Analysis of chromosome segregation mechanisms and cell division dynamics by designing innovative Haspine kinase-specific probes

The following thesis project is proposed by the teams of Sandrine Ruchaud and Sylvain Routier involving the co-supervision of a PhD student.

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State of the art

During mitosis, chromosome misalignment and loss of spindle checkpoint have been shown to be associated with chromosomal instability leading to cancer development. Mutations in genes encoding different components of these mechanisms have been repeatedly observed in cancer cells. An important number of these components are protein kinases whose functions are not yet fully understood. We are particularly interested in Haspin kinase for its essential functions in mitosis [1,2,3]. Its depletion by siRNA prevents proper chromosome alignment in metaphase and disrupts cohesin binding with chromatids at the centromere [1,2]. Haspin phosphorylates Histone H3 on Threonine 3 (H3T3ph) during early mitosis [1] and this phosphorylated residue serves as an anchoring site for the Aurora B kinase complex, leading to the accumulation and activation of the latter at the centromere creating a positive feedback loop [4,5,6,7]. Centromeric localization of Aurora B is essential for correct chromosome alignment and maintenance of chromosome stability.

Despite recent advances, the biological functions of Haspin remain poorly understood and represent an interesting challenge.

Haspin inhibition or depletion triggers severe mitotic defects leading to cell death by mitotic catastrophe [8,9,10]. Haspin has been found to be overexpressed in several highly proliferative malignancies and this overexpression is often correlated with the most aggressive stages of cancers [11-16]. In addition, Haspine inhibition has been shown to inhibit the development of several cancers in animal models [14,15,17-19]. This kinase is thus considered a relevant target for anti-cancer therapy.

The search for pharmacological inhibitors of protein kinases is booming. In 2021, seventy-two kinase inhibitors have reached the drug market (FDA approved), the majority of them in oncology [20]. A number of Haspin inhibitors have been described in the literature, some of them have been co-crystallized but remain not very selective [9,21].

In recent years, thanks to a strong support from the Ligue Contre le Cancer and the Cancéropole Grand Ouest, we have made progress in determining the mechanisms of action of Haspin, in particular its involvement in the correct segregation of chromosomes and in the dynamics of the cell division process. Today, we need to develop new tools in order to pursue this issue. In parallel, by combining the skills of two laboratories, one in fundamental biology in Roscoff and the other in medicinal chemistry at ICOA in Orleans, we have developed new chemical series, of the imidazopyridazine type, targeting Haspine, improving the existing [9]. These compounds have been validated for their mode of action confirming their potential for anti-cancer therapy. The published results [22] also showed their potential as tools for dissecting Haspin's mechanisms of action, prompting us to use them as a basis for generating innovative and more efficient probes, better suited to answer our biological question.

Objectives and scientific bottleneck

Commonly used reverse genetics methodologies (siRNA and knockout) allow the depletion of a protein of interest to study its functions. However, these approaches are limited by the half-life of the protein, offer a reduced window of study and an important variation of phenotypes from one cell to another. These phenotypes are often intermediate, making interpretation difficult and sometimes biased. As a result, chemobiology approaches have developed considerably, providing fundamental biology with complementary tools that are often more precise, making it possible to uncover and dissect new mechanisms of action and to validate or clarify already known mechanisms.

In this context, we propose to combine the skills of our two teams, in fundamental biology and medicinal chemistry, in order to answer precisely to an important biological question: by which mechanisms does Haspin kinase regulate the correct segregation of chromosomes in mitosis? To help us answer this question, we propose to recruit and train a young scientist at the interface between our two fields of expertise.

The objectives of the project are:

- to develop and characterize new series of Haspin inhibitory and selective macrocycles,
- to develop innovative chimeric molecules of PROTAC type that will selectively and rapidly activate Haspin degradation by the proteasome,
- to develop genetically invalidated (KO) and conditional cell lines for Haspin and a clonal line expressing an analogue-sensitive mutant Haspin (in KO condition)

- to determine with precision the molecular mechanisms responsible for the mitotic functions of Haspin using the models, probes and tools generated.

Methodologies to be put in place

1. *Development and characterization of specific and selective macrocyclic compounds*

The biological and physicochemical properties generated by macrocycling for kinase inhibitors confer favorable structural changes by constraining their conformation. Having at hand a series of molecules with side chains in key positions imidazopyridazine C-5 and pyrrazole C-3 that position themselves towards the solvent in the active site, we will synthesize a series of macrocycles and evaluate them for their biological properties (Fig. 1). We have recently obtained very encouraging preliminary results on a first synthesized macrocycle, giving us an IC_{50} on Haspin of 4 nM as well as a clear inhibition of endogenous Haspin in human cells, validating the proof of concept. The chemical demodulation of this structure should give us selective and highly specific inhibitors of Haspin and allow us to analyze the associated cellular phenotype in particular in "live imaging".

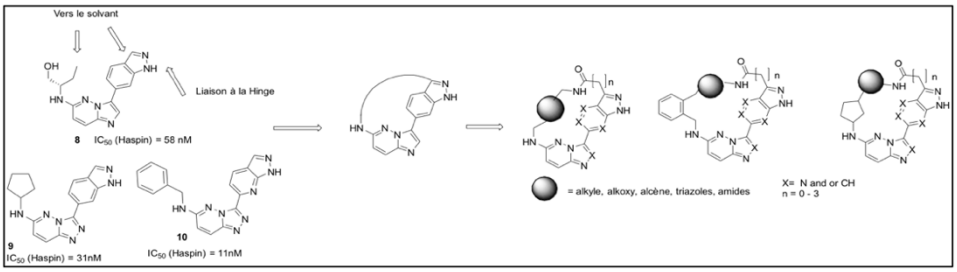


***Figure 1**: Macrocycling strategy for our hits.*

The compounds will be characterized according to their efficiency and their selectivity towards Haspine. The Haspine IC_{50} will be determined and the selectivity will be evaluated on the KISSf platform in Roscoff. The IC_{50}s and selectivity indices will guide the choice of compounds for further analysis on cells. The in-cell activity of the compounds will be evaluated on endogenous Haspine by immunofluorescence on fixed cells, by quantifying a specific fluorescent signal of the kinase activity (H3T3ph).

2. *Development, functional validation and characterization of selective PROTACs*

We also plan to design innovative PROTACs (PROteolysis-TArgeting) probes targeting Haspin. PROTACs chimeras exploit the intracellular ubiquitin-proteasome system to selectively degrade target proteins [23]. PROTAC is a bi-functional compound consisting of a target ligand and an E3 ubiquitin ligase ligand (CRBM, VHL), with the two entities connected by a linker (Fig. 2). These probes trigger degradation of a target protein in as little as one hour, a clear advantage over a half-life dependent protein depletion approach [24].

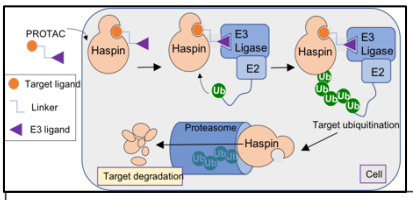
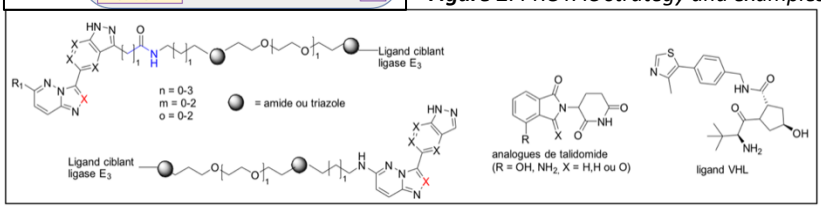


***Figure 2**: PROTAC strategy and examples of envisaged PROTACs structures.*



The efficiency of PROTACs chimeras on Haspin degradation will be measured in a dose and time-dependent manner in U-2 OS cells (Western blotting) and U-2 OS cells stably expressing GFP:Haspin (time lapse and flow cytometry). Haspin degradation will be quantified (DC_{50}) using a cell line expressing Haspin fused to NanoLuc by bioluminescence. A fine phenotypic analysis of cells treated with our best candidates will be performed by high resolution end point microscopy and live imaging.

3. *Analysis of Haspin functions during mitosis*

In order to finely analyze Haspin functions, we will generate, in addition to PROTACs and macrocycles, cell lines expressing a mutant "analog-sensitive" for Haspin (AS) as a replacement for the endogenous protein in order to control its activity at a specific time of the cycle and mitosis by the addition of a specific ATP analogue (Fig. 3) [25]. The mutation is made on the gate keeper residue and we mutated the so-called residue on Haspin (F605A/G). The activity as well as the inhibition by the ATP analogue 1NM-PP1 of these mutated forms were validated in vitro, constituting an encouraging preliminary result before generating the corresponding cell lines (Fig. 3).

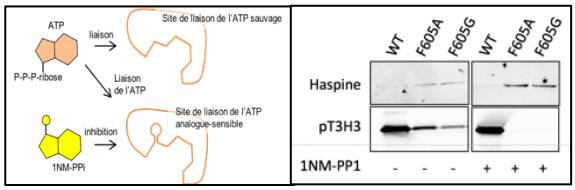


***Figure 3.** Représentation schématique de l'approche kinase analogue-sensible (AS) et validation in vitro des mutants AS de l'Haspine .*

We will first develop a conditional Haspin knockout model (Haspin KO^{c}) in U-2OS cells by CRISPR/Cas9 (in progress) before complementing this KOc with our AS mutants (Haspin $KO^{c/AS}$).

The Haspin KO^{c} line will allow us to compare phenotypes generated by depletion, inhibition or degradation of Haspin, and to detect possible off-target effects of our compounds. The phenotypes will be analyzed by high resolution microscopy and image analysis.

Expected results

The two Haspin KO^{c} and KO$^{c/AS}$ models associated with the new PROTACs and macrocycles will enrich our toolbox with unique and indispensable new tools for a fine and precise analysis of Haspin mechanisms of action and functions during cell division with an emphasis on chromosome segregation. They will help us consolidate and clarify the results already acquired and also allow us to identify new Haspin functions. The complementarity of our approaches will contribute to a better understanding of the role of Haspin in the orchestration of mitosis, which will help us to understand how the deregulation of this essential process can lead to genome instability and cancer. If successful, beyond their character as innovative tools, macrocycles and PROTACs may later trigger preclinical animal studies and will be protected by patent.

Complementarity of the teams and role of the doctoral student

This project combines the skills of two complementary and well-established teams specialized in fundamental biology and medicinal chemistry. Both are working in the field of protein kinases.

The Roscoff team, Sandrine Ruchaud, "Mitotic kinases, cytokinesis and cancer", UMR8227, focuses on the analysis of the molecular mechanisms underlying the regulation of essential mitotic kinases using powerful reverse genetics models and chemobiology approaches, all coupled with high resolution microscopy. The team uses this knowledge for the targeted design of new anti-cancer drugs using the kinase inhibitor screening platform of the Station Biologique de Roscoff (KISSf).

The Orleans team, Sylvain Routier, "Heterocyclic chemistry for innovation in therapeutics and PET imaging", UMR7311, ICOA, works on the development of new organic synthesis methods and their application for the preparation of bioactives for therapy and imaging. Their research focuses on rare heterocyclic structures.

The PhD student will be co-supervised by Sandrine Ruchaud and Sylvain Routier and will work on both sites in Roscoff and Orléans. The PhD student will have a background in biochemistry or at the biology-chemistry interface.

In Roscoff: The PhD student will be supervised by Sandrine Ruchaud, DR, and assisted by Béatrice Josselin, IE, both working on the project. With the support of the team, he/she will have to: 1- Finalize the Haspin conditional knockout (Haspin KO^{c}); 2- Generate the Haspin AS model in the Haspine KO^{c} line; 3- Analyze the mitotic functions of Haspin using the models and tools generated at ICOA.

At ICOA Orléans: The PhD student will be supervised by Sylvain Routier, PR. and Frédéric Buron, MCF, both working on the project. He/she will be required to: 1- Re-synthesize the heterocyclic bases of Haspin inhibitors; 2- Synthesize macrocycles by modulation of the flexible arm; 3- Synthesize PROTAC chimeras.

Additional chemistry resources will be brought to the project by the team as well as molecular modeling resources allowing faster optimization of the compounds (Pascal Bonnet team).

The risk-taking on the project is controlled by the diversity of the approaches and tools proposed by the two teams and a certain number of encouraging preliminary results. There is no risk associated with the understanding between two partners who have worked in collaboration for more than ten years on various projects for which they have obtained funding (ANR, Ligue Contre le Cancer), published results and filled patents together. In addition, the two teams are part of the "Marine Molecules, Metabolism and Cancer" axis of the Cancéropôle Grand Ouest and meet during annual colloquia to exchange and set up joint projects, structuring the two laboratories and research in the Grand Ouest. Both teams are part of the GDR ChemBio network for which Sylvain Routier is responsible for axis 2 "Chemical tools and molecular approaches" while Sandrine Ruchaud is part of axis 1 "Chemical targeting and modulation, understanding biological functions". The recruited PhD student will be immerged in these networks, giving him multiple opportunities for presenting and discussing his results amongst the community.

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