatidylinositol (GPI) molecule to plasma surfaces of expressing cells [1]. A scanning microscopy of CD73 shows a relatively rigid GPI-hooked C-termini linked to flapping N-terminal domains via flexible α-helix chains, a typical feature of the AMP-preferred nucleotidase in its open conformation. The dimeric subunits of CD73 interact through noncovalent bonding at its C-terminal domains with a considerable degree of plasticity. To form its catalytically active site, which comprises of amino acid residues of both domains and two zinc ions, the N and C-termini go through conformational rotations of about 114^0 aided by the flexibility of the α-helix linker and an intrachain domain movement at its dimerization interface, forming the closed conformation of CD73 [1].

While the uniqueness of its structure and these domain movements is believed to increase substrate specificity and permit release of product, they inevitably serve as its Achilles heel to inhibitor design strategies. Consequently, most non-competitive mAbs designed against CD73 either obstruct its necessary conformational movement by interacting with the

dimerization interface and the α-helix linker or binding to allosteric pockets on the N-terminus, mostly close to and shielding the interactiveness of the catalytically required Zinc ions. MedImmune's MEDI9447, currently in phase 1 clinical trial, is a typical example of a first-in-class anti-CD73 mAb designed to exploit these functional weaknesses [12]. Mechanistically, MEDI9447 is reported to inhibit the enzymatic activity of CD73 by binding to a discontinuous epitope within CD73's N-terminus and engaging in steric hindrance monovalent interactions, consequently preventing the necessary conformational rotations. Similar mAb inhibitory mechanisms have been demonstrated by Surface Oncology's SRF373, Bristol Myers Squibb's BMS98679, PT199 from Phanes Therapeutics and Corvus Pharmaceuticals' CP1-006 in multiple types of cancers [6].

Of all forms of inhibition strategies targeted at CD73, substrate site competitive inhibition is the most studied and well-advanced structure-based drug development template for future identification and design of novel small molecule CD73 inhibitors. In line with the first objective of the project therefore, we sought to identify new scaffold CD73 inhibitors that could significantly compete with AMP through virtual screening of a compound database platform and cell-based lead identification testing.

2.2 MATERIALS AND METHODS

Molecular Docking Platform

Structure-based drug candidate identification mostly requires a specialized, parameter-twitching screening platform that allows for the selection of favorable compounds from a large database of virtual molecules. Mcule (https://mcule.com/), an online molecular modeling and library screening platform designed for hit identification, lead optimization and compound sourcing was used for the virtual screening of potential CD73 inhibitors.

CD73 X-ray crystal structure preparation

Prior to structure-based compound screening, it is necessary to refine and optimize X-ray crystal protein structures. 4H2I (PDB DOI: 10.2210/pdb4H2I/pdb), the human CD73 X-ray crystal closed form III in complex with AMPCP was prepared using Schrodinger's Maestro software v13.3 (figure 2.1). During the preparation process, hydrogen atoms were added to the structure, spatial water and atomic clashes were removed, and hydrogen bonds optimized in addition to other default parameters that maximize the refinement process and enhance proper docking. Moreover, the AMPCP ligand and the accompanying zinc metal ions were removed. The resultant optimized protein was saved and made ready for virtual screening.
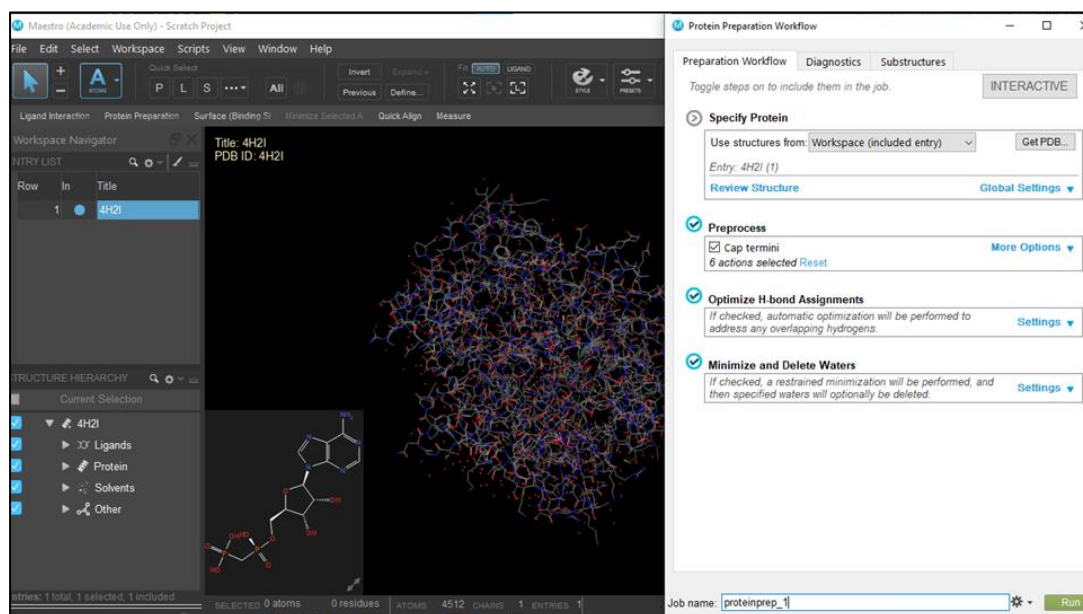
Fig 2.1: *Schrodinger Maestro's Protein Preparation Workflow*. Imported CD73 4H2I crystal structure is optimized for virtual screening by optimizing hydrogen bonds, and removing bound ligands, spatial water and atomic clashes. The integrated preprocess assigns bond orders, create disulfide bonds and zero-order bonds to metals as well as generating het states with Epik at a pH of 7.4.

Selection of inhibition strategy

The unique features of CD73 and its necessary conformational rotation for catalysis present varied hotspots for drug identification and design strategies. Substrate competitive binding inhibition, obstruction of the dimer interface movement, alpha-helix linker targeting, and allosteric inhibition are among the notable few. Computer-aided structure-based competitive substrate inhibition strategy was chosen as the preferred inhibitor identification approach.

Virtual Screening for new scaffold CD73 inhibitors

Mcule molecular docking screening platform was used to identify potential new CD73 inhibitors. The Input database functionality of the workflow was set to purchasable (in stock), allowing for quick order of potential compounds. To screen through the large database, the basic property feature was applied to filter for appropriate compounds. Respective ligand parameters were adjusted per instructor's protocol as shown below. Docking Vina search engine was selected to screen for the best 100, 000 compounds with the highest docking score and a diversity coefficient of 0.85.

Fig. 2.2: *Mcule has a simplified and intuitive workflow for hit identification, lead optimization and compound sourcing*. Four main workflow parameters were selected: basic compound properties, sampler size, diversity selection and Vina Docking. Whereas the basic properties ensured inclusion of recommended Lipinski rule, the diversity selection optimized the identification of ligands with diverse scaffolds as much as possible. The in-stock Mcule purchasable database allowed for quick ligand order where possible.

Library Molecular Docking Scoring

Mcule platform does not allow for refined pocket and pose assessment. To narrow down the obtained 100K compounds to the best 30-50 potential inhibitors, the obtained library was further subjected to a molecular docking process using Schrodinger's Maestro software v13.3. Prior to docking, the compounds were prepped using Maestro's Ligprep according to manufacturer's instructions and recommended default parameters. Docking was sequentially done, starting with Glide High throughput Virtual Screening (HTVS). By trading sampling breath for higher speeds, the first 1000 compounds with the highest docking scores were selected and further subjected to Glide SP docking screening. SP's performance is exhaustive and balances both speed and accuracy. After this, top 100 compounds were selected and further docked unto optimized 4H2I using Glide XP. This last docking process is way slower compared to the previous two, favoring accuracy over speed. It utilizes a unique functional form and an anchor and grow sampling approach for the Glide Scoring system.

Pose and Pocket Conformation Assessment

Despite the order of docking scores provided by Glide XP, each pose was manually assessed and further scored based on the number of essential interactions and hydrogen bonds made at the protein's catalytic site. This was based on the preamble that for a potential ligand to significantly compete with AMP, it must engage in similar interactions at the catalytic site and/or potentially form extra bonds that are stronger and essentially displace AMP from binding. A ligand's interaction is termed therefore essential if it forms hydrogen bonds with amino acid residue R354, R395 and D506, and a hydrophobic pi-stacking interactions with F417 and F500 residues. A ligand is preferentially chosen when other molecular interactions are possible with spatial water molecules as well as with residues of the N-terminal domain of CD73. Other significant parameters such as ligand-pocket fit and pose flexibility were also taken into consideration.

Ligand-CD73 inhibition assay

500K MEC-1 cells, previously cultured in RPMI + 10% FBS to homogeneity, were harvested and washed gently with PBS at 1500 RPM for 5 mins at 37°C in 1.5 ml Eppendorf tubes. Cell pellets were then gently resuspended and preincubated with 100 µM inhibitors for 30 minutes prior to the addition of 200 µM AMP at 37^{0}C in a water bath, making a total volume

of 500 ml. Cells were spun down at 1500 RPM for 5 mins and 100 µl of supernatant was pipetted into clean tubes after 5, 10, 15 and 30 minutes of AMP incubation. Pellets were gently resuspended after each centrifugation. Supernatants were either stored at -80°C for later assessment or 95 µl were mixed with 5 µl 50% Trichloroacetic acid (TCA) for nucleotide extraction and HPLC measurement according to manufacturer's instructions. Briefly, TCA-sample solutions were centrifuged at high speed and then 90 µl was mixed with 110 µl water in extraction glasswares. The sample solutions were vortex-washed with ether under the fume hood 3 times, and surface ether was then evaporated away with nitrogen gas. Samples were centrifuged further to remove any remaining ether and to settle down debris. 100 µl of extracted nucleotide solutions were then pipetted for HPLC measurement.

2.3 RESULTS AND DISCUSSION

Structure-based virtual screening identified 35 new scaffold potential CD73 inhibitors

Structure-based AMPCP-derived inhibitors are the most common small molecule antagonists of the enzymatic activity of CD73. Though in vitro studies have reported compounds with nanomolar activities, few have seen significant benefits in animal models and clinical trials, raising the need for new potent inhibitors [5]. Recognizing the substantial impact of the CD73-dependent nucleotide-metabolizing pathway in the microenvironment of both solid and hematological malignancies, the project was aimed at identifying novel antagonists of CD73 with scaffold variant to AMPCP-derivatives. By means of a computer-aided substrate-based virtual screening, we identified 35 potential inhibitors (figure 2.3D) that may significantly compete with AMP for binding at the catalytic sites of dimeric CD73. Compound scaffolds were markedly different from each other, a coefficient of 0.85; increasing the chance of finding a novel potential inhibitor.

Reports of X-ray crystal structure of CD73 in complex with AMPCP and molecular docking stimulation with AMP reveal ligands' essential pharmacophore at the substrate and catalytic sites (figure 2.3A and B) [1]. The ligands make strong hydrophobic pi-stacking interactions with the side chains of two phenylalanine amino acid residues F417 and F500 (yellow, fig. 2.3B) via their nucleobases, forming a clamp-like ligand sandwich. Hydrogen bonds between the phosphoribosyl moiety and two arginine (R354 and R395; purple, fig. 2.3B), aspartate (D506; red, fig. 2.3B) and asparagine residues (N245; green, fig. 2.3B) help to further stabilize them in position. To identify potential competitive inhibitors therefore, we took into consideration these essential interactions and devised a pose conformation assessment and a manual docking scoring scheme. Maestro Glide XP docked compound was selected if it made at least 3 hydrogen bonds, interacted with 3 or more of the essential amino acid residues, formed the classical pi-pi bond and generally had a good instructor-assessed pocket fit. Hydrogen bond strength, functional groups, chirality and drug toxic moieties were also taken into consideration. Additional interactions involving the classical asparagine-histidine dyad of the N-terminus was an advantage. Figure 2.3C illustrates ligand-pocket interactions

of an included ligand. The classical pi-stacking interaction, at least 3 HB, and more than 3 essential residues' involvement could be seen.
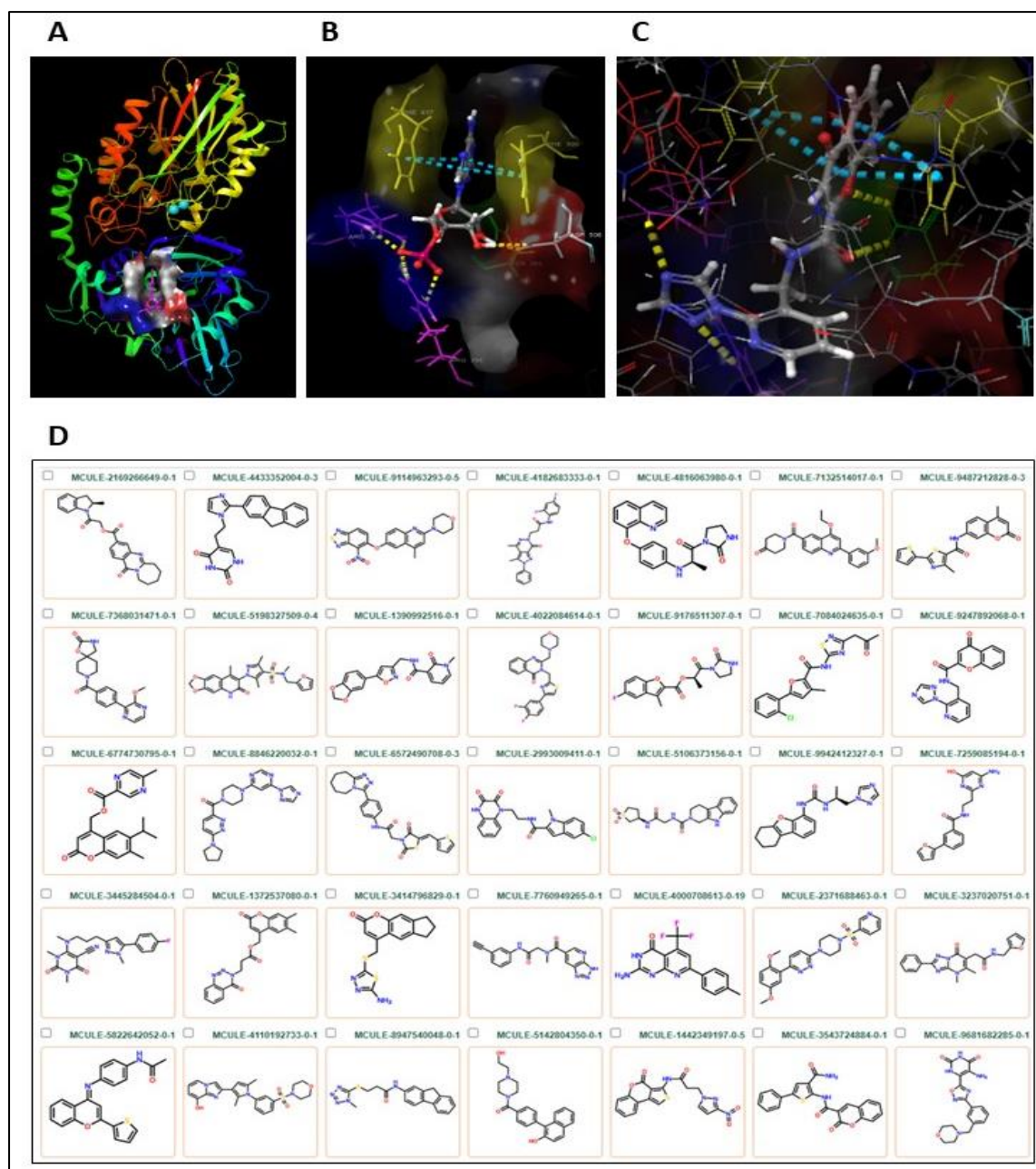
Figure 2.3: *Computer-aided structure-based identification of novel CD73 inhibitors.* (A) Monomeric CD73 docked with AMP (purple). Structure illustrates the N- (yellow and orange ribbons) and C-terminal (deep and light blue ribbons) domains linked by a flexible α-helix chain (green ribbon), and the two light blue Zinc ions essential for catalysis. (B) AMP's essential amino acid residue interactions at the substrate binding site of CD73. (C) A typical example of a lead compound satisfying the scoring criteria. (D) Top-ranked 35 identified compounds that made it through the exclusion criteria.

None of the identified compounds significantly antagonized CD73 like AMPCP

The 5'-nucleotidase, CD73, classically converts AMP to adenosine and serves as the rate-determining enzyme modulating adenosine-mediated immunosuppression in the TME [13]. A simple excellent assay to therefore validate the potency of potential inhibitors is the adenosine production inhibition assay using CD73 expressing cells or purified recombinant CD73 protein. To assess the inhibitory profiles of the identified 35 compounds, we incubated MEC-1 cells with the potential inhibitors and AMP, and then measured the amount of adenosine produced at several time intervals (figure 2.4A). Disturbingly, almost all the identified compounds converted AMP to adenosine at a similar rate as the negative control (figure 2.4B). While that represented a bracket range of 0-50% inhibition of CD73's enzymatic activity for most of the compounds, AMPCP, the positive control, abrogated the action of the ectoenzyme completely (figure 2.4C).
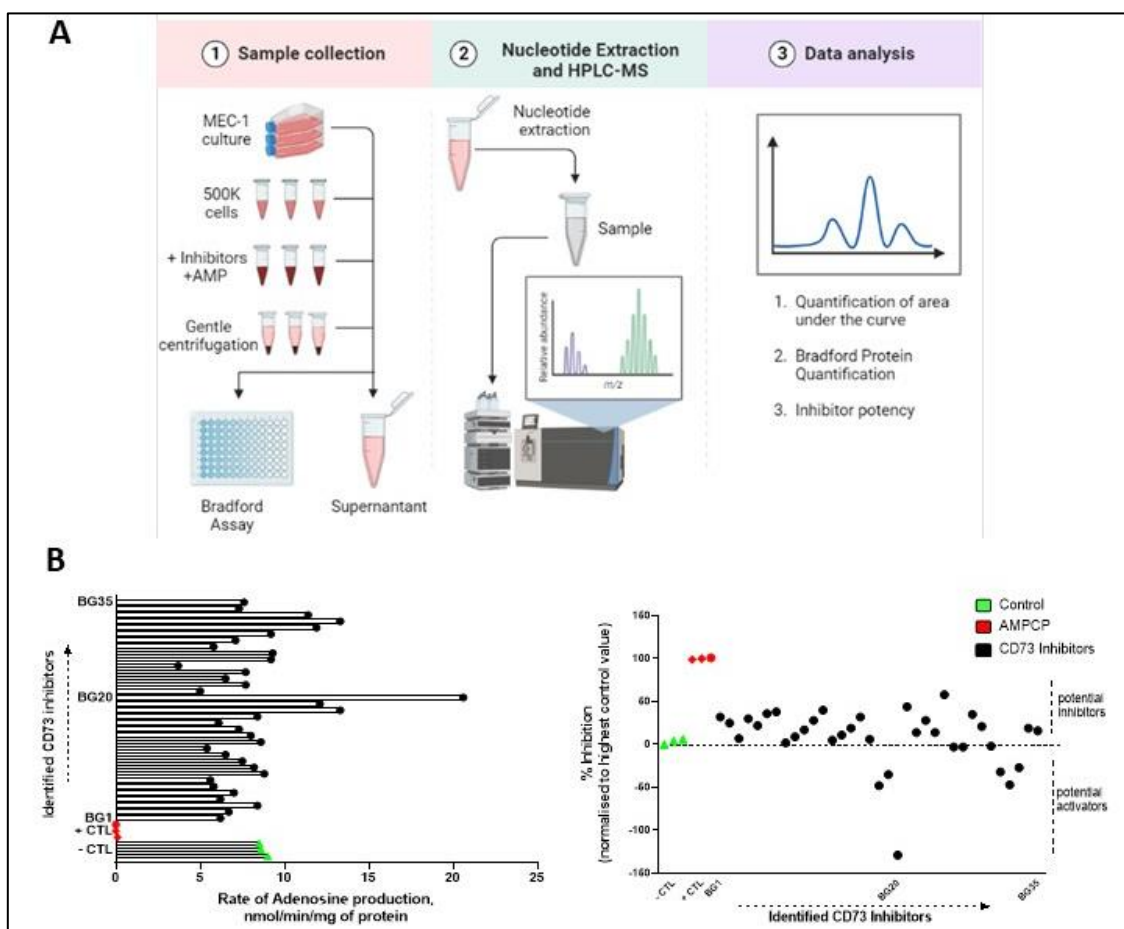
Fig. 2.4: *Identified compounds did not significantly inhibit CD73's action compared to AMPCP*. (A) Workflow showing MEC-1 treatment, sample preparation, nucleotide measurement and data analysis. (B) Measurement of inhibitory profiles of the identified compounds. Almost all ligands converted AMP to ADO at similar rates to the negative control. BG20, an exceptional one, could be a promising activator of CD73.

The effectiveness of virtual screening in identifying new drug compounds, among several factors, depends on the drug target strategy, molecular docking software and to a greater extend, on the library of compound database [14, 15]. Consequently, the quality of Mcule database and the robustness of the docking algorithm could affect the identification of potential drug molecules, and hence the above results. Since the intent of virtual screening and preclinical assays is to identify lead compounds from which further structural optimization processes could be carried out, ligand BG25 with 58% inhibition could be a potential lead CD73 inhibitor. Further structural optimization to engage in further hydrogen bonding with essential residues of the catalytic site and spatial water molecules could significantly improve the potency of the lead compound.

BG20 significantly activates, not inhibits, CD73

May the serendipity of science be with us! Intriguingly, the cell-based inhibition assay revealed one far outlier; a potential activator of the enzymatic activity of CD73. The rate of adenosine production more than doubled in the presence of BG20, recording an activation rate as high as 129% compared to the control (figure 2.4B). It is arguably impossible to compete for binding with the natural substrate of an enzyme and at the same time, increase the catalysis of the enzyme for the same substrate [16]. Therefore, the mechanism of action of BG20 is most likely through a non-competitive allosteric binding where it may enhance effective substrate binding, conformational changes and substrate release, ultimately enhancing the rate of catalysis.

The tumor-associated immunosuppressive and cancer promoting roles of CD73-derived adenosine have been well documented. Accumulating evidence however highlights beneficial roles in the central nervous [17, 18], cardiovascular [19] and respiratory systems [20], and in tissues such as the kidney and liver where preclinical studies have reported significant protection against ischemia-reperfusion injury [20, 21]. During spinal cord injury for example, CD73 safeguards against inflammatory damage by mediating a p38 MAPK-dependent microglia/macrophage M2 polarization [18]. These clearly highlight potential use cases for CD73 activators and hence BG20. Further studies to optimize and characterize these potential CD73 ligands (BG20 and BG25) is a laudable pursuit in the immediate future.

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CHAPTER THREE

Tumor-immunomodulation studies of a novel A2A receptor antagonist in combination with Venetoclax in Chronic Lymphocytic Leukemia (CLL) models.

3.0 Background

Because of its dominant immunosuppressive role in the TME of most tumors, components of the adenosinergic pathway have been validated as potential therapeutic targets. For example, small molecule and monoclonal antibody-mediated inhibition of CD73 has been shown to suppress tumor growth independent of its effect on immune cells, inhibit angiogenesis and attenuated tumor metastasis [1-5]. In addition, CD73 gene-silencing or pharmacological targeting improves antitumor immune responses of inhibitors of immune checkpoint molecules such as PD-1, CTLA-4 and A2AR in colon, prostrate, breast, carcinoma, and melanoma tumor mice models [3, 6, 7]. The A2A receptor is particularly of great interest as a cancer therapeutic target because of its broad distribution, high affinity for adenosine, consideration as a master regulator of several checkpoint pathways and the fact that quite several compound antagonists of A2AR are already in clinical trials for the treatment of Parkinson's disease [8-12]. Though many small molecules such as Corvus' ciforadenant (CPI-444), iTeos' inupadenant (EOS100850) and Novartis' taminadenant (NIR178) and monoclonal antibody A2AR inhibitors have been developed and are in clinical trials for the treatment of diverse cancer indications, none has made it to the FDA's drug approval list, partly, owing to complicated trial design problems and agonizing effects of high concentrations of A2AR inhibitors.

a. A2AR monotherapy blockade in preclinical models

Monotherapy strategies to target the hostile, hypoxia partly-driven, A2AR adenosinergic signaling in the TME have been extensively studied and validated in hematological malignancies like CLL and in some solid tumors [13, 14]. The Sitkovsky group has been very instrumental in this regard. Been the first to demonstrate the complete rejection of immunogenic RMA T lymphoma and CL8-1 melanoma cell lines in A2AR double KO mice, the

group's work also showed that pre-treatment of CD8+ T cells with A2AR antagonist or siRNA significantly improved tumor growth inhibition and abrogated tumor metastases when adoptively transferred into tumor bearing mice [15]. CD73 expression is known to promote aggressive metastasis through A2AR and A2B receptor activation in most tumors [5, 16, 17], and has been identified as a marker for poor clinical treatment outcomes [18-20]. The report of Beavis *et al* and others have shown that blocking A2AR or A2B receptors with small molecule antagonists markedly reduced metastasis in both CD73+ breast cancer and melanoma mice models in a perforin-dependent manner [17, 21, 22]. Moreover, A2AR, not A2B, blockade markedly enhanced the maturation, granzyme B expression and cytotoxic activities of NK cells [21], suggesting a possible metastasis-promoting mechanism independent of NK cells.

b. A2AR combination therapy blockade in preclinical models

Efforts to downplay therapy-acquired adenosine-mediated immunosuppression and improve the impressive clinical responses of immune checkpoint blockade (ICB) therapies have received much traction in the last decade. Accordingly, a number of studies combining inhibitors of A2AR and PD-1/PD-L1 or CTLA-4 have been performed; altogether demonstrating a significant reduction in tumor growth and metastases and increased antitumor immune responses mostly by enhancing CD8+ T cell activity and recruitment into tumors.

The first preclinical study involving these 2 axes was published in 2014. In both melanoma and breast cancer mouse models, Mittal *et al* [23] elucidated the effects of blocking A2AR and PD-1 with SCH58261 and RMP1-14 respectively on experimental and spontaneous lung metastases. Largely by INFγ- and NK cell-mediated mechanisms, the team reported a significant reduction in metastatic burden and prolonged survival in mice that received the combination therapy compared to single agent treatments. The mechanistic pathways in play are not quite surprising as A2AR signaling blockade has been reported to suppress metastases in a perforin-NK cell-dependent manner [21] and PD-1 inhibition results in an INFγ-mediated increased migration of T cells into tumors [24]. A year after, Beavis and Colleagues [25] documented a similar observation with the same inhibitors used in the previously discussed study in addition to a Phase 11b A2AR inhibitor SYN-115, but this time, in well-established mouse breast carcinoma AT-3, colon cancer MC38 and less immunogenic, highly metastatic 4T1.2 breast cancer cell lines. Though combination treatment *in vitro* enhanced the cytokine

production of CD8+ T cells co-cultured with cancer cell lines, tumor proliferation was unaffected, suggesting a coordinated effort of the immune cell repertoire may be required for a significant impact on tumor growth. Intriguingly, blocking PD-1 with mAb RMP1-14 in mice bearing tumors increased A2AR expression on tumor-infiltrating CD8+ T cells. Upon additional blockade of A2AR with either SCH58261 or SYN-115 however, the group noted a significant reduction in tumor burden, and INF-γ and Granzyme B production by CD8+ T cells compared to each monotherapy, signifying that the efficacy of A2AR/PD-1 blockade on tumor growth might be mainly mediated by activated CD8+ effector T cells.

Following these two preclinical studies, two independent experiments were published in 2018, using a newly discovered, selectively potent oral A2AR inhibitor CPI-444, currently known as ciforadenant. Leone *et al* [26] titrated the effect of antagonizing A2AR signaling alongside blocking PD-1 with RMP1-14 while Miller's team [27] explored a combination therapy with anti-PD-L1 or anti-CTLA-4 antibodies. Leone and Colleagues, in addition to similar results as their predecessors, noted that antagonizing A2AR signaling in mice-bearing tumors significantly repressed LAG-3 and PD-1 expressions on both CD4+ Tregs and effector CD8+ T cells, lending further evidence to A2AR signaling as a master regulator of several checkpoint pathways and the rationale to aggressively consider A2AR targeting as the missing piece to a more effective CLL treatment strategy. Miller's lab on the other hand reported an intriguing observation: mice with cleared tumors after combination therapy completely rejected the growth of a later tumor challenge even in the absence of further treatment. Since antitumor responses were mainly mediated by CD8+ effector T cells with breaks on their A2AR signaling, ciforadenant as a monotherapy may have induced the observed systemic immune memory.

c. A2AR blockade in Clinical Trials

The first and current clinical trial combining inhibitors of A2AR (ciforadenant) and PD-L1 (atezolizumab) was performed in 68 renal cell cancer (RCC) patients, who at the time of recruitment, were resistant to multiple former therapies; 72% been refractory to anti-PD-1/PD-L1 mAbs [28]. The study funded by Corvus Pharmaceuticals, developers of ciforadenant, reported durable clinical responses, however, in a considerably low proportion of patients in both arms of treatment, even in those who were previously resistant to anti-PD1/PD-L1 therapy. While 3% and 11% of the patients responded partially to ciforadenant monotherapy

and combination therapy respectively, a 6-month disease control rate (DCR) of 39% was confirmed in ciforadenant plus atezolizumab-treated patients compared to 17% when ciforadenant was used alone. Strikingly, the overall survival rate at 25 months for the combination therapy arm exceeded 90% and was at least 69% after 16 months of ciforadenant monotherapy treatment. The team discovered such a response positively correlated with increased activation and tumor infiltration of effector CD8+ T lymphocytes, broadened repertoire of circulating T lymphocytes and adenosine-related gene signature expression. Although combination-treated patients developed immune-related adverse events, grade 3/4 events were not frequent in patients who received ciforadenant alone. Dose tolerability and/or drug safety is indeed a thing of critical consideration here, but the clinical trial nonetheless demonstrated the relevance of targeting A2AR signaling, especially in cancer models or patients with acquired resistance to immune checkpoint therapies. It remains, however, to be seen how patients with different cancer types, especially CLL, would respond to dual targeting of A2A receptor and PD-(L)1/CTLA-4. Putting together, we are of the opinion that current frontline CLL therapies such as inhibition of BTK and BCL-2 may improve profoundly when combined with A2AR signaling inhibitors since adenosine-related immunosuppressive acquired resistances often develop with these therapies [29].

iTeos' inupadenant has had some successes as a monotherapy and in combination with pembrolizumab in adult patients with castrate-resistant prostate cancer, BRAF wild-type cutaneous melanoma and in several immune resistant advanced solid tumor patients with multiple indications [30, 31]. In these previously treated patients, inupadenant was active, well tolerated and showed a manageable safety profile even at the maximum dose of 160 mg twice a day (BID). Plasma concentrations of inupadenant increased with dose escalation, and impressively sustained and prolonged inhibition of A2AR signaling over the dosing interval. After a year of treatment, 6 patients with multiple indications had a stable disease, and the 24-month clinical response analysis showed 3 patients with stable disease and 2 durable partial responses in castrate-resistant prostate cancer and BRAF wild-type cutaneous melanoma patients [31]. Further evaluation of efficacy, tolerability and safety of inupadenant as a monotherapy and in combination with pembrolizumab or chemotherapy is ongoing in the expansion cohort of selected indications, particularly in PD-1 resistant melanoma [30].

d. Research hypothesis and Goals

Small molecule and antibody-mediated therapeutic targeting of adenosine-A2AR signaling has enjoyed the limelight glories over decades. Blocking A2AR in combination with immune checkpoint blockades (ICB) like anti-CTLA-4 and anti-PD-(L)1 in preclinical melanoma, breast cancer and MC38 colon cancer mouse models and in renal cell cancer patients have been shown to significantly reduce tumor growth and metastases and increased anti-tumor immune responses in combination therapy compared to single treatments. Recently, we demonstrated profoundly the restoration of immune competence by pharmacologically inhibiting A2AR signaling in a TCL1 adoptive transfer CLL mouse model [32]. By gavage administering SCH58261 every other day, our lab observed a significant reduction in the CD4+ regulatory T cell population, increased numbers and secretome of cytotoxic CD8+ lymphocytes, and a marked shift of infiltrating monocytes towards the inflammatory subtype. The downer, however, was that no measurable effect on tumor burden was observed. Being the first of its kind in a CLL model, our work has strengthened the therapeutic benefits of targeting the A2AR receptor on host immune cells and arguably heightened combination strategy attempts incorporating A2AR inhibitors as an emerging effective treatment option for CLL.

CLL is particularly characterized by an overly increased expression of BCL-2, an anti-apoptotic protein of the BCL-2 family of apoptosis regulator proteins. Consequently, venetoclax – a selective, highly potent inhibitor of BCL-2 had the first FDA approval for the treatment of CLL upon showing deep clearance of disease burden in bone marrow and peripheral blood [33, 34]. As a monotherapy, and in combination with anti-CD20 mAbs and ibrutinib, venetoclax has demonstrated impressive clinical responses, making it a go-for-drug in the management of CLL, especially in high risk patients [35, 36]. Despite these outcomes, CLL still remains incurable, partly, due to the high degree of targeted therapy and immune cell resistances in the overly hypoxic, increased A2AR signaling, immunosuppressive proliferating centers of leukemic B cells.

Taking cues from the limited combination studies involving these axes, we hypothesize that a dual-combination approach of disrupting the CLL anti-apoptotic machinery with venetoclax and inhibiting A2AR signaling with iTeos EOS850 on both tumor and immune cells present a

potential, more effective therapeutic intervention in significantly enhancing antitumor immune cell responses and drastically clearing total disease load.

3.1 MATERIALS AND METHODS

CLL patient samples, TCL1 clones and culture conditions

CLL blood samples were obtained in accordance with University of Turin Institutional Guidelines and Declaration of Helsinki. PBMCs from these were isolated according to established protocols. Eight T-cell leukemia/lymphoma 1 (TCL1) murine clones were obtained from the Biobank of Prof. Deaglio's lab. By adoptively transferring TCL1 leukemic cells into normal immunocompetent C57BL/6 mice, these recipients, due to the genetic overexpression of the TCL1 oncogene, develop lymphoproliferation resembling CLL late in life. Freshly harvested peritoneal cavity (PC) TCL1 leukemic cells are available for *ex vivo* assays within 1-6 weeks of lymphoid and peripheral tissue full colonization. Cells were cultured in AIM V medium (Thermofisher) at normoxic conditions of 21% O_2, 5% CO_2 and 37°C. Where storage cells were used, cells were quickly thawed on ice in RPM1-1640 supplemented with 10% FBS and allowed to recover at 37°C for at least an hour before seeding and treatment.

Reagents and Antibodies

EOS100850 (A2AR antagonist), hereafter referred to as EOS850, EOS or inupadenant, was generously provided by iTeos Therapeutics. Venetoclax and ibrutinib were purchased from Selleckchem. ATP, ADP, ADO, INO, APCP (CD73 inhibitor), SCH58261 (A2AR inhibitor) and HPLC-grade acetonitrile and methanol were bought from Sigma-Aldrich and pentostatin and 5-iodotubercidin from Tocris Bioscience, Bio-Techne. Western blotting rabbit polyclonal unconjugated antibodies (Parp 1, Caspase 3, BCL-2, MCL-1) were obtained from Cell Signaling Technology. Full list of antibodies used for flow cytometry are provided as supplemental information.

Structural comparison, mechanism of inhibition and A2AR inhibitory analysis of EOS850

iTeos Therapeutics, per company guidelines, were restricted in disclosing the structure of EOS850 to us. Notwithstanding, to better appreciate the nature of the compound we were working with, extensive literature review on reported patented structures, its mechanism of inhibition and both preclinical and clinical studies was conducted.

Apoptosis Assay

Freshly harvested TCL1 leukemic cells and thawed CLL primary cells were seeded at a density of $5x10^6$ cells/mL AIM V, allowed to recover and treated with 5µM EOS850, 10µM SCH58261, 10µM Ibrutinib and 25nM (TCL1) or 1.5nM (CLL) Venetoclax at normoxic culture conditions. For TCL1 cells, EOS850 and Venetoclax were replenished daily, and SCH58261 and Ibrutinib, every other day. Venetoclax was however not replenished for CLL primary cell cultures. Rate of apoptosis at 24-72 hours were then measured with an Annexin V/PI apoptosis kit (eBioscience, Thermofisher) according to established protocols. Briefly, collected cells were washed twice with PBS at 1500 RPM for 5 mins, washed once with 1x Annexin buffer and incubated with 3 in 100 µL diluted Annexin V reagent for 15 mins at room temperature in the dark. After washing, cells were incubated with 3 in 100 µL PI for 5 mins in the dark and read with FACSCelesta flow cytometer (BD Bioscience). Data was analyzed using FACS gating strategy.

MS/UPLC measurement of extracellular adenosine

Cells were cultured at $5x10^6$ cells/mL AIM V in 24 well-plates at normoxic conditions and treated as indicated in apoptosis assay above. To prevent excessive adenosine breakdown, Adenosine Stabilizing Buffer, ASB (10 µM Pentostatin + 1 µM 5-Iodotubercidin) was added daily. Culture supernatants were carefully collected on ice, centrifuged twice at 1500 RPM for 5 mins and either stored at -80°C or prepared for nucleotide separation. Standard calibration curve for Adenosine was obtained in freshly prepared AIM V medium supplemented with ASB. Separation and quantification of the adenosine was carried out using UPLC (Ultra Performance Liquid Chromatography)/ESI-MS on an Acquity H-class UPLC system coupled to an AcquityQDa Detector. A Kinetex F5 1.7µm, 100 Å, 50 x 2.1 mm with Van Guard Pre-column was used by isocratic elution with mobile phase A (water 0,1 % TFA) set at 98% and mobile phase B (acetonitrile) set at 2%. Flow rate was 0.2 ml/min for a total UPLC/MS analysis time of 5 min (for each sample). The column was kept at 40°C. ESI (+)-MS was carried out either in the full scan mode (*m/z*250-800) or in Selected Ion Monitoring (SIM) mode. Instrumental MS conditions were: Capillary voltage 0.8kV, Cone voltage 20V, Source Temperature 120°C, Probe temperature 600°C. The signal of adenosine was acquired in SIM (Selected Ion Monitoring) mode to improve sensitivity, at *m/z* = [M+H]$^+$= 269.1. The set-up of the method involved the acquisition of calibration curves obtained by adding to freshly prepared AIM V medium and

H$_2$O-TFA 0,2% (1:1 v/v) aliquots of an appropriate adenosine standard solution to a final concentration in the range 0.015 µM- 2.5 µM. The solution was filtered through a 0.22 µm PP filter membrane. A 4 µL aliquot of the filtrate was subsequently injected into the UPLC system. Relative peak areas were plotted against the concentrations of each analyte, a good correlation coefficient (r = 0.953) was obtained.

Western blot analysis

5x10^6 cells from apoptosis assay experiments were twice washed with PBS at 4500 RPM for 5 mins and then lysed for at least 30 minutes on ice with lysis buffer supplemented with a cocktail of protease inhibitors. Lysates were then resolved using a standard SDS-PAGE apparatus (Bio-Rad), electroblotted onto nitrocellulose membranes (Bio-Rad) and developed according to manufacturer's instructions. Proteins assayed are indicated under reagents and antibodies above. Chemiluminescent bands were detected using HRP-conjugated goat anti-mouse antibody (PerkinElmer). HRP-conjugated mouse anti-actin antibody (Santa Cruz) was used as the loading control. Images were acquired using ChemiDoc MP imaging system and analyzed with the Image Lab software (Bio-Rad).

FACS analysis

Single cell suspensions in PBS were washed with RIA buffer and then adjusted to a concentration of 1-5 x 10^6 cells/mL in ice cold PBS, 10% FCS, 1% sodium azide or RIA solution. About 0.1-10 µg/mL of conjugated primary antibodies were then added and incubated for at least 30 min in the dark at 4°C. Cells were then washed at 1500 RPM for 5 min, resuspend in 500 µL to 1 mL of ice-cold PBS, 10% FCS, 1% sodium azide and analyzed with FACSCelesta flow cytometer. Data was analyzed using the FACS gating strategy approach according to established protocol.

In vivo analysis of tumor burden and immune cell activity

8-week-old female C57BL/6 mice were injected intraperitoneally with 10^7 cells of #355 and #2009 TCL1 leukemic clones. Within 3 days to 4 weeks of engraftment (depending on the clone) when peripheral blood leukemia is > 10%, mice were randomly assigned into treatment arms (control, EOS850, Venetoclax and EOS850+Venetoclax) and administered with 10 mg/kg EOS850 and 50 mg/kg Venetoclax by gavage for 14 consecutive days. Control mice received

the solvent-vehicle in which EOS850 was solubilized. All mice were euthanized same day and their respective spleens, bone marrows, PBS-washed peritoneal cavity fluid and peripheral blood were collected. Spleens were dissected into two equal parts along the long axis; part formalin-fixed and other half dismantled to obtain a single cell suspension from which immune cell populations were analyzed using FACSCelesta (BD Bioscience) according to already established protocol [32].

Statistical analysis

Data were analyzed using GraphPad Prism version 8 software. Pairwise comparisons were computed with Student *t* test. One/two-way ANOVA was used to test differences among continuous variables of experimental groups with equal amount of data. Where otherwise, mixed-effects analysis was applied as specified in the figure legends for each graph. Results are shown as mean ± SEM. A comparative test is significant when P-value is ≤ 0.05.

3.2 RESULTS AND DISCUSSION

EOS850-mediated A2AR signaling blockade potentiates venetoclax's apoptotic effects *in vitro*

Mechanistically, A2AR signaling by tumor cells, in part, reinforces survival pathway stimuli and upregulates immune escape molecules such as PD-1 and CTLA-4 [37]. Consequently, antagonizing A2A signaling in tumors has been viewed as a potential strategy to shut down these mechanisms and ultimately induce apoptosis. To explore the apoptotic effects of inhibiting this axis in CLL and examine any potential synergy with venetoclax, a dose-escalation titration assay with venetoclax (Selleckchem) was first performed using freshly harvested murine TCL1 leukemic cells. Within a concentration range of 0.3 to 5μM, inupadenant as a single agent had no marked apoptotic activity (figure 3.2A). However, a combination treatment with 25nM venetoclax significantly increased annexin V/PI-double positive apoptotic cells, especially at a dose concentration of 2.5μM and above (figure 3.2A). Overall, the rate of apoptosis of both primary CLL and TCL1 leukemic cells was significantly higher in combination treated wells compared to single agents after 48 hours (figure 3.2B and C). In addition, inupadenant-venetoclax combination treatment led to more CLL cytotoxicity compared to SCH58261-venetoclax treatment (figure 3.2E), highlighting inupadenant's strong cooperativity and ability to highly potentiate venetoclax's apoptotic effects over SCH58261.

Though EOS-850 treatment alone showed minimal to no apoptosis induction at the tested concentration, a potential synergistic mechanism may bridge the two pathways to produce the observed significant rate of apoptosis in the combination treatment. Initially, it was hypothesized that apoptosis induction by venetoclax causes excessive cellular stress, and in a survival attempt to offset this, CLL cells overly upregulate A2AR expression and signaling, which, when antagonized by EOS-850, would significantly potentiate venetoclax's apoptotic effects. Contrary, preliminary RT-qPCR results (data not shown) showed that A2AR expression levels remain unchanged irrespective of drug treatments, suggesting a venetoclax-induced A2AR upregulation independent mechanism of cooperativity between the two pathways. Since receptor activity could increase despite no variations in expression levels, further studies elucidating modulatory changes in activity of A2AR and stimulatory G proteins upon venetoclax treatment may aid in redefining the above hypothesis.
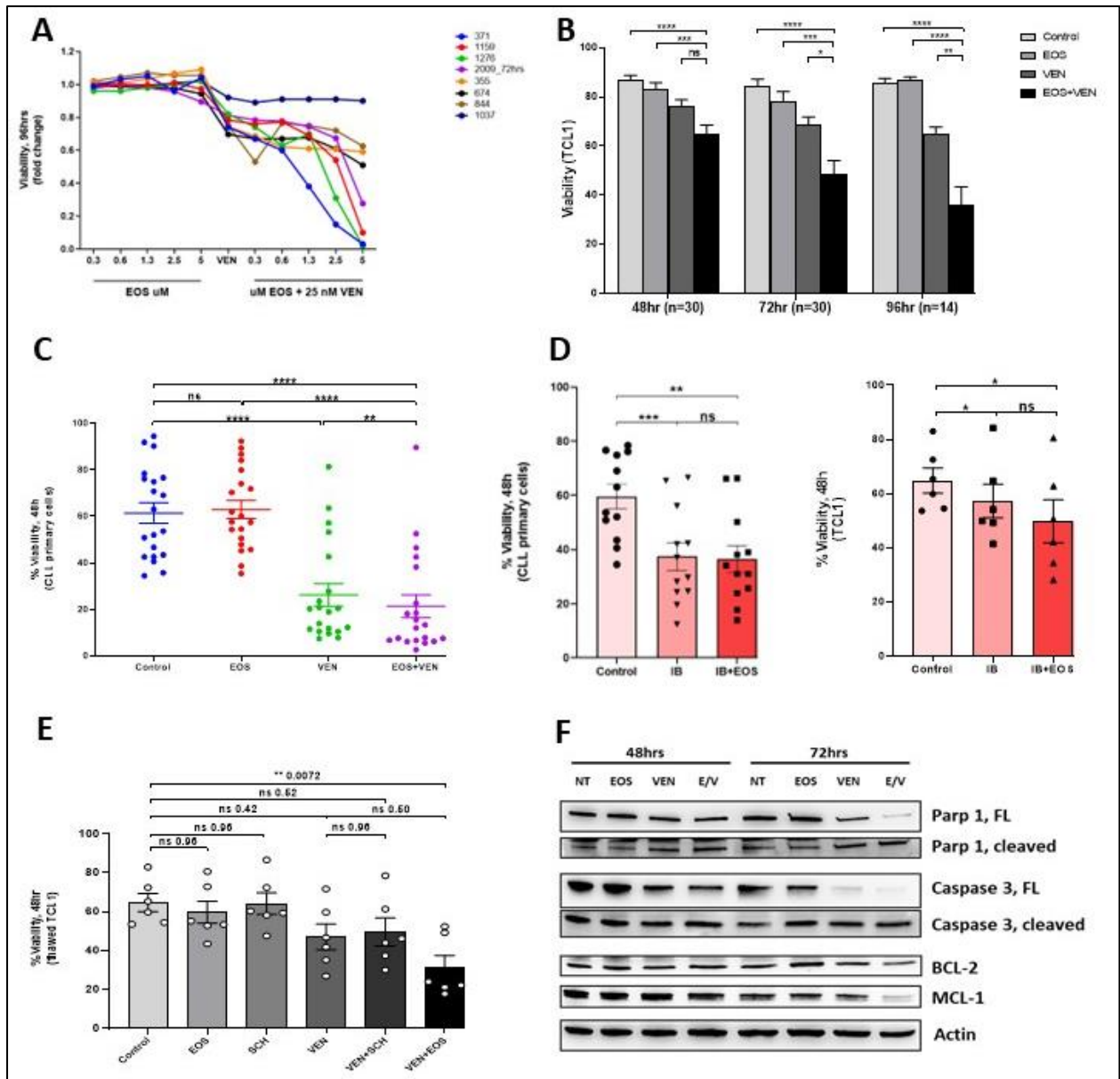
Figure 3.2. Blocking A2AR signaling improves venetoclax effects in primary CLL and TCL1 cells. (A) Dose-escalation assay of EOS-850 in combination with 25 nM venetoclax in 8 TCL1 leukemic cell clones cultured under normoxic conditions for 96hrs except #2009. 5 nM EOS-850 concentration was chosen for further ex-vivo assays. **(B-C)** EOS-850 and venetoclax combinational treatment of primary CLL (n=20) and TCL1 cells (n=30) under normoxic conditions. Percent viable cells and statistics were analyzed with Annexin/PI kit and mixed-effects model (REML) Turkey's multiple comparisons test respectively. **(D-E)** Comparable viability assay of TCL1 (n=6) and CLL primary cells (n=12) treated with EOS-850 and ibrutinib **(D)** and EOS-850 or SCH58261 and venetoclax **(E)**. Statistics were calculated using the student two-tail paired t test **(D)** and ordinary one-way ANOVA Turkey's multiple comparisons test **(E)**. Western blot analysis of induction of apoptotic proteins over 48- and 73-hour time course (FL = full length, CL = cleaved). Statistical significance was measured at P ≤ 0.05 with the following designations of significance: * (0.05≥P>0.01); ** (0.01≥P>0.001); *** (0.001≥P>0.0001) and **** (0.0001≥P).

Pharmacological effects of EOS-850 may be most amplified in adenosine-rich environment

The eventual hydrolysis of extracellular ATP and other nucleotides to adenosine normally outweighs its deamination to inosine in the tumor niche, leading to adenosine's extracellular accumulation [38]. By antagonizing adenosine's binding to A2AR, levels of extracellular accumulated adenosine could further increase as more of the molecule would exist in the receptor-unbound state. In line with this, we sought to additionally examine whether EOS-850 is on target by measuring adenosine levels of culture supernatants using mass spectrometry/high performance liquid chromatography (MS/HPLC). Venetoclax induces high rate of cell death and consequently, generated elevated levels of adenosine in both primary CLL cells and TCL1 leukemia (figure 3.3A). Intriguingly, inupadenant-venetoclax dual incubation further markedly increased adenosine levels, suggesting that inupadenant is on target and can potently antagonize adenosine binding to A2A receptor and/or can significantly induce apoptosis of venetoclax-primed or stressed cells. Surprisingly, though non-significant, extracellular adenosine concentrations were relatively lower in inupadenant single agent-treated cells compared to the control (figure 3.3A). An allosteric binding site for EOS-850 that could effectively lock adenosine to its receptor, reduce its extracellular levels and concomitantly impede its signaling may explain this contrasting observation. In fact, report by iTeos Therapeutics suggests a non-competitive binding mode of inhibition for EOS-850. Moreover, *ex vivo*/*in vitro* treatment with 5uM inupadenant for an extended duration may slightly agonize A2AR signaling, leading to trivial increments in cellular viability and reduced extracellular production of adenosine (figure 3.2B). Since adenosine receptors in different tissues in general could respond differently to varying concentrations of small ligand molecules, the dosing amounts of inupadenant need to be carefully considered, at least, for their use cases in CLL.
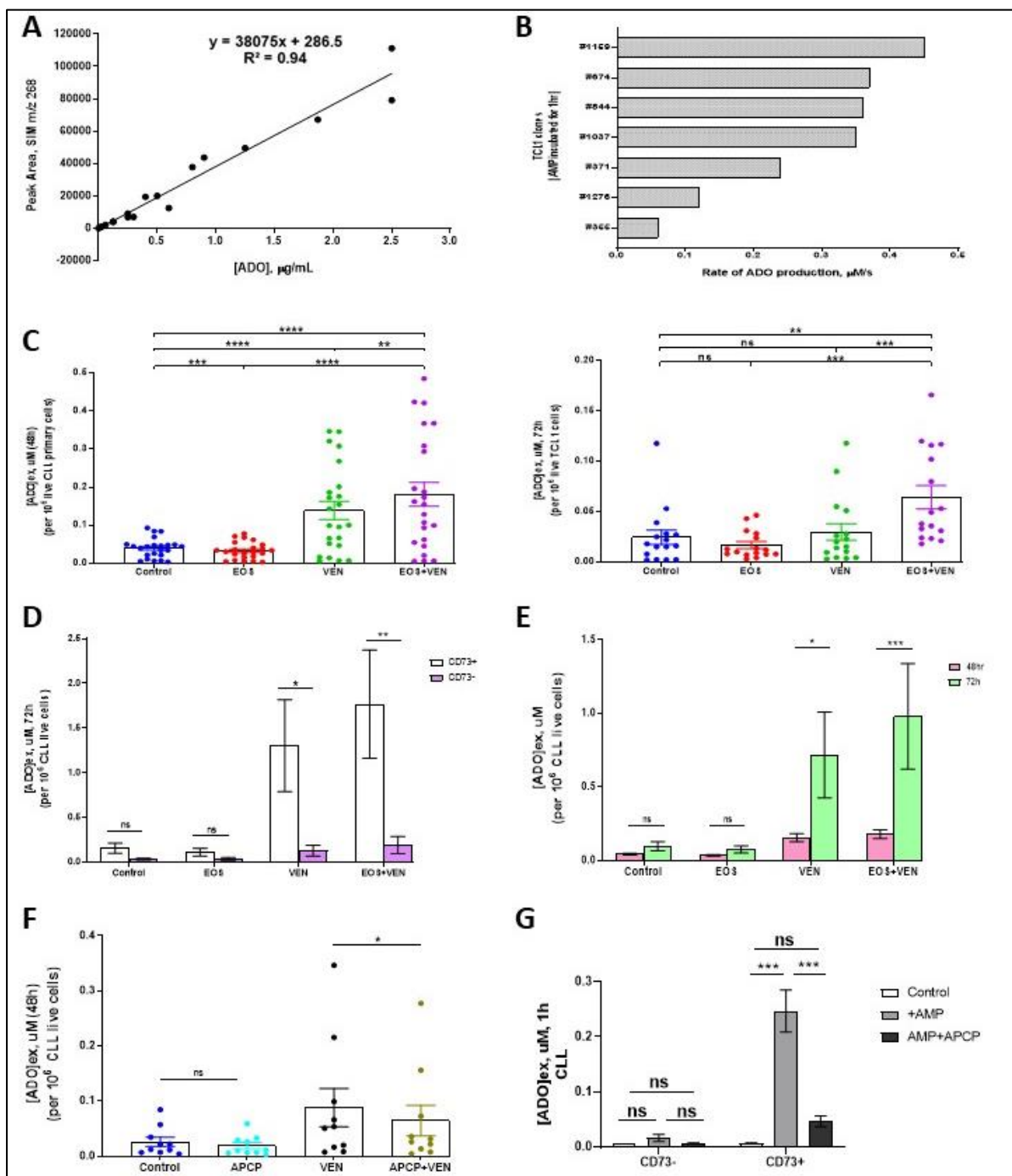
Figure 3.3. Antagonizing A2AR signaling builds up CD73-derived extracellular adenosine in the presence of venetoclax. (A) Calibration curve for standard concentrations of adenosine in AIM V. Concentrations of adenosine in culture supernatants were extrapolated from the obtained line of best fit **(B)** Assessment of the activity and/or levels of CD73 expressed by different clones of TCL1 leukemic cells by measuring the rate of conversion of AMP to adenosine. **(C-E)** Measurement of adenosine by UPLC/MS in culture supernatants of EOS-850 and venetoclax treated cells: **(C)** of TCL1 clones and CLL patient samples under normoxic conditions for 72 and 48 hours respectively, **(D)** of primary CLL samples distinguished by the expression levels of CD73. Samples with CD73 levels below 20% by immunofluorescence staining were designated CD73⁻, and **(E)** of primary CLL samples cultured for 48 and 72 hours. **(F-G)** Assessment of the effect of blocking CD73's enzymatic activity on the accumulation of extracellular adenosine in CLL primary cells **(F)** treated with or without venetoclax for 48 hours and **(G)** stratified based on CD73 expression levels and incubated with AMP for 1 hour. Statistical significances were analyzed with ordinary one-way ANOVA Turkey's multiple comparisons test (C), two-way ANOVA Turkey's multiple comparisons test (D, E and G) and student paired t test (F). A statistic is significance when P ≤ 0.05 with the following designations of significance: * (0.05≥P>0.01); ** (0.01≥P>0.001); *** (0.001≥P>0.0001) and **** (0.0001≥P).

Both venetoclax and ibrutinib have demonstrated profound induction of CLL apoptosis in both preclinical and clinical studies as single agents and in combination [39-43]. Indeed, pharmacological and protein profiling *ex vivo* and *in vitro* studies of the apoptotic effects of idelalisib, bendamustine, ibrutinib and venetoclax on CLL primary cells obtained from patients on ibrutinib suggests venetoclax and ibrutinib as optimal partners in inducing the highest cytotoxicity [44]. Because A2AR-mediated immunosuppression is a common feature of the CLL proliferation niche, and a well-known mechanism of counteracting drug-induced apoptosis [45], we additionally tested the level of cooperativity between A2AR signaling blockade and BTK inhibition. In both primary CLL and TCL1 cells, dual ibrutinib and EOS-850 incubation produced no greater cell death compared to ibrutinib alone (figure 3.2D). This partly suggests that the apoptotic effects of inupadenant are most amplified in an adenosine-rich environment as demonstrated by the high accumulation of extracellular adenosine in venetoclax treated wells or its effects are significantly heightened by a venetoclax-mediated soluble factor cross-talking with the A2AR signaling axis and/or vice versa. Venetoclax can cause BCL-2 independent metabolic reprogramming [46]. Consequently, whether the observed significant cooperativity between venetoclax and inupadenant is venetoclax specific or not remains an important question to be addressed. Is the synergy directly modulated by BCL-2 inhibition or venetoclax-mediated mitochondrial reprogramming and/or another pathway if cooperativity is venetoclax-specific? Pre-clinical studies combining A2AR blockade with other apoptosis-inducing agents, and in BCL-2 knock-out models would aid to clarify these questions.

EOS-850 synergies with VEN in inducing a significant patrolling-to-inflammatory monocyte switch *in vivo*.

Our recent report on antagonizing A2AR signaling with SCH58261 in the TCL1 transgenic mice model was phenomenal in demonstrating a significant re-awakening and activation of the tumor-attacking host immune cell repertoire [32]. Because disease burden remained unchanged, we reasoned that directly inducing tumor death with venetoclax and suppressing the undesired effect of A2AR signaling on both tumors and infiltrating immune cells with EOS-850 present a better, potential treatment approach to such CLL-like late-stage murine leukemia.

To test the above hypothesis, 8-week-old female C57BL/6 mice were injected intraperitoneally with #355 and #2009 TCL1 leukemic clones, allowed to engraft and then treated with EOS850 and Venetoclax as specified (figure 3.4A). Like our previous SCH58261 *in vivo* experiment, we observed a marked switch in the monocyte population from a patrolling phenotype to an M1-like tumor-attacking inflammatory subtype and a considerable broadened repertoire of CD4+ T cells in the venetoclax-administered mice, and even much profound in the combination arm (figure 3.4 D and E). Since CLL disease progression is characterized by a greater population of patrolling monocytes, the observed switch suggests, though the main driver is venetoclax, inupadenant can significantly aid in CLL disease control when combined with drugs that induce tumor apoptosis.

In the e*x vivo* incubation of TCL1 leukemia with drug candidates, EOS-850 single treatment was shown to have minimal to no apoptotic effect but enhanced venetoclax-induced cell death, suggesting a scenario where venetoclax treatment may sensitize and predispose CLL cells to be overly dependent on adenosinergic signaling for survival. Accordingly, we anticipated a marked reduction in disease burden in the combination treated mice compared to venetoclax monotherapy. Surprisingly, the dual treatment had a slight, non-significant reduction in spleen CD5+/B220+ leukemic total cell count, spleen weight and volume compared to single agent venetoclax, though these parameters were markedly lower when compared to the controls (figure 3.4B and C). Taking the massive colonization of spleens into consideration after treatment, our preliminary data suggests the effects of antagonizing A2AR with inupadenant on CLL burden may be much appreciated when tumors are not in their late stages.
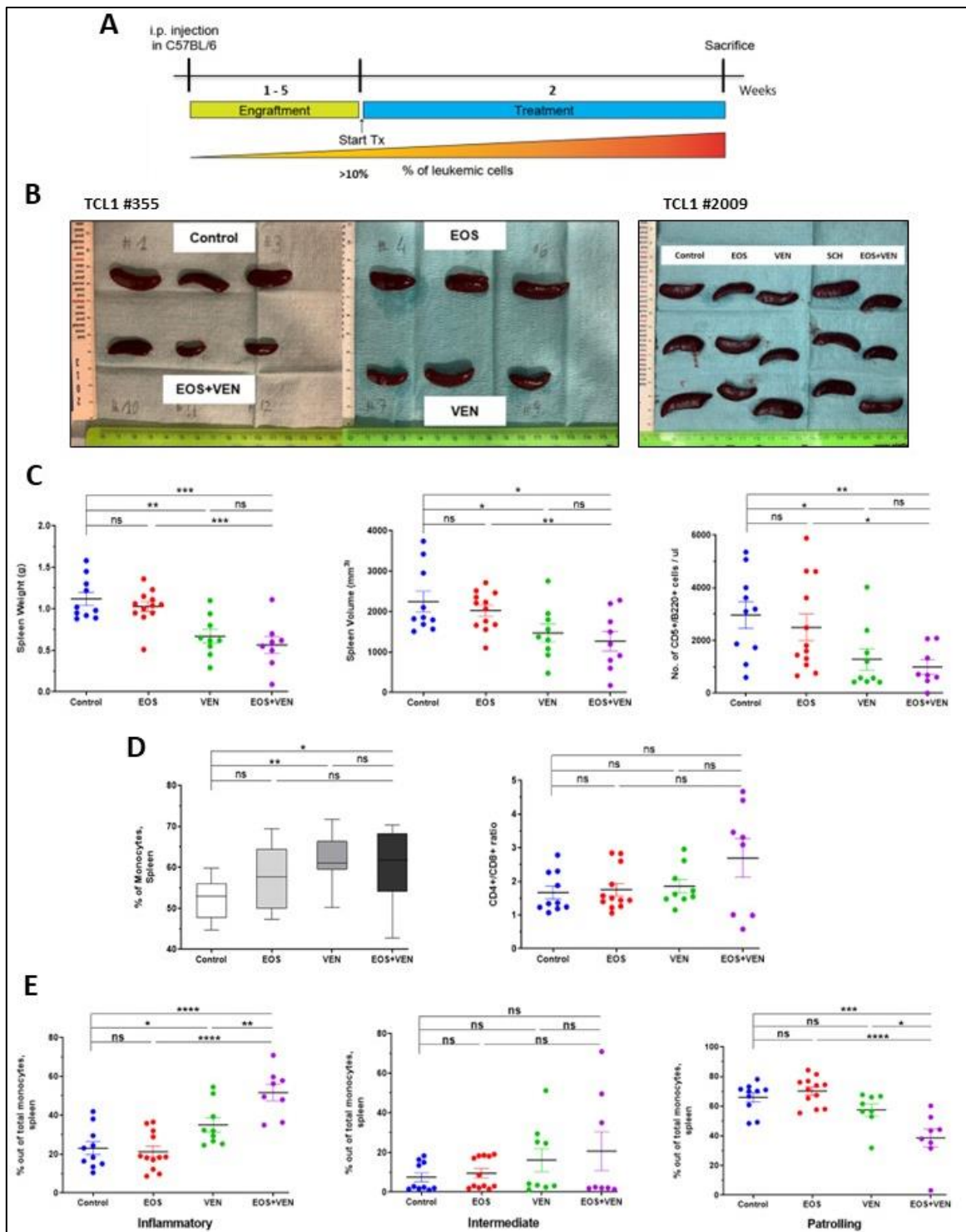
Figure 3.4: EOS850-mediated A2AR signaling blockade potentiates venetoclax's effects *in vivo*
(A) Schematic illustration of the length of leukemic cell-spleen engraftment, experimental settings, and treatment schedule. **(B)** Representative spleens of female C57BL/6 mice (n≥8 per group) treated with 10 mg/kg EOS850 and 50 mg/kg Venetoclax by gavage for 14 consecutive days compared to untreated leukemia-injected mice. **(C)** Analysis of CLL disease burden in mice spleens show considerable decrease in spleen weight, spleen volume and number density of CD5+/CD220+ B cells after treatment, the main driver being venetoclax. **(D)** left panel: box plot estimation of spleen-infiltrated total monocytes; right panel: dot plot analysis of regulatory CD4+ T lymphocytes in comparison to cytotoxic CD8+ T cells. **(E)** Assessment of monocyte subpopulations shows a marked rate of treatment-mediated M2-to-M1 monocyte switch. A2A receptor inhibition significantly potentiates effects of venetoclax in sustaining inflammatory monocytes within the TME. All statistics were analyzed with student unpaired t test with the following designations of significance: * (0.05≥P>0.01); ** (0.01≥P>0.001); *** (0.001≥P>0.0001) and **** (0.0001≥P).
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CHAPTER FOUR

Conclusions and Future Perspectives

a. A2AR Targeting – preclinical and clinical advances

Tumors are masters at escaping antitumor immune strategies and surviving the harsh, highly hypoxic heterogeneous tumor microenvironment (TME). Mounting evidence has solidified the suppressive roles of adenosine, a key component of the TME, on tumor-infiltrating immune cells and targeted therapy, while enhancing tumors to proliferate, thrive and metastasis. Consequently, CD39 and CD73; enzymatic components of the canonical pathway for adenosine production, as well as adenosine receptors, particularly A2AR, regulating its signaling have been extensively studied and validated as potential therapeutic targets [1]. A2AR therapeutic targeting has garnered much interest because of its perceived role as a master regulator of several immune checkpoint pathways, its ubiquitous expression, and high specificity for adenosine. Mechanistically, adenosine-A2AR signaling promotes stanniocalcin 2 (STC2)-mediated epithelial-mesenchymal transition (EMT) via ERK/MEK and PI3K/AKT signaling pathways to foster tumor survival, migration, and invasion [2-4]. Co-inhibition of multiple components of the adenosinergic axis, for example, blockade of CD73 and A2AR on leukocytes [5], have been shown to markedly suppress tumor growth and metastasis, and enhance immune cell tumor infiltration and activity compared to single target antagonization, suggesting that dual component inhibition holds more promise for the foreseeable future.

A2ARs are the most abundantly expressed adenosine receptors in most solid tumors and hematological malignancies. Consequently, several small molecule and monoclonal antibodies targeting the receptor are in clinical trial development for the treatment of a variety of cancers. Though monotherapies have demonstrated encouraging antitumor immunity responses mainly via increased CD8+ lymphocyte activity and tumor infiltration , combination strategies with ICBs such as anti-PD-1 and anti-CTLA-4 have shown better durable disease control compared to single agents [6, 7]. A number of these drug agents include MK-3814 by Merck, Corvus', AZD4635 (AstraZeneca/Heptares), PBF-509 by Novartis/Pablobiofarma and iTeos' EOS100850.

Recently, preclinical study reports on the impact of A2B receptor signaling on infiltrating immune cells have been very instructive [8-11]. While concerted efforts are geared toward identifying and optimizing A2AR inhibitors, there is the need to accelerate studies in developing inhibitors that could effectively target both receptors for a more robust inhibition of adenosine signaling and overall disease control.

b. Precautions to A2AR Targeting

Though under the tight regulation of HIF-1 [12], inhibition of adenosine signaling by adenosine deamination is a potential downstream target pathway worthy of the limelight. Therapeutic strategies to decrease adenosine accumulation in the TME by adenosine deamination to inosine have been proposed. Opponents to these strategies, however, have shown that inosine can preferentially trigger the ERK1/2 signaling pathway by directly engaging A2A receptors and sustaining tumor survival [13]. Moreover, as a metabolite, it can quickly be processed back to adenosine or be fed into the purine salvage pathway, potentially reinforcing tumor growth. The rationale here is to take care of the potential detrimental effects of inosine as we intend to degrade adenosine as a therapeutic strategy. Comprehensive preclinical studies on the effects of inosine on tumor growth and antitumor immune cell re-awakening/activation and cytokine secretion, particularly via A2A receptors in the TME would doubtless be eye-opening.

Caution though as the potential of bacteria-derived inosine adjuvant A2AR signaling therapy is equally enormous [14, 15]. Using three bacteria species in murine colon cancer models, Mager and his team [15] found that these intestinal microbes significantly enhanced efficacy of anti-CTLA-4 and anti-PD-(L)1 therapies via inosine production, and that inosine's activation of antitumor T lymphocytes was dependent on T cell A2AR expression and co-stimulation. The danger to this, however, is that in the absence of a robust T cell activation, (microbe-derived) inosine therapy could be counter-productive. The upside, though, is that adenosine deamination strategies could be a sweet combination therapy to ICBs or other therapeutic options that elicit a strong T cell activation. The advantage could be hugely synergistic as immunosuppressive adenosine would be eliminated from the TME, T cell activation and infiltration would be optimized, and inosine would complement the whole cascade signaling under such a context of robust T cell activation.

Truly, these ICBs have been shown to elicit significant INF gamma-mediated increased migration of T cells into tumors [16, 17]. On the contrary, blocking A2A in combination with anti-CTLA-4 and anti-PD-(L)1 treatment in preclinical melanoma, breast cancer and MC38 colon cancer mouse models and in renal cell cancer patients have been shown to significantly reduce tumor growth and metastases, and increased anti-tumor immune responses in combination therapy compared to single treatments [6, 18-20]. Were the beneficial microbes identified by Mager *et al* absent in these models and patients? If not, were their beneficial effects counteracted by the benefits of blocking adenosine-A2A signaling? Potentially, for high adenosine concentrated tumors, inosine-A2AR signaling activation might be less beneficial compared to A2AR blockade, and vice versa, as even Mager *et al* found considerably low adenosine concentrations in their tumor models. In most tumors, concentrations of adenosine could rise from nM to mM ranges. Because of the high affinity of adenosine for A2AR compared to inosine, consideration of inosine-A2AR signaling therapy must be taken with a grain of salt, especially in high adenosine concentrated tumors.

c. **Improving Clinical Outcomes**

Combination therapy is the "go-to" option in most cancer therapeutic interventions. In fact, the effectiveness of A2AR monotherapy clinical trials have been hampered by immune cell TME exclusion and inactivity through the expression of tumor-induced immunomodulatory markers such as Foxp3, LAG-3, TIGIT, PD-1, KIR and BTLA [21-23]. A striking observation we made with our recent work on the pharmacological inhibition of A2AR in a CLL adoptive transfer model was a classical significant switch of M2 patrolling to proinflammatory (M1) macrophage subtype population, revealing the potential for combination therapy involving macrophage-mediated antitumor immunity [24]. Antibody-mediated cellular phagocytosis (ADCP) is mainly mediated by macrophages. Adenosine-A2AR signaling dampens macrophage ADCP functionality through the expression of CD47 "don't eat me signal" on tumors and enrichment of the M2 macrophage phenotype in the TME. Pharmacological inhibition of CD39 markedly augmented anti-CD20 (rituximab) and anti-CD47 (daratumumab) therapies in a P2X7 receptor-dependent manner in an aggressive B-cell lymphoma in vivo model by enhancing macrophage-mediated tumor engulfment [25]. Because CD39 is upstream adenosine receptor signaling, such momentous observation suggests that A2AR signaling

blockade could potentially overcome current resistance to rituximab and other antibody drug therapies and revolutionize macrophage-mediated antitumor strategies.

Adenosine-A2AR signaling has been implicated as a key immunosuppressive arsenal invented by tumors to repress immune checkpoint inhibitor (ICH) efficacy. A recent study by Takao and Colleagues involving 60 metastatic renal cell carcinoma (RCC) patients highlighted the inverse correlation between A2AR receptor expression and patients' response to inhibitors of vascular endothelial growth factor (VEGF), PD-1 and CTLA-4 [18]. Indeed, several studies combining inhibitors of A2AR and PD-1/PD-L1 or CTLA-4 have been performed; altogether demonstrating a significant reduction in tumor growth and metastases and increased antitumor immune responses mostly by enhancing CD8+ T cell activity and recruitment into tumors [6, 19, 26-28]. CLL presents similar elevated levels of A2AR signaling and a concomitant dysregulated expression of immune markers and checkpoints [29]. These, in part, highlights the need for a greater concerted re-focus on A2AR antagonization strategies in our efforts to treating CLL.

d. The Case of CLL – Exploring Optimal Partners

Currently, CLL can only be managed despite the plethora of treatment options available to clinicians. Both venetoclax and ibrutinib; inhibitors of BCL-2 and BTK respectively, and their combinations with anti-CD20 mAbs have distinguished themselves as the golden standard of choice for the treatment of CLL, especially for high-risk patients. More interestingly, venetoclax and ibrutinib clinical trial dual treatments have demonstrated far more impressive outcomes, strengthening the pair as optimal partners for the management of CLL [30-33]. Notwithstanding, A2AR-mediated immunosuppressive resistance networks mostly develop under these settings, partly contributing to observed disease progression and relapse during and after treatment. Surprisingly, data combining A2AR inhibitors with current treatment options for CLL is limited. The use of A2AR antagonists such as SCH58261 and CPI-444 (ciforadenant) have shown significant results in preclinical models of CLL and solid tumors, and encouraging clinical outcomes in renal cell cancer patients when used in combination with inhibitors of PD-(L1) [27]. Since adenosine-derived immunosuppression is arguably perceived as the missing piece of the fight-against-CLL puzzle, the field would most likely benefit tremendously with more treatment combination studies involving A2AR antagonists.

EOS-100850 (inupadenant) is a clinical stage 2 drug candidate designed to antagonize A2AR signaling potently and selectively in solid tumors. It has shown substantial clinical outcomes in castrate-resistant prostate cancer, BRAF wild-type cutaneous melanoma and in several immune resistant advanced solid tumor patients with multiple indications. Though the chemical structure has not been disclosed by its developers, a review of the candidate drug patent application publication identifies inupadenant to have a high degree of structural similarity to preladenant (MK-3814), a derivative of SCH58261 [34, 35]. Analysis of the preferred substituted forms of the novel 5-aminothiazolo[5,4-e][1,2,4] triazolo[1,5-c]pyrimidin-2(3H)-one core structure may give a compound of chemical formula C25H26F2N8O4S2 (+)-5-amino-3-(2-(4-(2,4-difluoro-5-(2-(methylsulfiny)ethoxy)phenyl)piperazin-1-yl)ethyl)-8-(furan-2-yl) thiazolo[5,4-e][1,2,4] triazolo [1,5-c]pyrimidin-2(3H)-one(Thiazolo (5,4-E) (1,2,4) triazolo (1,5-C) pyrimidin-2(3H)-one,5-amino-3-(2-(4-(2,4-difluoro-5-(2-((S)-methylsulfinyl)ethoxy)phenyl)-1-piperazinyl) ethyl)-8-(2-furanyl)-), most likely in its salt form [34]. According to iTeos Therapeutics, inupadenant antagonizes adenosine signaling in a non-competitive mode [36]. Speculatively, based on structural similarities with preladenant, competitive assays of A2AR substrate analogue SCH58261 and analysis of crystal structures of similar ligand-bound A2AR, inupadenant may also competitively and reversibly antagonize adenosine binding, most likely through van der Waals forces, aromatic stacking interactions and hydrogen bonding within the orthosteric substrate-binding pocket of A2A receptor [35, 37, 38].

To explore its use cases in CLL, we assayed its apoptosis-inducing combination effects in CLL patient samples and murine models (TCL1 leukemia). As a single agent, inupadenant had minimal to no apoptotic effect at the tested dose but synergized with venetoclax in inducing significant cell death *ex vivo* and a durable switch of monocytes from a patrolling population to an inflammatory subtype in Eμ-TCL1 adoptively transferred transgenic mice. The dual treatment reduced disease burden but was significantly indifferent from inhibiting BCL-2 alone, establishing venetoclax as the main driver. Targeting BCL-2 and A2AR in CLL is very promising. Establishing the mechanism of action and cooperativity between these two pathways would pave the way for more rigorous exploratory efforts. Moreover, taking the optimal partnership between venetoclax and ibrutinib, a triple combination of these two with an A2AR antagonist could be the long-sought-after treatment modality for curing CLL.

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