

## Summary

The scope of this PhD thesis was an evaluation of mechanism of action of compounds designed at the Ryvu Therapeutics for immunotherapeutic purposes. Immune cell-based instead of cancer cell-based therapy is one of the most extensively studied treatment possibility within modern medicine and biotechnology. Anti-CTLA-4 and anti-PD-1 antibodies are the most successful examples of that so far. Studies on these antigens have begun in 1990s and the authors were awarded with Nobel Prize in medicine in 2018. Despite the preliminary success, clinical trials revealed that some of the patients remained unresponsive to this therapy, which proved the need for further development in immunooncology and new drugs proposals in this field. Neoantigen presentation, recognition and tumor infiltration by immune cells are not effective because of plethora of mechanisms negatively regulating the immune response. One of these mechanisms is an immunosuppression caused by adenosine. This nucleoside acts through one of its four receptors present on the surface of most human cells. These receptors belong to GPCR family and share their common features.

The aim of the doctoral dissertation was a characterisation of new  $A_2$  adenosine receptor antagonists since these receptors are the most important in the context of adenosine-driven immunosuppression. The concept of the project led by Ryvu Therapeutics was creating a dual  $A_{2A}/A_{2B}$  antagonist with unique profile, that will be effective even at high adenosine concentrations. In order to characterize over 400 of newly synthesized compounds high throughput screening assays had to be developed and used to select the best ones. In the first step, the level of intracellular cAMP was measured in cell lines with overexpression of  $A_{2B}$  and high level of  $A_{2A}$ . All experiments were performed as head-to-head comparisons with competitors' compounds. Then, selected molecules were tested in radioligand binding assay. Among them, SEL330-639, which is the subject of this thesis, is one of the most potent nanomolar inhibitor of cAMP synthesis with activity on both  $A_{2A}$  and  $A_{2B}$ . The dual antagonist sustains these features even at high adenosine concentrations. Additional kinetic parameters of target binding confirmed its extraordinary properties such as long residence time.

The next goal of the study was testing the top compounds in immune cells setting: PBMCs, particular immune cells subpopulations and whole blood. The initial step was again a measurement of intracellular cAMP but in  $CD4^+$  and  $CD8^+$  cells after  $A_2$  antagonist treatment. Additionally, adenosine receptors were investigated at protein and transcript levels in T lymphocytes and dendritic cells. In  $CD4^+$  and  $CD8^+$  cells transcripts for both  $A_2$  receptors,

*ADORA2A* and *ADORA2B* were detected together with proteins  $A_{2A}$  and  $A_{2B}$ , and their levels depend on lymphocytes activation status. Second messenger measurement revealed that  $A_{2A}$  is the receptor mainly responsible for T cells response to adenosine. Dendritic cells in turn have the expression of both receptor subtypes dependent on their maturation status. Due to dual affinity to both  $A_{2A}$  and  $A_{2B}$  receptors and exceptional binding properties our compound – SEL330-639 – is potent in broad range of immune cells including those of lymphoid and myeloid origin.

The next event in signaling cascade after  $A_2$  adenosine receptor activation is phosphorylation of transcription factor CREB. The influence of the most active compounds on pCREB (Ser133) was tested in human whole blood and measured via flow cytometry in T cells subpopulations. SEL330-639 compound not only inhibits CREB phosphorylation but also adenosine induced genes expression.

In immunooncology project, the effect on functionality of immune cells is the key parameter of potential drug candidate. Therefore, SEL330-639 was tested in two types of functional assays: cytokine production by lymphocytes and dendritic cells and NK cells cytotoxicity. Dual antagonist released cells from immunosuppression mediated by adenosine at 100  $\mu$ M what corresponds to the highest concentrations detected in solid tumors. The extent of the effect depended on cell and cytokine type. SEL330-639 is the most potent in restoration of IL-2 production by  $CD4^+$  cells and TNF $\alpha$  secretion by moDCs. In case of NK cells, our antagonist is the most effective among tested and able to fully reinstate their cytotoxicity against target cells that has been inhibited by adenosine analog. The newly established models enabled confirmation of compound effectiveness in cells dependent on  $A_{2A}$  (lymphocytes and NK cells) and  $A_{2B}$  (dendritic cells). *In vivo* activity of SEL330-639 was also evaluated in murine syngeneic model. It revealed a beneficial influence of the compound on animal long term survival in combination with anti-CTLA-4 antibody.

The results that have been presented in the thesis have been obtained thanks to newly developed and optimized methods specifically for this project purposes. In addition to compounds activity analysis there were also plenty of experiments performed in order to better understand the basic biology of adenosine receptor and the adenosine itself. It was detected that adenosine concentration in cell culture medium depends mainly on duration of cell culture and T cell activation. Moreover, it was shown that during TCR activation  $A_{2A}$  level increases in time dependent manner on the surface of T lymphocytes. On top of that, the desensitization of  $A_{2A}$  receptor on the surface of  $CD4^+$  cells was confirmed at indicated conditions. All of the

results lead to conclusion that our dual  $A_{2A}/A_{2B}$  antagonist SEL330-639 with long residence time effectively prevented or released cells from adenosine mediated immunosuppression what have been proved in *in vitro* experimental models. The broad spectrum of activity on different subsets of immune cells and unique features makes it a very attractive drug candidate to be used in immunooncology.