Targeting Aspartate Transcarbamoylase: A Versatile Approach to Multispecies Drug and Herbicide Discovery

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CHAPTER 1

Introduction and Scope of the Thesis

INTRODUCTION AND SCOPE OF THE THESIS

The discovery of the pyrimidine biosynthesis pathway can be tracked back to 20_{th} century, with significant contributions from various researchers[1, 2]. This pathway plays a fundamental role in DNA and RNA replication, and other crucial living processes, such as assembly of biological membranes and protein glycosylation and glycogen synthesis[3, 4]. As a critical part of living organisms, this process consists of two pathways: the nucleotide salvage pathway and *de novo* pyrimidine synthesis pathway. The nucleotide salvage pathway allows recycling of nucleotides and nucleobases that helps conserve energy and resources by converting nucleic acid breakdown products (nucleosides and bases) from both intra- and extra-cellular sources back into fully functional nucleotides for reuse in DNA and RNA synthesis[5, 6]. The *de novo* pyrimidine synthesis pathway is defined as the formation of uridine monophosphate (UMP) from glutamine and carbonic acid through six catalytic steps[7]. The *de novo* pyrimidine synthesis pathway is relatively high in energy consumption, but capable of accumulating a large-scale nucleotide acid pool for cell needs, such as proliferation[7]. These two pathways can help to build a balance of the need for nucleic acids in living organisms.

Different species have varying needs for the nucleotide salvage pathway and the *de novo* pyrimidine synthesis pathway. These differences can be attributed to factors such as the organism's evolutionary history[1, 8], specific metabolic requirements[9, 10], and environmental conditions[11]. In this thesis, our primary focus is on the *de novo* pyrimidine synthesis pathway across various living species, with particular emphasis on the enzyme responsible for the second catalytic step: Aspartate transcarbamoylase (ATCase), which catalyzes the formation of carbamoyl-aspartate (CP-Asp) from L-aspartate (Asp) and carbamoyl phosphate (CP)[12].

In **chapter 2**, we review the *de novo* pyrimidine biosynthesis pathway, mainly focusing on the potential variations in the availability of the second step of this pathway across different species. Malaria is significant due to the unavailability of the salvage pathways in *Plasmodium falciparum* (*P. falciparum*). While the details of tuberculosis pyrimidine metabolism are not fully understood, inhibiting this pathway could hold promise against *mycobacterium tuberculosis* (*Mtb*). The *de novo* pyrimidine synthesis pathway is also involved in proliferating human cells, including human tumor cells, making it relevant for anticancer studies[13]. Additionally, we discuss certain pathogens related to neglected tropical disease and ESKAPE pathogens. Notably, this pathway is also a target of herbicides, with more details to be discussed in **chapter 7**.

As we mentioned in **chapter 2**, the *de novo* pyrimidine synthesis pathway is considered to be a significant antimalarial target. In **chapter 3**, our primary focus is on the screening of preliminary fragments and the identification of new inhibitors that target an unknown allosteric pocket on the *P. falciparum* ATCase (*Pf*ATCase), characterized by a combination of fragment-based X-ray crystallography and enzymatic assays that follow a cocktail approach. Together with the X-ray crystallography driven determination of the binding site location and associated ATCase conformational changes, a compound library (named BDA series) was designed. Enzymatic assays, differential scanning fluorimetry (DSF), microscale thermophoresis (MST) and cellular activity were performed with the most potent compounds. These details are further expanded upon in **Chapter 4**, in which the fragment screening of discovery and design methods were described, highlighting the initial and intermediate stages of fragment-based drug discovery through the example of the allosteric inhibitor of *Pf*ATCase and the value of multicomponent reaction chemistry in fragment-based drug design.

Given the long history of tuberculosis and its significant impact on mankind, along with the essential function in pyrimidine biosynthesis for mycobacterial survival, clear applications can be drawn from our previous work. In C**hapter 5**, we report on the inhibition of ATCase of *Mycobacterium tuberculosis* (*Mtb*ATCase) from members of the BDA series which were developed against *Pf*ATCase. Through *in vitro* enzymatic assays, we demonstrate that the BDA series compounds are able to distinguish between *Pf*ATCase, *Mtb*ATCase and Human ATCase (as described in **chapter 6**). These results also indicate that members of the BDA series exhibit inhibition of *Mtb*ATCase in the single digit μM range through noncompetitive inhibition, with a particular highlight being demonstrated by kinetic assays on compound BDA-06. Moreover, we not only conducted assays on Mtb in culture growth to evaluate the anti-TB activity of the best compounds, but also constructed a computer model and performed *in silico* docking experiments to explain the observed species selectivity exhibited by this compound series.

In **chapter 6**, we present findings on the same BDA series inhibitors. which also exhibited low nanomolar inhibition against human ATCase in *in vitro* enzyme assays. BDA-41 was selected for kinetic assays, and the resulting curves conform to Michaels-Menten kinetics, indicating a non-competitive mechanism. Furthermore, we conducted a comparative analysis of the inhibition provided by the BDA series and N-phosphonacetyl-L-aspartate (PALA), previously reported as a transition state analog of the ATCase catalytic mechanism[14, 15]. Our compounds demonstrated more complete inhibition results. While we are in the process of obtaining a co-crystal, a computer model was again constructed to further illustrate the compound binding

state. Finally, we report on cytotoxicity studies and demonstrate the impact of BDA-41 on the cell cycle using U2OS cells.

In **chapter 7**, we report screening on inhibition of the BDA series against *Arabidopsis* ATCase (*At*ATCase) in *in vitro* enzyme assays. ATCase plays an important role in *Arabidopsis thaliana*, affecting leaf and root growth, as well as chlorosis[16]. We explored the potential impact of BDA series compounds on this plant species. The results revealed inhibition even extending to the plant kingdom. Similarly, kinetic assays were performed, confirming a non-competitive mechanism of the BDA-41compound. Interestingly, in the plant assays, BDA-03 and BDA-12 showed surprising results, inhibiting the growth of both wild-type and ATCase overexpressing plants. Finally, since co-crystallization experiments are still in process, a computer model was again constructed to generate a model of the binding mode.

In C**hapter 8**, we provide a summary of this thesis. Furthermore, we briefly discuss further research directions and perspectives in the inhibition of ATCase across various species, including *Trypanosoma cruzi* ATCase (*Tc*ATCase), *Chaetomium thermophilum* ATCase (*Ct*ATCase), and possibly Neglected tropical diseases and ESKAPE pathogens as well. The optimization of BDA series compounds which target these species may also be considered. Given the emergence of drug resistance strains in many species, the identification of new drug targets and candidates has become imperative. The significance of targeting essential cellular activities cannot be overstated. Therefore, we will explore research focusing on this pathway, both at the molecular and cellular level, in the future.

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