### Table I: Introduction of the program

<table>
<thead>
<tr>
<th>Acronym</th>
<th>CIAS</th>
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<tbody>
<tr>
<td><strong>Proposal title (in French)</strong></td>
<td>Inhibition combinée d'AKT et de STAT5: une nouvelle approche thérapeutique des leucémies myéloïdes chroniques résistantes aux inhibiteurs de tyrosine-kinase et des mastocytoses systémiques KIT D816V+.</td>
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<tr>
<td><strong>Proposal title (in English)</strong></td>
<td>Combined inhibition of STAT5 and AKT - A novel approach for therapy of drug-resistant chronic myeloid leukemia and systemic mastocytosis</td>
</tr>
<tr>
<td><strong>Type of research</strong></td>
<td>X Basic Research  □ Industrial Research  □ Experimental Development</td>
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Table II: **French and Austrian partners involved in the project:**

<table>
<thead>
<tr>
<th>Country</th>
<th>Organisation</th>
<th>Role in the project (coordinator or partner)</th>
<th>Requested funding to the ANR (euros)</th>
<th>Requested funding to the foreign agency (euros)</th>
</tr>
</thead>
<tbody>
<tr>
<td>France</td>
<td>Laboratoire de Biologie et Pharmacologie appliquée (LBPA) CNRS UMR 8113 Team: Molecular Oncology and Pharmacology Ecole Normale Supérieure de Cachan (ENS-C) 61, Ave du Président Wilson 94235-CACHAN CEDEX FRANCE Member of the Labex (Laboratory of Excellence) LERMIT</td>
<td>French Partner</td>
<td>350 668,00</td>
<td></td>
</tr>
<tr>
<td>Austria</td>
<td>Department of Internal Medicine I Division of Hematology and Hemostaseology (DIM-DHH) And Ludwig Boltzmann Cluster Oncology Medical University of Vienna Wachringer Guertel 18–20 A-1090 Vienna AUSTRIA</td>
<td>Austrian Partner Coordinator</td>
<td></td>
<td>349 377,00</td>
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Table III: **French and Austrian people involved in the project:**

<table>
<thead>
<tr>
<th>Organisation</th>
<th>Last name</th>
<th>First name</th>
<th>Current position</th>
<th>Involvement in the project (person.months)</th>
<th>Role and contribution to the project</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBPA CNRS UMR 8113 ENS-C</td>
<td>AROCK</td>
<td>Michel</td>
<td>Professor (ENS-C)</td>
<td>18</td>
<td>Supervision of the French team. Data analysis and reporting. Writing of publications.</td>
</tr>
<tr>
<td>LBPA CNRS UMR 8113 ENS-C</td>
<td>TCHERTANOV</td>
<td>Luba</td>
<td>Research Director (CNRS)</td>
<td>9</td>
<td>Supervision of <em>in silico</em> modeling and screening.</td>
</tr>
<tr>
<td>LBPA CNRS UMR 8113 ENS-C</td>
<td>X1</td>
<td>X1</td>
<td>Post-Doc to be recruited</td>
<td>36</td>
<td><em>In silico</em> modeling of AKT and screening of virtual chemical library on the docking sites of interest identified on AKT.</td>
</tr>
<tr>
<td>LBPA CNRS UMR 8113 ENS-C</td>
<td>LANGENFELD</td>
<td>Florent</td>
<td>PhD student</td>
<td>12</td>
<td><em>In silico</em> modeling of STAT5 and screening of virtual chemical library on the docking sites of interest identified on STAT5.</td>
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<tr>
<td>LBPA CNRS UMR 8113 ENS-C</td>
<td>BIBI</td>
<td>Siham</td>
<td>PhD student</td>
<td>24</td>
<td><em>In vitro</em> testing of selected drugs on CML and SM cell lines – Analysis of cell signaling, cell cycle and apoptosis.</td>
</tr>
<tr>
<td>Medical University of Vienna</td>
<td>VALEN T</td>
<td>Peter</td>
<td>Associate Professor</td>
<td>3.6</td>
<td>Principal Investigator in Vienna (Coordinator). Project Coordination, logistics and management Data analysis and reporting. Writing of publications.</td>
</tr>
<tr>
<td>Medical University of Vienna</td>
<td>SPERR</td>
<td>Wolfgang R.</td>
<td>Associate Professor</td>
<td>3.6</td>
<td>Clinical sample pipeline and data set management, coordination of translational studies and statistics</td>
</tr>
<tr>
<td>Medical University of Vienna</td>
<td>GLEIXNER</td>
<td>Karoline V.</td>
<td>Staff Hematologist</td>
<td>9</td>
<td>Clinical sample pipeline Coordination of research, <em>in vitro</em> studies on patients cells.</td>
</tr>
<tr>
<td>Medical University of Vienna</td>
<td>X2</td>
<td>X2</td>
<td>Post-Doc to be recruited</td>
<td>36</td>
<td>Purification of neoplastic stem cells by cell sorting, shRNA analyses, <em>in vivo</em> studies in NSG mice.</td>
</tr>
<tr>
<td>Medical University of Vienna</td>
<td>X3</td>
<td>X3</td>
<td>Technician to be recruited</td>
<td>18</td>
<td>Cell isolation, Western blotting, qPCR, ELISA, and flow cytometry and cell sorting. Technical assistance in animal care, sampling and analyses.</td>
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Combined inhibition of AKT and STAT5: a new therapeutic approach for tyrosine-kinase inhibitor-resistant chronic myeloid leukemia and KIT D816V+ aggressive systemic mastocytosis or mast cell leukemia, with special focus on neoplastic stem cells in these malignancies.

Chronic Myeloid Leukemia (CML) and Aggressive Systemic Mastocytosis (ASM) are myeloproliferative neoplasms characterized by a recurrent molecular abnormality leading to the constitutive activation of a tyrosine kinase (TK), namely BCR/ABL for CML and KIT D816V for ASM. In both diseases, unregulated TK recruits and activates downstream signaling. In CML, TK inhibitors (TKIs) specific to BCR/ABL can control the disease progression, but become ineffective when additional acquired mutations of BCR/ABL lead to resistance, as for the T315I mutant or compound mutations. In SM, KIT D816V mutant is immediately resistant to most TKIs specific to KIT. Thus, combined targeting of downstream molecules activated by TK in CML or ASM/MCL, such as AKT and STAT5, could be a new therapeutic approach in TKI-resistant patients.

Regarding STAT5, the Austrian partner (Pr P. Valent, Vienna) and others have recently shown that i) STAT5 is constitutively activated in CML cells, ii) high STAT5 levels mediate imatinib (the most widely used TKI) resistance and indicate disease progression and iii) chemical inhibition of STAT5 activation decreases survival of TKI-resistant BCR/ABL+ cells. The P. Valent team has also shown that STAT5 is constitutively phosphorylated in neoplastic MC in patients with SM, whereas the French partner (Pr. M. Arock, Cachan) obtained similar results in a human KIT D816V+ MC cell line.

Regarding AKT, it has been shown that: i) it is constitutively activated in CML cells, as demonstrated ex vivo in patient-cells and in BCR/ABL T315I+ cell lines; ii) chemical inhibition of AKT induces decreased proliferation and increases apoptosis of BCR/ABL T315I+ CML cells and iii) long-term exposure of CML cells to TKIs induced AKT phosphorylation, accounting in part for acquired resistance. It is also known that AKT is constitutively phosphorylated in neoplastic MCs isolated from patients with KIT D816V+ ASM. The French partner achieved comparable results with the KIT D816V+ human MC cell line. Finally, the Austrian partner has shown, using manipulated cell lines, that i) STAT5-PI3-K-AKT-cascade controls the neoplastic development of KIT D816V+ MCs; and ii) knock-down of either STAT5 or AKT activity results in growth inhibition.

The above described data strongly suggest that downstream STAT5-PI3-K-AKT signaling cascade is critical for BCR/ABL- or KIT D816V-mediated growth and survival of neoplastic precursors in CML or neoplastic progenitors and MCs in ASM, respectively. Taken together, these results also suggest that combined pharmacologic inhibition of STAT5 and AKT might represent new avenue to circumvent TKI resistance in CML and ASM. However, currently available inhibitors of STAT5 or AKT are not active at pharmacologic concentrations or/and they have an unfavorable toxicity profile.

Based on this knowledge, we propose the following collaboration between the French and Austrian partners:
1) To model in silico the different states of STAT5 and AKT, to characterize their structural, dynamics and functional features, and to identify a set of putative target structures. These structures will be used for the virtual screening of several chemical libraries of compounds to select hits likely to inhibit STAT5 or AKT.
2) To screen in vitro the effects of STAT5 or AKT inhibitors on BCR/ABL T315I+ CML and KIT D816V+ MC to determine i) their activity alone on cell proliferation, ii) their selectivity of action on STAT5 or AKT, iii) to define most potent synergistic combinations of STAT5 and AKT inhibitors with each other or with other targeted drugs on the above cited parameters (we aim at selecting compounds producing synergistic effects at a 1-100 nM range) and iv) the lack of toxicity of these combinations on normal hematopoiesis in vitro.
3) Once identified in vitro in cell lines, these combinations will be tested ex vivo on cells of TKI-resistant CML cells or MC in ASM/MCL as well as on malignant stem cells. If ex vivo data confirm the selection of the best
inhibitory combinations, these combinations will be tested in vivo on NSG mice grafted with bulk cells or MSC of CML or ASM patients.

The ultimate goal of our work is to develop combinations of potent AKT and STAT5 inhibitors, and possibly of other critical pathway inhibitors, providing a new therapeutic approach to TKIs resistant CML or KIT D816V+ ASM, which also target neoplastic/leukemic stem cells (LSC).

2. Context, Position and Objectives of the Proposal

2.1. Objectives, Originality and Novelty of the Project

During the past few years, several new targets have been identified in neoplastic mast cells (MCs), and various targeted drugs have been tested, with the aim to improve anti-cancer therapy in malignant MC disorders. However, unfortunately, most drugs, including new KIT D816V-blocking agents and various ABL kinase inhibitors that also block KIT activity, have failed to achieve long-lasting remissions in advanced SM. A similar problem exists in Ph+ CML, where many of the imatinib-resistant patients cannot be cured even with second-line or third-line BCR/ABL TKIs. An essential point is that during disease progression, many different, additional signal transduction pathways, apart from BCR/ABL and KIT, become activated in neoplastic cells. However, little is known about these pathways. We and others have recently shown that AKT and STAT5 are critical downstream signal transduction molecules that are constitutively phosphorylated in Imatinib-resistant CML and KIT D816V+ SM (see 2.2.). This has been demonstrated in vitro using KIT D816V+ and BCR/ABL+ imatinib-resistant, human cell lines. In addition, inhibition of the phosphorylation of these targets has shown their crucial involvement in malignant cell proliferation in both diseases. More importantly, during disease progression, the levels of STAT5 increase and STAT5 production and activation are constitutively phosphorylated in Imatinib-resistant CML and KIT D816V+ SM (see 2.2.). This has been demonstrated in vitro using KIT D816V+ and BCR/ABL+ imatinib-resistant, human cell lines. In addition, inhibition of the phosphorylation of these targets has shown their crucial involvement in malignant cell proliferation in both diseases. More importantly, during disease progression, the levels of STAT5 increase and STAT5 production and activation are constitutively phosphorylated in Imatinib-resistant CML and KIT D816V+ SM (see 2.2.). This has been demonstrated in vitro using KIT D816V+ and BCR/ABL+ imatinib-resistant, human cell lines. In addition, inhibition of the phosphorylation of these targets has shown their crucial involvement in malignant cell proliferation in both diseases.

The originality and novelty of the project lie on several different points:

- Develop molecules that act specifically and potently as inhibitors of STAT5/Phosho-STAT5, AKT/Phospho-AKT or other key molecules involved in deregulated proliferation of malignant cells in CML and ASM, and are devoid of significant toxicity against normal human hematopoietic cells.
- Use these molecules in combination in order to i) obtain a synergistic effect allowing to decrease the dose of each individual partner-drug, to ii) overcome resistance that occurs quite frequently during treatment with single agents and to iii) target and finally eliminate malignant stem cells.

The originality and novelty of the project lie on several different points:

- Demonstration that AKT and STAT5 act as most critical signaling pathways crucial for intrinsic resistance of malignant stem cells to conventional chemotherapy in CML or/and ASM/MCL.
- Investigation of other signaling molecules potentially involved intrinsic resistance of malignant stem cells to conventional chemotherapy in CML or/and ASM/MCL.
- Large scale application of in silico modeling and screening of chemical libraries to i) identify new molecular structures on AKT and on STAT5, that are involved in the function of these proteins (ligation to signaling molecule-partners, phosphorylation, and, in case of STAT5, binding to DNA), as well as on other signaling molecules newly identified, that could serve as targets and, ii) to select and synthesize new chemical compounds active at the nanomolar and selective of these targets.
- In vitro, ex vivo and in vivo parallel testing of the selected compounds in order to validate or improve the more relevant molecules that have been selected through the filter of in silico screening.
- Use of these new highly selective compounds in combination, with the aim to i) obtain a synergistic effect allowing a better efficacy with low toxicity; ii) overcome resistance due to pre-existing or acquired mutations in BCR/ABL or to KIT D816V and to target malignant stem cells. If such anti-malignant stem cell therapies can be defined, they may pave the way for new curative treatment strategies. Today, targeted drugs are used mostly as monotherapy-agents, a fact that could explain i) the occurrence of acquired mutations that render malignant cells progressively insensitive to these inhibitors and, ii) the intrinsic resistance of malignant stem cells to this monotherapy, responsible for relapse.
- Willing to patent not only one or several molecules for their biological and therapeutic properties, but also to patent a new concept of combined targeted therapy.

A- French Partner (FP):

1) Parallel in silico modeling of the different states of STAT5 and AKT, or possibly of other critical signaling molecules identified by the Austrian partner (AP), to characterize their structural, dynamics and functional features, and to identify a set of putative target structures. These structures will be used for the virtual screening of several chemical libraries of compounds to select hits likely to inhibit STAT5, AKT or the other critical targets identified.
2) Synthesis, by the chemist’s (service provider) of the Labex (Laboratory of Excellence) LERMIT of specific hit compounds targeting STAT5, AKT or of other signaling molecules, selected in the previous step of in silico modeling.

3) In vitro testing of STAT5, AKT, or of other identified specific hit compounds, alone or in combination, on tyrosine kinase inhibitors (TKIs)-resistant human chronic CML cell lines or on KIT D816V+ human MC lines. The effects of the targeted specific hit compounds used alone or in combination will be evaluated on i) cell proliferation; ii) cell cycle; iii) induction of apoptosis and iv) phosphorylation state of AKT and STAT5 in the cell lines treated by the selected compounds. Only compounds i) highly selective of STAT5 or of AKT and ii) acting alone or in combination at the nanomolar range on these cell lines will be retained for subsequent ex vivo and in vivo experiments conducted by the Austrian partner.

The major objective of the French partner is to discover new highly active and specific STAT5 or AKT targeted inhibitors, and possibly potent targeted inhibitors acting on other critical signaling molecules evidenced by the Austrian partner, that could be effective in vitro in combinations to kill TKIs-resistant CML or KIT D816V+ SM cell lines, without affecting normal hematopoiesis.

**B- AUSTRIAN PARTNER (AP):**

1) Analysis of production and activation of AKT and STAT5 in neoplastic cells in patients with ASM and TKI-resistant chronic myeloid leukemia (CML).

2) Analysis of upstream oncogenic signaling networks contributing to overproduction and hyperactivation of AKT and STAT5 in neoplastic cells in Ph+ CML and advanced SM.

3) Analysis of downstream effector events and pathways triggered by activated AKT and STAT5 in neoplastic cells in Ph+ CML and advanced SM.

4) Purification of neoplastic stem cells from the bone marrow of patients with CML and advanced SM and analysis of expression and activation of STAT5 and AKT, and of other critical signaling molecules in neoplastic cells.

5) Evaluation of effects of AKT and STAT5 inhibitors in combination with other targeted drugs, in order to achieve synergistic growth inhibitory effects in vitro and in vivo.

The major objective of the AP is to identify most relevant cooperating signaling pathways and networks in cloned and primary neoplastic cells in advanced SM and CML and to apply combinations of new targeted drugs that act synergistically with STAT5- or AKT inhibitors in blocking the growth of neoplastic cells ex vivo and in vivo, thereby complementing research efforts made by the French partner and to demonstrate that certain drug combinations are able to kill LSC in advanced SM and CML.

Finally, the major goals of this cooperative work are:

A) to discover and develop several combinations of potent AKT and STAT5 inhibitors (PASI), and of potent inhibitors of critical upstream signaling molecules, providing a new therapeutic approach to TKIs resistant CML or KIT D816V+ ASM, that could not only target the “more differentiated” malignant highly proliferative elements in both diseases, but also their malignant “low proliferative” chemotherapy resistant stem cells;

B) to patent, together with our supervisory authorities (CNRS and ENS-CACHAN for the French partner; Medizinische Universität Wien for the Austrian partner; as we have already done for another common patent) these combinations not only for their application in CML or ASM but also for any other malignancies where AKT, STAT-5 and critical upstream signaling molecules have been shown to play a critical role in mechanisms leading to uncontrolled proliferation, in mechanisms leading to defects in the normal scheme of apoptosis, or in maintenance of malignant stem cells.

C) to license, together with our supervisory authorities, these combinations of PASI and of other targeted drugs to a major pharmaceutical company which has already a portfolio of antileukemic and/or antitumor molecules, so that the firm can ensure the different pre-clinical and clinical phases.

**2.2 STATE OF THE ART**

**2.2.1 MAST CELLS AND MASTOCYTOSIS**

Mast cells (MCs) are multifunctional immune cells derived from hematopoietic stem cell in the bone marrow, which undergo terminal maturation in their ultimate tissue destinations under the influence of Stem Cell Factor (SCF). The specific receptor for SCF is KIT (CD117)\(^1\), a transmembrane receptor with intrinsic tyrosine kinase (TK) activity. Binding of SCF to KIT leads to receptor dimerization and activation of TK activity\(^2\). The receptor autophosphorylates at specific tyrosine residues during activation; the resulting phospho-tyrosine residues serve as docking sites for signal transduction molecules. Activated KIT also catalyzes the phosphorylation of substrate proteins. It induces multiple signal transduction pathways as a result of its interaction with several enzymes and adaptor proteins\(^3\). Namely, KIT recruits the phosphatidylinositol 3-kinase (PI3-K) leading to the activation of AKT\(^4\). Activation of KIT leads also to the recruitment of Ras/mitogen-activated protein kinases (Ras/MAPK) and of the Janus kinase/signal transducers and activators of transcription (Jak/STAT) pathways\(^5\), which are involved in apoptosis, cell survival, proliferation, migration and differentiation\(^6\). Mutations that cause
constitutive activation of KIT may give rise in humans to a spectrum of hyper-proliferative and cancer disorders such as mastocytosis.

Mastocytosis is a term used to collectively describe MC hyperplasia/neoplasia in one or more organs. It is a heterogeneous and relatively rare disease (estimated incidence in developed countries is around 1 new case for 250000 people/year) characterized by an abnormal accumulation of MCs in one or several organs. Clinical symptoms are the result of MC-derived mediators and less frequently of destructive infiltration of several organs by MCs. The disease can affect children and adults, although most cases occur during the first two years of life or after puberty. Mastocytosis in childhood is often self-limited and involves only the skin, whereas the course in patients with adult-onset disease is normally chronic and includes systemic involvement. The WHO (World health Organization) classification describes several subcategories of this disease broadly divided into cutaneous or systemic disease. Systemic disease is subsequently divided into indolent and aggressive disease. The clinical course of SM may range from benign (indolent SM (ISM)) to a more aggressive, life-threatening clinical course (aggressive SM - ASM), SM associated with clonal hematologic non–MC lineage disease (SM-AHNMD), and MC leukemia (MCL). Interestingly, the majority of patients with SM carry KIT mutations, most frequently D816V; a point mutation in the catalytic domain of KIT (ASP816VAL) which is found in up to 85% of all patients with SM. This mutation causes constitutive activation of KIT in the absence of SCF, leading to the recruitment of several signal transduction pathways.

The KIT D816V mutation has been demonstrated not only in MCs of SM affected patients, but also in the peripheral blood mononuclear cells of the patients. Particularly, KIT D816V mRNA has been detected in non-MC hematopoietic lineages in the peripheral blood and bone marrow in patients with ASM. Thus, it appears that mastocytosis, especially in its aggressive subtype, is a disorder of a pluripotent hematopoietic progenitor. Of note, a recent study has shown that KIT D816V mutation is a hallmark of ASM, where it is found in pluripotent hematopoietic stem cell. As for other hematopoietic malignancies such as CML, where the existence of CML stem cells (SC) has been extensively established, this observation also suggests that malignant SC may exist during mastocytosis, whose phenotype has been identified by P. Valent group as a CD34+/CD38-/CD13+/CD33+/CD44+/CD117+/CD123+ cell that harbors the KIT D816V mutation and that might constitute an interesting target for combined therapy in the aim to cure the disease.

Treatment of SM is based first on amelioration of symptoms with pharmacologic agents targeting MC mediators and therapy of skin lesions. So far, no standard antineoplastic therapy for patients with ASM has been defined. IFN-α shows variable efficacy in patients with ASM but induces numerous side effects that limit its use. Cladrabine or 2-CdA may induce clinical remissions in patients with ASM. Nevertheless, 2-CdA may induce pancytopenia and immunosuppression, and its potential oncogenicity remains largely unknown.

Newer evidence-based approaches to the treatment of ASM have focused on KIT inhibitors because of the ubiquity of KIT mutations in patients with advanced SM and the importance of this receptor in normal MC functions. The TKI imatinib is not generally indicated in patients with typical KIT D816V mutation because of resistance. The drug has been reported to reduce MC load and symptoms in patients with KIT mutations at other sites of the receptor. Other TKIs, such as dasatinib, PKC412 (midostaurin) and at higher concentrations, nilotinib (AMN 107), inhibit KIT D816V mutant. However, the efficacy of these drugs on ASM is limited and/or they have significant toxicity, thus multiple drug approach using combination therapy with targeted agents that have different mechanisms of actions should be proposed in ASM.

### 2.2.2 CHRONIC MYELOGENOUS LEUKEMIA (CML)

CML is a myeloproliferative neoplasm characterized by proliferation of a pluripotent hematopoietic stem cell (HSC). The programmed cell death (apoptosis) failure observed in this neoplasm allows for the growth advantage of the CML cells, which progressively overcome bone marrow (BM) and blood, possibly resulting in leukocytosis, splenomegaly or anemia and other related clinical symptoms. In more than 90% of the CML cases, the patients’ BM cells are featuring the Philadelphia chromosome. This major DNA translocation usually occurs between the chromosome 9 and 22 long arms, t(9;22)(q34;q11), leading to a new BCR-ABL chimeric gene, whose detection is a widely used diagnosis tool. This hallmark has been the historically first direct link between genes and disease to be chromosomally characterized by an abnormal accumulation of MCs in one or several organs. Clinical symptoms are the result of MC-derived mediators and less frequently of destructive infiltration of several organs by MCs. The disease can affect children and adults, although most cases occur during the first two years of life or after puberty. Mastocytosis in childhood is often self-limited and involves only the skin, whereas the course in patients with adult-onset disease is normally chronic and includes systemic involvement. The WHO (World health Organization) classification describes several subcategories of this disease broadly divided into cutaneous or systemic disease. Systemic disease is subsequently divided into indolent and aggressive disease. The clinical course of SM may range from benign (indolent SM (ISM)) to a more aggressive, life-threatening clinical course (aggressive SM - ASM), SM associated with clonal hematologic non–MC lineage disease (SM-AHNMD), and MC leukemia (MCL).

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The KIT D816V mutation has been demonstrated not only in MCs of SM affected patients, but also in the peripheral blood mononuclear cells of the patients. Particularly, KIT D816V mRNA has been detected in non-MC hematopoietic lineages in the peripheral blood and bone marrow in patients with ASM. Thus, it appears that mastocytosis, especially in its aggressive subtype, is a disorder of a pluripotent hematopoietic progenitor. Of note, a recent study has shown that KIT D816V mutation is a hallmark of ASM, where it is found in pluripotent hematopoietic stem cell. As for other hematopoietic malignancies such as CML, where the existence of CML stem cells (SC) has been extensively established, this observation also suggests that malignant SC may exist during mastocytosis, whose phenotype has been identified by P. Valent group as a CD34+/CD38-/CD13+/CD33+/CD44+/CD117+/CD123+ cell that harbors the KIT D816V mutation and that might constitute an interesting target for combined therapy in the aim to cure the disease.
ABL CML 26. Other more complex Philadelphia translocations [for instance t(8;9;22)(q13;q34;q11)] can also be found in rare CML cases 27.

Figure 1: Schematic representation of the Bcr, Abl and Bcr-Abl protein. 28

C-ABL is a non-receptor nuclear and cytoplasmic kinase involved in various cancers. It allows for the phosphorylation of critical signaling proteins involved in cell growth/proliferation, such as the Proliferating Cell Nuclear Antigen (PCNA) in breast cancer 29. Unlike its hybrid oncogenic homolog BCR-ABL, c-ABL is not constitutively phosphorylated and displays only low TK activity 30. The SH2 (Src Homology 2) and SH3 (Src Homology 3) domains of c-ABL connect to the kinase domain by a linker and participate to the regulation of c-ABL activity. Finally, the nuclear translocalization signal (NTS), DNA binding domain (DB) and actin-binding motif (AB) are found at the C-terminus.

The human Bcr protein contains multiple domains, including the oligomerization domain (OLI), the serine/threonine kinase domain (S/TK), the domain homologous to the human Dbl and yeast Cdc24 proteins (DH) and the domain with GTPase-activating activity for Rac (RacGAP) 28. Even though no complete structure of BCR/ABL has been published yet, the interactions between the Bcr- and Abl-domains are believed to be responsible for a significantly higher kinase activity (compared to c-ABL) related to leukemogenesis. The OLI domain up-regulates the activity of ABL domains to increase chimeric protein kinase activity. The SH3 domain, which negatively regulates the kinase activity of ABL, interacts with BCR and is also responsible for the constitutive activity of BCR-ABL 31.

As a central unregulated protein, BCR-ABL effect is mediated through phosphorylation events, either by autophosphorylation or phosphorylation of various signaling transducers. Thus, BCR-ABL activates several signaling pathways including but not limited to RAS 32, RAF, JAK/STAT 33 and PI3-Kinase-AKT 34 in direct link with the leukemogenesis and disease progression (figure 2). These findings have led to the development of BCR-ABL-specific tyrosine kinase inhibitors (TKIs) for the last 13 years, which have considerably improved patients’ therapy.

Figure 2: BCR-ABL downstream signaling effectors. Note the central place occupied by both AKT and STAT5 (red circle) in the signal transduction pathways leading to altered apoptosis and enhanced proliferation of CML cells 35.

CML symptomatology can be summarized in 3 clinico-biological phases: one chronic phase followed by an accelerated phase and eventually the blastic phase. Increased myelopoiesis, presence of BM cells, basophilia, eosinophilia or micromegakaryocytes are the main features of peripheral blood analysis in the chronic phase. Such markers are increased once the patients entered a more aggressive stage of the disease, and bone marrow or peripheral blasts with a myeloid phenotype can be found in a majority of cases 36, reflecting the loss of differentiation ability. If untreated, the CML inevitably evolves to an acute leukemia.

The first clinically used drug with survival benefit was an alkylation agent, Busulfan, in 1953. The ribonucleotide reductase inhibitor Hydroxyurea was then used from 1972, improving the median survival rate from 44 to 58 months, but had no effects on preventing progression to blast crisis. Allogeneic hematopoietic stem cell transplant appeared in the mid-70’s, but is still offered to patients in accelerating or blastic phase, when HLA
eligible and resistant to other treatments. Cytogenic response was first obtained with Interferon-alpha (IFN), which improve the survival rate, especially in combination with cytarabine from the mid-90’s. These considerable therapeutic improvements remained however insufficient as most patients eventually progressed to blast crisis.

In the early 2000’s, Introduction of the TKI imatinib in the treatment algorithms has considerably improved patients survival rate, and small compounds thus became the first-line therapy for CML. Imatinib binds the ATP-binding site of the chimeric BCR-ABL TK in a competitive manner, decreasing progression to accelerated/blastic phases and drastically increasing remission at 18 months 37. Following, two others TKIs were discovered and approved in CML treatment. Dasatinib binds BCR/ABL, as well as other kinases such as SFK (Src-Family Kinases), KIT and PDGFR-α/β 38, in both the active and inactive state 39. This former ability leads to a broader inhibition of ABL, independent of the protein conformation; thus, dasatinib was demonstrated to be more potent in advanced CML. Last, Nilotinib (AMN107) displays a higher binding affinity and selectivity than imatinib, favoring ABL inhibition in a much more potent manner. It is approved for CML in chronic and accelerated phases resistant to imatinib treatment, with significant results in terms of remission 40.

Bosutinib (SKI-606) shows a diminished off-target spectrum, as it does not inhibit KIT or PDGFR 41, whereas remission at 12 months in newly diagnosed chronic phase CML is similar to imatinib 42. By showing effects on imatinib-resistant patient, it is now approved by FDA as second-line therapy for the treatment of Ph+ CML in chronic, accelerated, or blast phase.

Despite TKIs greatly improved survival rates and remission, transforming a fatal disease in manageable chronic condition, they also result in drug resistance. Drug-resistance mechanisms are heterogeneous and comprise overexpression, epigenetic activation, upregulation of downstream signaling effectors, or missense mutations that reduce drug affinity or effects 43. This latter global mechanism can be observed in imatinib-resistant CML cells where downstream signaling effectors (including SFKs, PI3-K/AKT 44 and STAT5 45) are activated (figure 2).

Whatsoever, most relapsing or resistant patients show one or more acquired mutation(s) in the BCR-ABL gene. When different points mutations coexist, one can distinguish polyclonal mutations (mutations in separate clones) from compound mutations (one clone exhibits all the mutations). Recent results evidenced that this latter case occur with a higher frequency 46. The most common mutation is T315I, which represents approximately 20% of the retrieved mutations, and which is resistant to all currently approved TKIs. The third-generation TKI ponatinib (AP24534) latest clinical trial results recently claimed for high anti-leukemia activity on Ph+ patients without or with mutations (including the T315I) 47, suggesting a so-called “pan-BCR-ABL” activity. Also, ponatinib demonstrated activity on many others kinases such as FLT3, FGFR, VEGFR, KIT or PDGFR 48.

Finally, a new class of inhibitors targeting the allosteric phenomena in BCR-ABL is under clinical evaluation. DCC-2036, developed by Deciphera, belongs to this strategic class and its activity consists in forcing a conformational switch from the active to the inactive state of BCR-ABL 49. GNF-2 (Genomics Novartis Foundation) on the contrary targets the myristoyl binding cleft of BCR-ABL, therefore enhancing the TKIs efficacy against mutated and non-mutated BCR-ABL 50.

Last but not least, a new challenging field deals with eradicating cancer stem cells (SC) 51 to achieve disease cure. Current therapies provide remission but usually results in disease recurrence. There are now evidences that TKIs fail in eliminating primitive CML SC, allowing for the persistence of minimal residual disease (MRD), but that combined targeted therapies might lead to the elimination of CML SCs at all stages of disease 52,53.

2.2.3 THE PHOSPHOINOSITIDE 3-KINASE (PI3-K)-AKT PATHWAY

In mammals, AKT, also known as protein kinase B (PKB), is present under 3 different isoforms, namely AKT1 (or PKBα), AKT2 (PKBβ), and AKT3 (PKBγ). These 3 isoforms, products of distinct genes, are highly related, exhibiting greater than 80% of homology at the amino acid level. Each isoform possesses an N-terminal pleckstrin homology (PH) domain of approximately 100 amino acids, followed by the kinase catalytic domain, which shows a high degree of similarity to those found in PKA and PKC 54 (figure 3). Also present in this region is a threonine residue (T308 in PKBα/AKT1) whose phosphorylation is necessary for activation of AKT. Following the kinase domain is a hydrophobic C-terminal tail containing a second regulatory phosphorylation site (S473 in PKBα/AKT1).

![Figure 3: general structure of the AKT protein](image-url)
Phosphorylation at T308 and S473 occurs in response to growth factors (GF) and other extracellular stimuli and is essential for maximal activation of AKT. Following GF activation of receptor tyrosine kinases (RTKs), or other cell surface receptors, the p110 subunit of PI3-K is activated and phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) on the 3-OH group, generating phosphatidylinositol-3,4,5-triphosphate (PIP3) at the inner side of the plasma membrane. AKT interacts specifically with PIP3, which causes its translocation to the inner membrane and conformational changes, resulting in the exposure of the two main phosphorylation sites at T308 and S473. Phosphorylation of these sites by the protein serine/threonine kinase 3-phosphoinositide-dependent kinases 1 and 2 (PDK1 and PDK2), also recruited by PIP3, is required for maximal activation of AKT. Activated (phosphorylated) AKT mediates the activation and inhibition of various targets like forkhead family transcription (FKRH), NF-κB and the cyclic AMP response element binding protein (CREB), the proapoptotic protein BAD, and cyclin D by activating transcription factors downstream of mTOR/FRAP, resulting in cellular survival by anti-apoptotic effects, cell growth and proliferation. Through these interactions, AKT contributes to cancer progression such as in BCR-ABL-positive CML, KIT D816V+ SM, melanoma, or invasion and metastasis in Hepatocellular Carcinoma (HCC). Thus, AKT is an attractive target to inhibit for cancer therapy, and recent efforts in the development and biological evaluation of AKT inhibitors have led to identification of novel inhibitors with various heterocyclic scaffolds.

**AKT and mastocytosis**

The PI3-K/AKT is a major signaling pathway involved in growth and activation of MCs. Recently, the PI3-K/AKT has been identified as a major signaling molecule responsible for KIT-dependent differentiation and growth of neoplastic MCs harboring oncogenic KIT mutants. P. Valent team in Vienna showed that constitutively activated AKT promotes abnormal development of MCs in vivo and in vitro. More importantly, the same group showed that AKT is constitutively phosphorylated in neoplastic MCs from patients suffering from KIT D816V+ SM and in the HMC-1 cell line, suggesting that persistent AKT activation has a critical role in mastocytosis. This hypothesis is supported by the observation that abrogation of AKT activity was followed by growth inhibition of neoplastic MCs expressing the oncogenic KIT D816V mutant. Consistent with this data, a recent study showed that deficiency of the phosphatase and tensin homolog (PTEN, a tumor suppressor factor that inactivates AKT by phosphorylation sites at T308 and S473) in MCs causes deregulated proliferation with many features of systemic mastocytosis.

Moreover, a study performed by the team of M. Arock in Cachan on a newly isolated human KIT wild-type (WT) MC cell line transfected with the KIT D816V mutant, showed AKT phosphorylation in the absence of SCF in these KIT D816V+ cells, contrasting with the human KIT WT MC line counterpart which required the presence of SCF to express P-AKT. In addition, the same group demonstrated that the treatment with an AKT inhibitor decreased much efficiently proliferation in the KIT D816V+ cell line than in the KIT WT cells, suggesting that phosphorylation of AKT is critical for the transforming effect of KIT D816V.

**AKT and CML**

The downstream effectors of BCR-ABL in CML include the JAK/STAT, Raf/MEK/extracellular signal-regulated kinase (ERK) and PI3-Kinase (PI3-K)/AKT/mammalian target of rapamycin (mTOR) pathways, all of which affect cell viability, cell-cycle progression and leukemogenesis. Activation of PI3-K has emerged as one of the essential signaling mechanisms in BCR/ABL mediated leukemogenesis. PI3-K proteins and enzyme activity can be detected in BCR/ABL immunoprecipitates, which led to the initial assumption that activation of PI3-K occurred mainly from its direct association with ABL.

The essential role of AKT in BCR/ABL-mediated leukemogenesis was established by experiments demonstrating that the kinase-deficient AKT K179M mutant, which acts in a dominant-negative manner, inhibited BCR/ABL induced transformation of BM cells in vitro and suppressed leukemia development in mice. The residual leukemogenic potential of wild-type BCR/ABL in the presence of the dominant-negative AKT mutant is most likely due to AKT-independent mechanisms of transformation, although one cannot exclude incomplete suppression of AKT activation in cells coexpressing wild-type BCR/ABL and K179M AKT mutant. Consistent with the critical role of AKT in BCR/ABL leukemogenesis, the constitutively active AKT E40K mutant rescued the defective transformation mediated by BCR/ABL SH2 mutants (delta SH2 and R1053L) in vitro. The importance of AKT as a signal transducer from the SH2 domain of BCR/ABL established in the in vitro experiments was confirmed in vivo using retrovirally infected BM cells injected into SCID mice. Compared with wild-type BCR/ABL, cells expressing delta SH2 BCR/ABL have markedly decreased leukemic potential, only occasional involvement of non-hematopoietic organs, and diminished frequency of blastic transformation. Coexpression of the constitutively active AKT E40K, but not of AKT, restored the leukemogenic properties of DSH2 BCR/ABL in vivo. On the other hand, all the BCR/ABL mutants able to activate PI-3K also activated AKT, as it was demonstrated in presence of the T315I mutation in KBM-5 cell line. Thus, AKT appears to be the primary target of PI-3K in the signaling pathway activated from the SH2 domain of BCR/ABL. In summary, AKT appears to be a major target of PI-3K, which is constantly activated by BCR/ABL.
AKT inhibitors

AKT is viewed as an attractive target for cancer therapy and inhibition of AKT alone or in combination is currently evaluated. However, to date, no result exists about the effect of AKT inhibitors on SM or CML.

AKT inhibitors include MK-2206, Tricibine (API-2), GSK690963, GSK2141795, KP372-1, Perifosine, Enzasturin (LY317615), PBI-05204, Erucylphosphocholine (ErPC), erucylphosphohomocholine (ErPC3) and RX-0201 66. Most of these inhibitors have effects on solid tumors and a minority target hematologic malignancies. GSK2141795 is an AKT inhibitor which shows activity in various cancer models, including blood cancers and solid tumor models. It has been investigated further in clinical trials 66. KP372-1 induces mitochondrial dysfunction and apoptosis in AML cells at concentrations ranging between 0.5 µM and 1 µM, but not in normal hematopoietic progenitor cells. The effects of perifosine have been evaluated on many different tumor types, resulting with an IC 50 varying between 8 µM and 20 µM after 24h on T-acute leukemia cells 68 and between 1,25 µM and 6 µM after 72h on human endometrial cancer cell lines 69. Perifosine is or has been in at least 43 clinical trials to treat various cancers, either by itself, or in combination with other agents 66. MK-2206 inhibits auto-phosphorylation of both AKT T308 and S473. MK-2206 decreased T-acute lymphocytic leukemia (T-ALL) cell viability by blocking the cells in the G0/G1 phase of the cell cycle and inducing apoptosis with IC50 varying between 1.7 µM and 5.1 µM 62. It is in at least 43 clinical trials either as a single agent or in combination with other small molecule inhibitors or chemotherapeutic drugs in diverse types of cancer patients 66.

All in all, these data show that most AKT inhibitors exhibit IC50 in the micromolar range, suggesting that there is a need to develop AKT inhibitors acting at the nanomolar range. In addition, the use of AKT inhibitors in combination might increase their effects on cell proliferation. For instance, it has been recently demonstrated that treatment of STAT5shRNA17-cells with LY294002 (PI3-K inhibitor) resulted in an 80% inhibition of proliferation on leukemia cells, which was superior to that induced by either STAT5-shRNA17-cells alone (60%) or Ly294002 treatment alone (55%) 70. This result indicates that PI3-K/AKT and STAT5 cooperate to stimulate HS2-cell proliferation. In addition, the effects of STAT5 and PI3-K/AKT on cell cycle are additive 70. Thus, it appears that simultaneous targeting of PI3-K/AKT and JAK/STAT5 signaling pathways may enhance inhibition of malignant cell proliferation.

2.2.4 STAT PROTEINS/STAT5

The Signal Transducer and Activator of Transcription (STAT) family of proteins was discovered in the 90’s as a key cytokine signaling transducer 71. Since, seven mammalian STAT proteins have been identified, called STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. They all share the ability to transmit a cellular signal from the cell membrane to DNA, by promoting the transcription of different specific genes, but are activated by distinct ligands 72. STATs proteins mainly target genes involved in cell growth and cell cycle regulation, including but not limited to Bcl-Xl, 73,74 Cyclin D1 75, or Ly-6E 76. To fulfill their physiological role, the STATs proteins: (i) undergo an activation consisting of a tyrosine-phosphorylation step, (ii) dimerize through reciprocal interaction mediated by the phosphor-tyrosyl residue and the SH2 domain of the STAT monomers, (iii) is internalized into the cell nucleus via associating with importins, (iv) binds a specific DNA sequence and (v) activate the transcription through protein partners recruitment. Interestingly, in leukemia induced by constitutive activation of STAT5, the molecule can also be found in tetrameric activated forms linked to DNA, and it seems that these STAT5 tetramers in excess are involved in the process of leukemogenesis 77. Several STAT activators have been described, of which the canonical JAK-STAT pathway is overwhelming 78. The implication of the TK receptors 79 and non-receptors 80 as STAT activators has also been reported. Among the STATs proteins family, STAT5a/b are thought to be of primarily importance in myeloproliferative neoplasms such as SM 81 or CML 82. Thus, STAT5 is a potential major target for which no specific inhibitors exist 83,84. Indeed, current inhibitors cause decrease in STAT5 phosphorylation, whereas no kinases diminished activity occurs in the same time. Finally, Page et al. advanced through an in silico study that the SF-1-088 salicylic acid-containing inhibitor binds the SH2 domain of STAT5, inducing a lower phosphorylation level 84.

STAT5 and Mastocytosis

STAT5 has been demonstrated to be involved in normal MC growth and survival through the 2000’s 85. Consequently, several teams tried to elucidate the implication of STAT5 in neoplastic MC growth, survival and transformation. Furthermore, due to accumulating evidences of the major involvement of STAT5 as a pro-oncogene in myeloid cells, several recent studies have dealt with its implication in abnormal MC growth downstream of the main mastocytosis-driving agent, KIT D816V.

A first study led by Harir et al. showed that phosphorylated STAT5 (pSTAT5) is found in the cytoplasm of MCs from patients with mastocytosis. It further emphasized the molecular interactions between STAT5 and PI3-K, and that knock-down of STAT5 (or AKT) led to inhibition of cell growth 86. A second study also led by the Austrian partner has further explored this new pathway, showing that neoplastic MCs express cytoplasmic and nuclear pSTAT5. Furthermore, the same team showed that KIT D816V promotes STAT5-activation, which contributes to growth of neoplastic MCs 86. Thus, STAT5 is one major effector controlling KIT-mutant mediated aberrant growth signal in SM. The molecular mechanisms of STAT5 activation in MCs harboring a mutant KIT have been explored in details 81. Moreover, the expression levels of different STATs showed that only STAT5 is
transcriptionally active in HMC-1 and P815 MCs lines. Finally, an experiment on KIT D816V-transfected human MCs performed in the French partner team unambiguously indicated that P-STAT5 is present without SCF stimulation, by contrast to a wild-type human MCs line which was unable to express P-STAT5, even in presence of SCF (Saleh et al., submitted).

Altogether, these results strongly suggest that STAT5 is of primarily importance in the tumor development. However, the pharmacological inhibition of STAT5 remains a challenging field, and new STAT5 inhibitors active at pharmacological doses on both indolent and aggressive forms of mastocytosis are still needed.

**STAT5 and CML**

STAT5 pathway is involved in myeloid progenitor differentiation and growth of myeloid cells. It interacts with the fusion protein BCR/ABL which phosphorylates a specific tyrosine, allowing STAT5 to dimerize, to translocate into the nucleus and to activate the transcription of genes promoting cell survival/growth. Whether this interaction is direct or mediated by protein partners remains unclear and is still under investigation. Latest studies on STAT5 in a CML environment have proven that STAT5 is necessary for both transformation and the cell cycle progression. Also, STAT5 suppression induces a higher sensitivity of imatinib-sensitive K562 cells to imatinib, and sensitizes imatinib-resistant K562 cells to imatinib. More interestingly, high levels of activated STAT5 are correlated to TKIs resistance in vitro and in vivo, and to CML progression. Taken together, these results suggest that STAT5 would be an attractive target, as it could overcome drug resistance as well as disease progression.

**STAT5 inhibitors**

A very few papers describing the first STAT5 inhibitors have been recently published. First, cell lines or CD34+ cells from CML patients treated with pimozide revealed decreased pSTAT5 levels. Interestingly pimozide induces CML cell cycle arrest and apoptosis, with a synergistic efficiency in co-treatment with imatinib, by decreasing STAT5 phosphorylation. Finally, pimozide also exhibits growth inhibition on CD34+ CML cell, whereas non-CML CD34+ cells are only slightly affected. Such results allow us to assume that a STAT5-targeted therapy may act selectively on cells overexpressing activated STAT5. Two others classes of STAT5 inhibitors have also been published recently. They share common features in their inhibition profiles: suppression of STAT5 activation and induction of apoptosis. If no results on imatinib-resistant or primary cells are available for acid-salicylic-containing molecules, indirubin derivatives show activity on non-imatinib-resistant, T315I-imatinib-resistant and CD34+ primary CML cells. However, these results have been obtained with compound concentration around 5 μM, which is a concentration not achievable during administration to human.

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2.2.5 PRELIMINARY RESULTS

The preliminary results obtained by the Austrian partner concerning the constitutive activation of STAT5 and of AKT in various hematologic malignancies, including CML and mastocytosis, and the critical role of these pathways in malignant cell proliferation and in resistance to TKIs, are extensively detailed above (sections 2.2.1 to 2.2.4) and presented in the bibliography section, as well as his major contributions to the state of the art.

The preliminary data obtained by the French partner concern (i) the demonstration of the constitutive phosphorylation of STAT5 and AKT in a new human KIT D816V+ mast cell line (ROSA KIT D816V), the ability of these cells to engraft NSG mice, giving rise to a disease mimicking SM in human, the analysis of the effect of AKT and STAT5 inhibitors on the proliferation of both malignant human MCs and CML cell lines, and (ii) the first steps of in silico modeling of STAT5.

a) **ROSA KIT WT and ROSA KIT D816V** cells, but not ROSA KIT WT cells express constitutively P-STAT5 and P-AKT

To determine whether the ROSA cell lines exhibited constitutive activation of KIT, we examined by the phosphorylation of the tyrosine residue 703 of this receptor by in the presence or absence of rhSCF, and compared the responses of ROSA cells with that of HMC1.2 cells. As shown in Figure 4A, in the absence of rhSCF, ROSA KIT D816V and HMC-1.2 cells exhibited a constitutive phosphorylation of KIT, whereas ROSA KIT WT cells exhibited only low level of KIT phosphorylation. Stimulation with rhSCF resulted in higher level of phosphorylation of KIT in ROSA KIT WT cells, and increased its level of phosphorylation of in ROSA KIT D816V cells, but not in HMC-1.2 cells (Figure 4A).
We also examined the level of phosphorylation of the residue serine 473 of AKT on the presence or absence of SCF in the two ROSA cell lines as compared to the one of HMC-1.2 cells. We noticed that, contrasting ROSAKIT WT cells which required the presence of SCF to express phospho-AKT, ROSAKIT D816V cells exhibited a significant phosphorylation of AKT in the absence of rhSCF (Figure 4B). Interestingly, the level of phospho-AKT was even higher in ROSAKIT D816V cells than in HMC1.2 cells (Figure 4B).

Finally, we demonstrated the constitutive phosphorylation of STAT-5 in ROSAKIT D816V cells, but not in ROSAKIT WT, even in the presence of rhSCF (Figure 4C).

- **Analysis of the differential effect of a specific inhibitor of AKT on the proliferation of ROSA KIT D816V or ROSA KIT WT cells**

In order to evaluate the potential role of AKT in the proliferation of ROSA cells, we treated both cell lines with various concentrations (0.01 to 5 μM) of a potent and selective inhibitor of AKT1/AKT2 (compound A6730), which allowed us to determine the approximate IC50 of the molecule on ROSA KIT WT and on ROSA KIT D816V cells. Interestingly, while the IC50 of compound A6730 was found to be around 3 μM for ROSA KIT WT cells, the same compound inhibited by 50% the proliferation of ROSAKIT D816V cells at a concentration nearing 0.5 μM (figure 5), suggesting that phosphorylation of AKT is critical for the transforming effect of KIT D816V.

- **Analysis of the effects of STAT5 inhibitors on malignant human MCs and CML cells**

We have tested the effect of STAT inhibitors provided from Patrick Gunning (Toronto University – Canada) on the proliferation of malignant human MCs lines (ROSA and HMC1) and of CML cell lines (KCL22 sensitive and resistant to Imatinib, and ERY-1), as well as of CD34+ cells isolated from bone marrow samples from patients with CML. Using the STAT5 specific inhibitor BP-1-I02, we have observed a significant inhibition of the proliferation of the various cell types tested accompanied as expected by the inhibition of STAT5 phosphorylation (evidenced by phosphoflow cytometry), suggesting that the growth inhibitory effect of STAT5 inhibitor is truly the result of the decreased level of phosphorylation of STAT5.

Next, we analyzed the effect of combination of STAT5 and AKT inhibitors on malignant human MCs and, at the combined concentrations tested, we have observed a slight synergy between both types of inhibitors.
NSG mice grafted intravenously with ROSA KIT^{D816V} cells develop a systemic mast cell disease.

Mice were injected intravenously with ROSA^{KIT D816V} cells. As soon as disease symptoms appeared or after a maximum period of 6 months, the mice were sacrificed and, depending on the site of injection, BM samples were analyzed by flow cytometry for their content of CD117+/CD45+. Nearly all the mice injected intravenously presented a significant BM engraftment. The mean percentage of CD117+/CD45+ in BM was around 52%.

b) In silico modeling of STAT5

Here, we present preliminary results of the French partner on STAT5 modeling and in silico analysis. To date, as no crystallographic structures are available to perform an extensive docking study, we focused on the generation of various conformations (monomeric and dimeric bound to DNA) of STAT5 through homology modeling and Molecular Dynamics (MD) simulations. MD allows, from a starting conformation, to accurately study the dynamics/energetics of a biologic system by generating trajectories over time. First, the DNA-bound phosphorylated dimers (STAT5a and STAT5b) were simulated to define the protein-protein and protein-DNA interactions. The results evidenced the crucial role of the phospho-tyrosine in the monomer-monomer binding, but also defined the phospho-tyrosine binding site as being formed, for instance for STAT5a, by K600, R618, S620, D621 and S622. Similarly, we identified residues K347, R423, R425, K426, R430 and H471 as crucial for DNA binding. The role of the interfacial water molecules in the STTA5-DNA binding affinity is currently under study. In the same time, we simulated the non-phosphorylated and the phosphorylated monomers of STAT5a and STAT5b twice, to generate 12000 distinct conformations of each system. We then used MDpocket to detect potential inhibitor binding site over the generated trajectories.

As one might expect, MDpocket software detected the phospho-tyrosine binding site as a putative inhibitor pocket. But interestingly, it detected also close sub-pockets unexploited by the SF-1-088 STAT5 inhibitor, while not detecting the binding of the non-acid salicylic containing moiety of the inhibitor. Others putative binding pockets were also retrieved at the DNA binding site location and offer others opportunities for STAT5 inhibition. Altogether, these findings suggest that innovative compounds might target diverse sites of STAT5. The broad variety of inhibitory mechanisms observed for STAT3 inhibitors emphasized this concept.

Thus, the French partner is planning to perform a virtual screening using a subset of the generated conformations and virtual libraries of compounds or fragments to detect potential hits able to inhibit STAT5 activation. Ongoing studies are currently focusing on the dynamics and allosteric aspects of STAT5 to detect others potential binding sites to screen.

3 SCIENTIFIC AND TECHNICAL PROGRAM, PROJECT ORGANIZATION

3.1 SCIENTIFIC PROGRAM AND PROJECT STRUCTURE

3.1.1 Presentation of the structuration of the whole common project, including the different tasks, the input of each partner:

A synopsis of the whole proposed joint program of research is presented below. P1: French partner; P2: Austrian partner. Specific tasks for the French partner (FP) are located left to the dashed line in violin whereas the specific tasks for the Austrian partner (AP) are located to the right of the same line. Common exchange tasks are represented by green arrows crossing the violet dashed line. Each specific task for each partner is shown (for the FP in brown and for the AP in blue) with its corresponding number (Tx’ means additional tasks following the discovery by the Austrian partner of new critical signaling targets in malignant patient’s cells). The bottlenecks or contingencies that could jeopardize the project outcome are defined by “non-concluding results”. The numerous green arrows crossing between the two partners underline the strong complementarities between them with constant dialogs and goings and comings in the aim to increase efficiency and induce synergy.
3.1.2 Presentation of “who does what” and justification of the program work breakdown into tasks consistent with the objectives.

Our multidisciplinary and integrated program involves different scientific disciplines such as computational modeling \textit{in silico} (molecular modeling), chemical synthesis, \textit{in vitro} and \textit{ex vivo} cell biology and pharmacology, and animal models. However the program is designed so that there is a constant entanglement between all disciplines used. Indeed, as soon as the molecular structure of AKT and of STAT5 will be established \textit{in silico} by the FP (FP Task 1), he will go to Task 2, i.e.; \textit{in silico} modeling of the structures involved in the phosphorylation, dimerization or binding to DNA of STAT5, \textit{in silico} modeling of binding sites of AKT to PIP3, to PDK1 or to downstream partners, and \textit{in silico} modeling of phosphorylation sites of AKT. As soon as the structures of interests will be identified on STAT5 and AKT, FP will start the Task 3, i.e.; the virtual screening of chemical libraries to select molecules that will target AKT or STAT5. As soon as Task 3 will allow to select the first hits, the FP will ask his external service provider (the chemists at the Labex LERMIT; Service de Chimie Bioorganique et de Marquage, CEA, Institut de Biologie et de Technologies de Saclay; Director: Bernard Rousseau.) to synthesize (FP Task 4.1) these first hits, then following the first data obtained \textit{in vitro} (FP Task 5.1), to optimize these hits (FP Task 4.2). These first synthesized hits, then the optimized ones will be immediately tested in combinations \textit{in vitro} on TKIs-resistant CML cell lines and on the KIT D816V+ MC line (FP Task 5.1 and 5.2) to determine the best combination(s) of molecules giving a potent and synergistic antiproliferative effect. In parallel with Task 1 of the FP, the Austrian partner (AP) will begin his own tasks. AP Task 1 will be to first to recruit and sample TKIs-resistant CML or KIT D816V+ mastocytosis patients, and quite simultaneously to begin the purification and characterization of malignant cells \textit{ex vivo} (AP Task 2). AP Task 3 will consist in the analysis of the expression and functions of STAT5, AKT and of other signaling molecules in patients cells and in cell lines to identify additional targets of interest (we will limit voluntary to two additional targets: Tg1 and Tg2). The end of his Task 3 will be in phase with the Task 4 of the FP, so that the hits synthesized will be tested in combination in parallel by the FP on his \textit{in vitro} models of CML or mastocytosis cell lines (FP Task 5) and by the AP (AP Task 4) \textit{ex vivo} on cells from CML patients resistant to TKIs or on patients with KIT D816V+ mastocytosis. It is only if the results of \textit{in vitro} testing on cell lines (FP Task 5) and of \textit{ex vivo} testing on patients’ cells (AP Task 4) are consistent that the Austrian partner will begin the test of selected combinations of drugs in animal experiments (AP Task 5). In the case of discordant results, a meeting will be held between the two partners to analyze the reasons for this discrepancy and whether it is necessary to start again from the \textit{in silico} screening Task or from the chemical synthesis and optimization Tasks. In addition, biological data obtained by the AP for any additional signaling molecules of interest as potential additional targets (voluntary limited to the two most interesting additional targets (Tg1 and Tg2)) will induce the beginning of new tasks for both partners.
Indeed, as soon as the molecular structure of Tg1 and of Tg2 will be established in silico by the French partner (FP Task 1'), FP will begin Task 2', i.e.; in silico modeling of the structures involved in the biological functions of Tg1 and of Tg2. As soon as the structures of interests will be identified on Tg1 and Tg2, FP will start the Task 3', i.e.; the virtual screening of chemical libraries to select molecules that will target interesting structures on Tg1 and on Tg2. As soon as Task 3' will allow us to select the first hits, the French partner will ask his external service provider to synthesize these first hits and to begin optimization (FP Task 4'). Based on identified hits, optimized drugs will then be tested in combinations in vitro on TKI-resistant CML cell lines and on KIT D816V+ MC lines (FP Task 5') and by the AP (AP Task 4') ex vivo on cells from CML patients resistant to TKIs or from patients with KIT D816V+ mastocytosis. It is only if the results of in vitro testing on cell lines (FP Task 5') and of ex vivo testing on patients' cells (AP Task 4') are consistent that the AP will begin the test of selected combinations of drugs in animal experiments (AP Task 5'). In the case of discordant results, a meeting will be held between the two partners to analyze the reasons for this discrepancy and whether it is necessary to start again from the in silico screening Task or from the chemical synthesis and optimization Tasks.

Since the project includes a component of ex vivo experiments on cells from patients and in vivo on animals, the Austrian partner has obtained approval from the Ethics Committee of the Medical University of Vienna. In each case, written informed consent will be obtained from patients before sampling of their cells and, with regards to experiments performed in vivo in animals, they will be carried out in accordance with guidelines for animal care and protection and protocols approved by the Austrian law (GZ 66.009/0040-II/10b/2009).

3.2 DESCRIPTION BY TASK

Because Tasks 1', 2', 3', 4' and 5' for the French partner (FP) and 4' and 5' for the Austrian partner (AP) are totally replicative of FP Task 1, 2, 3, 4, and 5 and AP Task 4 and 5 in terms of objectives, of task leader and of the partners involved, of the deliverables, of the methods and technical choices and the way in which solutions will be brought, and of the risks and the back-up solutions envisaged, they will not be fully developed but are presented in details in the synopsis of the whole proposed joint program of research and in the Gantt diagram and in Table IV.

3.2.1 Organizational Task and methods of coordination

The coordinator of the transnational program, Peter Valent, will follow the advance of the tasks of his own team almost day to day. He will hold every two weeks (or more frequently depending on the duration of the task) meetings with every member of his team where the persons concerned will present by oral in detail the work progress on the task he is responsible for on: new data, difficulties, alternative methodology when difficulty, planning condition in relation to the deliverable. This presentation will be accompanied by the writing of a short report summarizing the key points. Similarly, a more comprehensive assessment will be conducted at mid-term for each task. Finally, at the date scheduled for the end of a task meeting of the whole team will be organized to ensure that all the objectives for this task have been met and, if not the case, the back-up solutions that can be implemented to make up for the delay. In addition, the coordinator will organize two preparatory meetings with the external service provider (chemists of the Labex LERMIT) to ensure the feasibility of chemical synthesis and optimization. These chemists will also be invited to the meetings at the middle and at the end of the task of virtual screening libraries of chemical compounds. They will then be asked to propose their first syntheses appearing quickly accessible and shall specify the time within which they will be able to deliver the compounds to be tested. Likewise, they will be invited to meetings held since the first results will be obtained in vitro with combinations of inhibitors. Based on these initial results, they will be asked to present the optimization protocols on already synthesized molecules, or to propose neighboring molecules which could be easier to manipulate in vitro, ex vivo and in vivo.

The French partner PI, Michel Arock, will organize, as requested by the coordinator, the same time table of meetings with his own team: he will hold every two weeks (or more frequently depending on the duration of the task) meetings with members of his team where the persons involved in the project will present by oral in detail their data and the work progress and any of the following: technical difficulties, alternative methodologies when difficulties are occurring, planning condition in relation to the deliverable. If major issues and deviations from the original plan emerge, results from the presentation and discussion will be documented in form of a short report summarizing key points. Similarly, a more comprehensive assessment will be conducted at mid-term for each task. Finally, at the date scheduled for the end of a task meeting of the whole team will be organized to ensure that all the objectives for this task have been met and, if not the case, the back-up solutions that can be implemented to make up for the delay.

Regarding the relationship between the two partners and the coordination of the two teams, Peter Valent will ask the French partner, each two months or more frequently depending on the duration of each task, a short written report including the following points for each on-going task: new data, difficulties, alternative methodology when difficulties occurred, planning conditions in relation to the deliverable. Furthermore, 2 joint meetings will be organized which will bring together all the people of the two teams. One meeting will be held in Cachan and one in Vienna (the cost of these consortium missions will be shared by the two teams, each team taking in charge 3
missions). The program, time-table and aims of these consortium missions are presented in the Table IV and in the Gantt diagram:

Obviously the frequency of the meetings will increase with time as expected data will become available in vitro, ex vivo then in vivo closely in time after a relatively long but necessary period of in silico modeling from the French side and of sampling and analysis of malignant cells from patients from the Austrian side.

Additional meetings, for which no funding is requested here, will be organized after the 36th month between the coordinator and the partners and will be devoted to future strategies of development of the program and to finalization of patenting and publications.

### 3.2.2 Tasks of the French partner (FP)

#### 3.2.2.1 FP Task 1: In silico modeling of the different states of STAT5 to characterize its structural, dynamics and functional features, and to identify a set of putative target structures. **Task leaders: Dr Luba Tchertanov, Mr Florent Langenfeld. Duration: 9 months.**

STAT5 a/b cycle of activation involves several states which are likely to be targeted in a new therapeutic strategy: the two main STAT5 steps are the tyrosine-phosphorylation and parallel dimerization. We need to fully understand the structural, dynamics and functional features of STAT5 in order to accurately define targetable sites. As no human crystallographic structures are currently available, a homology modeling step has already been performed (cf. 2.2.5.b).

The major cytoplasmic form is monomeric and tyrosine-unphosphorylated. The dimerization step implies a reciprocal interaction of the phosphor-tyrosine residue with the SH2 domain of the other monomer, which leads to the parallel, active dimer of STAT5. Whether the phosphorylation occurs in the monomeric or dimeric form remains unclear, thus to fully describe this receptor, we need to accurately model these three different states for each STAT5 isoform: monomeric and unphosphorylated, monomeric and phosphorylated, dimeric and phosphorylated. STAT5 DNA binding site is a putative inhibitor binding site, as the activity of STAT5 depends on its interaction with a specific DNA sequence. Preventing STAT5/DNA binding would stop the cell growth signal transduction and the activation of antiapoptotic gene transcription. Other modes of inhibition such as protein-protein inhibitors (PPIs), phospho-tyrosine binding inhibition or allosteric modulation are also conceivable. In both cases, the monomers would not be allowed to interact each others to form the active dimer. The current inhibitors are supposed to act this way. As assumed by Page and coworkers, SF-1-088 STAT5 inhibitor binds the SH2 domain of STAT5, in place of the phospho-tyrosine. This mechanism is in accordance with biological data revealing Src Family Kinases (SFKs) inhibition, knowing that SFKs include a SH2 domain as well.

Molecular Dynamics (MD) is a powerful tool that allows us to model the dynamic behavior of a system described at the atomistic level through a given amount of time, by iteratively solving Newton’s equation of motion. One can thus explore the energetic landscape, characterize the dynamics and produce multiple conformations of a given protein.

The deliverables for this task are the statistical analysis of the retained binding sites topology. Based on MD calculations, such analysis will allow us to cluster the generated conformations and thus to select a small subset of structures for the following step (task 3). The MD simulations are partially achieved and allow us to date to describe the different crucial functional sites. The phospho-tyrosine binding is mainly mediated through multiple, constant hydrogen bonding of polar residues to the phosphate group. DNA-STAT5 interactions are also mediated by polar residues forming hydrogen-bonds to the DNA phosphate backbone, but more specific bonds are also found: H471 forms hydrogen bonds with a specific nucleotide base involved in the STAT5-DNA recognition process. In the crystallographic study of the Stat3β homodimer bound to DNA, residue N466 has been described as an important determinant of base specificity at the DNA – STAT3 interface. Interestingly, H471 in STAT5 is the homolog residue of N466 in STAT3, which correlates well with our results and the critical role of STAT5 H471. Statistical analysis of the conformers’ structural flexibility in these areas (phospho-tyrosine and DNA binding site) is currently on-going to extract representative structures from MD trajectories.

#### 3.2.2.2-FP Task 2: In silico modeling of the different states of AKT, to characterize its structural, dynamics and functional features, and to identify a set of putative target structures. **Task leaders: Dr Luba Tchertanov, Dr XI (post-doc to be recruited). Duration: 9 months.**

AKT is a kinase protein whose activity is tightly linked to the transition between its inactive and active form trough major conformational changes. Numerous crystal structures of the Pleckstrin-Homology domain and the catalytic kinase domain, constituted of a N- and a C-lobe, are currently available, providing an extensive description of both forms of AKT in complex with inhibitors or unbound. The activation is primarily mediated by an activation loop (so-called A-loop), with a threonine residue (T309) whose phosphorylation is required for...
activation. Nevertheless, the allosteric transition mechanisms leading to the kinase domain activation remains unknown. Similar kinases, such as the cyclin-dependent kinase 5 or Src family kinase Lyn, undergo such motions of the kinase domain. This first protein has been recently studied, suggesting a two-step mechanism combining rotation of the αC helix (positioned in the N-lobe) and A-loop refolding. In parallel, Lyn has been showed to undergo complex A-loop motion and secondary structures changes, requiring a communication through the αC helix. Thus, internal communication is of major interest in kinases modeling.

Allostery occurs in proteins when structural changes are required for functionalization. Such phenomenon involves a succession of interacting residues that eventually build up a communication pathway. The modulation of such internal pathways can lead to allosteric inhibition of proteins or receptors by preventing mandatory inter-residues communication. Thus, Dr. L. Tchertanov team developed a new analysis tool dedicated to the characterization of communication paths through a Modular NETwork Analysis (MONETA). This method may provide the formalism required for a rational approach of kinases modulation. Combining multiple approaches (MONETA, MD…) to AKT will result in an extensive understanding of the key dynamic features.

The expected deliverables for this task are the complete dynamic analysis and description of AKT. This will help to detect putative binding or modulation sites.

3.2.2.3-FP Task 3: Virtual screening of several chemical libraries of compounds on selected structures from Task 1 and Task 2 to select hits likely to inhibit STAT5 or AKT. **Task leaders:** Dr Luba Tchertanov, Mr Florent Langenfeld, Dr X1 (post-doc to be recruited). **Duration:** 3 months.

One major issue in virtual screening is to take into account the receptor flexibility. Ensemble-based virtual screening (i.e. based on a small subset of target conformations) is one usual way to overcome this question. Even though the computational costs are increased, the accuracy of the predicted affinities is greatly improved. One major aspect of virtual screening is that it requires characterized targetable sites to be defined prior to the beginning of the process. The complete dynamic analysis of the different states of STAT5 and AKT (tasks 1 and 2) will lead to a subset of various conformations representative of all the conformational space explored.

Several current databases are currently freely available for virtual screening. Among these, the ZINC database contains over 21 millions of commercially-available compounds, whereas filters allow the users to select a subset of compounds based on rules such as Lipinski’s or on chemical scaffold diversity. We will combine such libraries with protein conformations in an ensemble-, structure-based virtual screening in order to detect possible hits compounds that have the better docking scores and thus the better predicted activity. A second round will then take place for the most promising compounds using more accurate (and more computationally expensive) methods to gain insights into the binding modes and to decrease the number of putative hit/lead to synthesize and test through biological assays. Then, using latest advances in scoring methods will help discriminating active and inactive compounds. For instance, the Automatic analysis of Poses using Self-Organizing Map (AuPosSOM) method for pose ranking is based on the assumption that active compounds should have specific contacts with their target to display activity. It is thus possible to differentiate active form inactive compounds using multiple conformations from docking results.

The deliverables will be a proposal of more than 20 compounds with distinct chemical scaffold thought to have an inhibition activity on STAT5 or AKT proteins.

3.2.2.4-FP Task 4: Chemical synthesis (2 months) and optimization (2 months) of the various hits identified by in silico screening on AKT and STAT5. **Tasks leaders:** Pr Michel Arock, Dr Luba Tchertanov. **External service provider (member of the Labex LERMITE):** Service de Chimie Bioorganique et de Marquage, CEA, Institut de Biologie et de Technologies de Saclay; **Director:** Bernard Rousseau. **Duration:** 4 months.

The task comprises two sequenced steps:

1) Synthesis of the various hits identified by in silico screening on AKT and STAT5.
2) Optimization of selected hits identified by in silico screening on AKT and STAT5 whose chemical structure predicts for druggability and limited toxicity

This task, which is essentially the responsibility of an external provider is closely related to the results of task 3 (in silico screening of compound libraries), but also for the optimization steps, upon the first results obtained during the startup of task 5. Indeed, according to the presence or absence of an antiproliferative effect in vitro of the twenty potentially anti-AKT and the twenty compounds potentially anti-STAT5 selected at the end of step 3 and synthesized, their IC50 value (we are targeting IC50 <100 nM for the best compounds, which will be our success indicator) and a possible potential synergistic effect between the two best of these two types of compounds (a second success indicator), our service provider will be required to provide optimization of compounds already obtained or either the synthesis of a series of related compounds. In case of failure at this step, i.e.; 1) drugability of the hits is subject to caution or 2) the synthesis of the hits is difficult or there is instability of the best molecules or 3) there is a potential toxicity of the major hits identified, the back-up will be to 1) go back to task 3 in order to find new series of compounds AND 2) to start a new campaign of optimization using the already published inhibitors of STAT5 and of AKT in order to improve their efficacy, their specificity and their drugability.
Obviously, to date, we have no clear idea of what kind of chemical structures will bind specifically the pockets of interest found in silico on STAT-5 and on AKT. Thus it is not possible at that time to provide the methods and techniques used for synthesis of these compounds, and for their optimization, as well as to analyze the risks and to provide the back-up solutions.

3.2.2.5-FP Task 5: In vitro testing combinations of optimized hits on CML or mastocytosis cell lines and on normal hematopoietic progenitors in order to select a limited number of synergistic combinations with no or non-significant toxicity on normal cells. Task leaders: Pr Michel Arock, Mrs Syham Bibi, Mrs Sylvie Jeanningros.

Duration: 4 months.

The task comprises four different steps:

1) Large-scale dose-response testing of optimized drugs targeting AKT or STAT5 in vitro in order to select the most potent drugs and determine their IC50 in malignant cells.
2) Testing the combinations of most potent drugs used at their IC50 in order to evidence synergistic effects on malignant cells. We will use as a model of TKIs-resistant CML cells the KCL22 cell lines, and as a model of KIT D816V+ cells our ROSA KIT D816V cell line. Both cell lines have been shown to over express P-AKT and P-STAT5.
3) Testing on normal hematopoietic progenitors or on non TKI-resistant cell lines of the best combinations selected for synergistic effects, in order to evaluate toxicity and specificity.
4) Once the best combinations of anti-AKT and anti-STAT5 will be identified in step 2, these combinations will be applied on the ROSA KIT WT cell line which does not overexpress P-STAT5 and P-AKT, even in the presence of SCF, and which is TKIs sensitive. We expect no or limited antiproliferative effect of the combinations selected on such cell line, used at the IC50, which will evidence the specificity of the inhibitors. In addition, the combinations selected will be applied in a dose escalation scheme on semi-solid medium cultures of normal CD34+ cells from human cord-blood (we have already access to such materials) treated with SCF, IL-3, GM-CSF, TPO and EPO. After 14 days in culture, total number of colonies of more than 50 cells and the % of each type of colony (CFU-GEMM, CFU-GM, CFU-G, BFU-E) will be analyzed. We hope that the best combinations of inhibitors selected will have an IC50 on such cell cultures at least ten times their IC50 on TKIs-resistant malignant cell lines and that these combinations will not have a particular toxicity on a specific hematopoietic lineage, which will be analyzed by the determination of the % of each type of colony in treated cultures as compared to non-treated control cultures.

The deliverables for such task is thus the identification of one (or best several) synergistic combinations of anti-AKT and anti-STAT5, each compound and the combination acting at < 100 nM on CML or mastocytosis cell lines, and with a security index > 10.

The risks are the following: i) IC50 of AKT or STAT-5 inhibitors > 1 μM, ii) no synergistic effects on malignant cells in vitro, or iii) Significant in vitro toxicity of the best combinations of drugs on normal hematopoietic progenitors or no specificity.

The back-up solutions in these cases are obviously the optimization of the compounds by the External Service Provider. This is the reason why the task 4 of the FP is divided in two periods of two months. The first period correspond to the synthesis of hits and the second period to the optimization of these hits. This is also the reason why Task 4 and Task 5 are synchronized in the time-table (please see the Gantt diagram).

3.2.3 Tasks of the Austrian partner (AP)

3.2.3.1 AP Task 1: Recruitment and Sampling of TKI-resistant CML patients and KIT D816V+ mastocytosis patients. Task leaders: Pr Peter Valent, Pr Wolfgang R. Sperr, Dr Karoline V. Gleixner, Dr X2 (Post-doc to be recruited). Duration: 9 months

During the first 9 months, the Austrian partner will establish a local project-related biobank of samples derived from patients with TKI-resistant CML and KIT D816V+ ASM and mast cell leukemia (MCL). Cells will be collected from the peripheral blood (PB) and bone marrow (BM). Isolated mononuclear cells (MNC) will be frozen and stored in liquid nitrogen. The aim is to collect cell samples (3-5 tubes per patient) from about 20-30 patients with TKI-resistant CML and about 15-20 patients with KIT D816V+ ASM/MCL. In control experiments, cell viability
and expression of key markers will be checked before and after freezing. In each case, cells will be examined for the presence of the disease-related driver mutation (*BCR/ABL* in CML and *KIT D816V* in ASM/MCL).

- **Objectives and success indicators:**
  
  - Established biobank after 9 months

- **Detailed work program:**

  Cells will be collected from patients during routine diagnostic investigations. In each case, the patient will provide written and verbal informed consent to donate blood and/or BM cells and that his/her cells are examined for responses to kinase blockers and other drugs, target expression, and *in vitro* and *in vivo* (xenotransplantation) stem cell assays. All studies have been approved by the local ethics committee (IRB) of the Medical University of Vienna. BM aspirates will be obtained by the clinical core team (P.V., W.R.S., K.V.G.). Control samples will be obtained from patients with indolent SM (ISM) or non-neoplastic BM. Cell isolation and storage as well as sample validation will be performed by Dr. X2 in collaboration with the local lab team of the PI. In most instances, MNC will be isolated (Ficoll). All cells will be stored in liquid nitrogen. An essential goal of the project is to connect the biobank data-set with our clinical data-set in order to determine clinical correlations (target expression with clinical endpoints such as TKI-response or survival) in the current project. In addition, we will collect follow-up (FU) samples. The clinical data-set, FU evaluation and clinical statistics will be coordinated by W.R.S.

  - **Deliverables:**
    a) Biobank ready for use (able to provide defined samples of primary cells) before month #9
    b) Biobank setup completed after 9 months
    c) Collection of follow up (FU) samples – FU biobank started
    d) Connecting the biobank data-set with the clinical data-set and clinical endpoints


  - **Description of the methods and technical choices and the way in which solutions will be brought, the risks and the back-up solutions envisaged.**

    In case more samples are needed or part of the samples are of suboptimal quality or cells cannot be induced to grow *in vitro* or *in vivo*, sampling and storage of patient-derived material (samples) will be extended beyond “month #9” and to more patients. Another point may be that the size of the samples (cell numbers) collected from individual patients is too small, so that more cells from more patients are required. Finally, the FU may take a longer time period. In each case, sampling of more probes from more patients may be required, and should be possible, based on the continuous flow of patients in our department. In this case we will also involve Mr or Mrs X3 who should assist in sample preparation and storage after “month #9”.

3.2.3.2-AP Task 2: Purification and characterization of malignant cells *ex vivo*. **Task leaders: Pr Peter Valent, Pr Wolfgang R. Sperr, Dr X2 (Post-doc to be recruited), Mr or Mrs X3 (Research Technician to be recruited)**

**Duration:** 10 months

The Austrian partner will purify and characterize neoplastic cells and cell subsets in patients with TKI-resistant Ph+ CML and *KIT D816V*+ ASM/MCL. The bulk of neoplastic cells will be purified as MNC using Ficoll. Neoplastic stem cells will be purified from MNC (either freshly obtained or obtained from the biobank) by magnetic cell-sorting (Lin- cocktail; CD34+) and (followed by) multicolor flow cytometry (CML: CD38-/CD25+/CD123+/IL-1RAP+; ASM/MCL: CD45+/CD34+/Lin-/KIT+/CD123+). Neoplastic mast cells will be isolated (from whole BM, buffy coat or from MNC) as CD45+/KIT++/CD34- cells.

- **Objectives and success indicators:**

  Purified stem cell samples and mast cell samples available for molecular and functional studies

- **Detailed work program:**

  Cells will be obtained from the local biobank (see above) or from freshly obtained samples. Sorting and re-sorting will be performed on a FACS Aria and will result in a high purity of isolated cell fractions. The Postdoc employed in this project (Dr X2) will attend a sort course and will be able to purify stem cells and mast cells. For the phenotypic characterization of isolated neoplastic stem cells and mast cells, the following assays will be performed: immunocytochemistry (ICC), flow cytometry, qPCR, FISH. In each sample, the presence of BCR/ABL (CML) or *KIT D816V* (ASM/MCL) will be confirmed.

  - **Deliverables:**

    a) Series of samples of purified stem cells in Ph+ CML and *KIT D816V*+ ASM/MCL
    b) Series of samples of purified mast cells obtained from patients with *KIT D816V*+ ASM/MCL

  Coordination and logistics: P.V., W.R.S. and K.V.G.; Multicolor flow cytometry, and cell sorting: Dr. X2; Preparation of cells for staining and sorting, ICC, qPCR, and FISH: Mr or Mrs X3.

  - **Description of the methods and technical choices and the way in which solutions will be brought, the risks and the back-up solutions envisaged.**
A major point may be that the numbers of isolated neoplastic stem cells in individual patients are too small, so that more cells from more patients are required. In this case, we will recruit more patients during the course of the project. Another pitfall may be that isolated stem cells rapidly change their kinase activation profiles during manipulation. Therefore, all isolation-steps will be performed at 2-8°C. After isolation, cells will be used in various assays. In the case of a kinase or STAT5 (activation) assay (ex vivo activity) we will keep the cells at a continuous “2-8°C-range” until the assay is performed or cells are frozen.

3.2.3.3-AP Task 3: Analysis of the expression and functions of STAT5, AKT and of other signaling molecules in patient cells and in cell lines; identification of additional targets Tg1 and Tg2
Task leaders: Pr Peter Valent, Pr Wolfgang R Sperr, Dr Karoline V. Gleixner, Dr X2 (Post-doc to be recruited), Mr or Mrs X3 (Research Technician to be recruited)
Duration: 7 months

In a first step, primary neoplastic cells (obtained from patients with TKI-sensitive and TKI-resistant CML and from patients with ISM or ASM/MCL) will be examined for expression of total STAT5, phosphorylated (p) STAT5, total AKT and pAKT as well as STAT5 and AKT mRNA levels. These experiments should confirm our earlier results and should provide a solid basis for consecutive experiments in the present study. We will also examine the expression and activation-status of STAT5 and AKT in human CML cell lines and SM-related cell lines. In addition, we will apply siRNA and shRNA against STAT5 and AKT in order to confirm the critical function of these signaling molecules in neoplastic cell growth and survival in advanced CML and ASM/MCL. In a next step, we will examine neoplastic cells (cell lines and primary neoplastic cells) for expression of additional drug targets, with emphasis on cooperating signaling networks and pathways. These studies will be performed by conventional omics (gene array and proteomics), synthetic lethality screens, phospho-proteomics, and phospho-RTK arrays. Target screens will focus on recently discovered oncogenic pathways in CML and ASM/MCL, including the RAS/MEK/ERK pathway, SRC, LYN and BTK. As a result of these screens novel interesting targets (Tg1 and Tg2) should be defined, and most suitable drug partners (for STAT5 and/or AKT blockers) will be identified.

- Objectives and success indicators:
  Panel of suitable drug partners for STAT5 and/or AKT blockers
- Detailed work program:
  Primary cells will be obtained from the biobank or from freshly aspirated PB or BM samples. Expression of STAT5 mRNA will be quantified by qPCR according to established protocols. Expression of total STAT5 and total AKT protein and pSTAT5 as well as pAKT will be quantified by Western blotting. Gene array analyses will be performed using Affymetrix algorithms (Affymetrix platform) and phosphoproteomics as well as synthetic lethality screens according to published techniques. Omics-based studies and bioinformatics will be performed in collaboration with the Department of Laboratory Medicine and the Center for Molecular Medicine in Vienna (both on Campus). In a first step, human cell lines will be tested. Depending on first results, primary cells will be examined in a second step in order to confirm cell line-results. SiRNA and shRNA analyses will be performed according to established protocols. ShRNA will be transduced by lentivirus-mediated gene transfer. Read out assays will include standard proliferation- and cytotoxicity assays (MTT, 3H-thymidine uptake) as well as standard apoptosis assay (morphology, caspase-3, Tunel). All assays required for long-term culture, transfection, and manipulation of cell lines and primary human target cells are established in our lab. A critical assay is the validation of target-partner-combinations by shRNA and siRNA. In these experiments, a step-wise approach will be applied using shRNA and siRNA (with suboptimal blocking effects) in combination. In a first step, shRNA transduced cells will be prepared and validated. Then, siRNA will be transfected into stable shRNA-transduced subclones. In these experiments cell lines will be tested and the combination effect will be measured by conventional read outs and by calculating combination index (CI) values using CalcuSyn software.

- Deliverables:
  a) Confirmed role of STAT5 and AKT as major signaling nodes in neoplastic cells in TKI-resistant CML and advanced KIT D816V+ ASM/MCL (as prerequisite to look for target-partners)
  b) Identification of potential new drug-targets in neoplastic cells in TKI-resistant CML and KIT D816V+ ASM/MCL
  c) Delineation of major cooperating signaling networks and nodes in neoplastic cells in TKI-resistant CML and KIT D816V+ ASM/MCL

  Coordination and logistics: P.V. and K.V.G.; Sample-selection, work plan, synthetic lethality screens, clinical correlates: W.R.S., K.V.G.; siRNA and shRNA analyses, phospho-RTK array, drug combinations studies: Dr. X2; Cell isolation, sampling and thawing and all read out bioassays: Mr or Mrs X3.

- Description of the methods and technical choices and the way in which solutions will be brought, the risks and the back-up solutions envisaged:
  In primary target validations, siRNA and shRNA will be tested. The experience of our lab is that depending on the type of molecular target and type of target cell, either the siRNA or the shRNA approach will yield better results (more complete knock-down of the target). Therefore, based on the better outcome, we will use either siRNA or shRNA in our primary target validations. The other (quite contrary) pitfall could be that survival of neoplastic cells is completely dependent on a certain target. To address this scenario, several different shRNA and siRNA species with different...
(suboptimal versus optimal) knock-down-potency, will be tested, and in some instances, it may be that an shRNA producing a suboptimal rather than a more complete knock-down of the target, will be the preferred agent. The same holds true for the shRNA+siRNA combination approach. We expect to identify a larger series of new potential targets in neoplastic cells in advanced CML and ASM/MCL, and the question may arise how to select the optimal ones for further validation. This question will be addressed by applying the following criteria: a) the target should be a new target (unknown so far), b) targeted drugs should be available, c) BCR/ABL-independent and KIT-independent targets are preferred.

3.2.3.4-AP Task 4: Testing of optimized combinations of AKT and STAT5 inhibitors on patients cells ex vivo
Task leaders: Pr Peter Valent, Dr Karoline V.Gleixner, Dr X2 (post-doc to be recruited), Mr or Mrs X3 (Research Technician to be recruited)
Duration: 5 months
After having defined the most effective (most critical) cooperating target pathways and signaling molecules, we will further validate these cooperation by applying shRNA and targeted drugs and various drug-combinations. These studies should reveal and validate novel most potent drug combinations through which growth and survival of malignant cells in TKI-resistant CML and KIT D816V+ ASM/MCL can be optimally suppressed or the malignant cells can even be eliminated. STAT5 and AKT will be examined as critical primary signaling molecules responsible for malignant expansion of mast cells. Studies will employ bioassays (optimized for studying in vitro cell growth) and examine cell lines, primary impure cells as well as purified (stem cell-enriched) fractions of neoplastic cells if possible.

- **Objectives and success indicators:**
  Identified drug combinations that synergistically block growth and/or survival of neoplastic cells

- **Detailed work program:**
  In a first step, cells transfected with STAT5-specific shRNA or AKT-specific shRNA will be exposed to various drugs known to block those signaling pathways or molecules that were identified as major cooperating partner-pathways in the current project. In a next step, drugs targeting STAT5 and AKT, synthesized by the FP) will be examined and combined with these designated drugs as well as with a series of other drugs known to interfere with malignant cell growth in CML or/and SM/MCL (drug-testing screen). Studies will be performed using human cell lines (K562, KU812, HMC-1.1, HMC-1.2) as well as Ba/F3 cells exhibiting KIT D816V, BCR/ABL, or mutant-forms of BCR/ABL. Depending on the results obtained, synergistic drug combinations will then also be tested on primary CML cells and primary neoplastic mast cells (ASM/MCL) as well as on purified neoplastic stem cells if possible. Read out assays will include standard proliferation and cytotoxicity assays (MTT, 3H-thymidine uptake) as well as standard apoptosis assay (morphology, caspase-3, Tunel). Drug- and shRNA-induced target-inhibition will be controlled by qPCR and Western blotting. Synergistic interactions between drugs (or between shRNA and certain drug candidates) will be determined by calculating combination index (CI) values using Calcusyn software.

- **Deliverables:**
  a) Identification of most potent drug combinations producing synergistic effects on malignant cell growth in various bioassays and cell line models
  b) Synergism of drug-combinations can be confirmed in primary cells in CML and ASM/MCL
  c) Synergism of drug-combinations can be confirmed in fractions enriched for neoplastic stem cells

  **Coordination or research and logistics:** P.V., K.V.G.; Drug synergism and shRNA+drug combination assays: K.V.G., Dr X2; Cell culture and preparation for bioassays: Mr or Mrs X3.

- **Description of the methods and technical choices and the way in which solutions will be brought, the risks and the back-up solutions envisaged.**
  It may turn out that despite clear-cut results in omics and synthetic lethality screens, no synergistic drug interactions can be demonstrated. In such a case, other additional targets and drug partners will be examined by further screening in omics (using different types of cells and primary cells if possible). Another caveat may be that certain drug combinations show a synergistic effect in cell lines, but not in primary neoplastic cells. In this case, it may be necessary to repeat omics data and lethality screens using primary cells which may be a very difficult approach and time- and cost-consuming. Based on results from such screens, we would then replace the non-cooperating drugs by new more effective agents.

3.2.3.5-AP Task 5: Engraftment of NSG mice with TKI-resistant CML or KIT D816V+ mast cells and treatment with optimized combinations of AKT and STAT5 inhibitors in vivo
Task leaders: Pr Peter Valent, Dr Karoline Gleixner, Dr X2 (post-doc to be recruited), Mr or Mrs X3 (Research Technician to be recruited)
Duration: 7 months
After having identified most potent anti-neoplastic drugs and defined most potent drug combinations, the in vivo effects of these drugs will be examined using a xenotransplantation approach employing NSG mice. In a first step, cell lines and single drugs will be applied, in order to confirm the in vivo anti-neoplastic activity of individual drugs. Then, drug combinations will be applied in order to learn whether the in vivo anti-neoplastic activity exceeds the effects of the
single drugs. In a next step, primary cells will be used, if possible, in order to confirm drug effects and drug-combination effects.

- **Objectives and success indicators:**
  Identification of drug combinations that produce synergistic anti-neoplastic effects *in vivo* on TKI-resistant CML cells and KIT D816V+ neoplastic mast cells in ASM/MCL. The primary goal is to identify most potent drug combinations that will not only kill the bulk of neoplastic cells but also the neoplastic stem cells in these malignancies.

- **Detailed work program:**
  Non-obese diabetic SCID-IL-2R-gamma^-/- mice (NSG mice) will be used in this study. Animal studies will be approved by the ethics committee of the Medical University of Vienna (Austria) and the ethics committee of the University of Veterinary Medicine Vienna (Austria), and will be carried out in accordance with guidelines for animal care and protection and protocols for experimental animal housing and studies approved by Austrian law (GZ 66.009/0040-II/10b/2009). NSG mice will be kept under stringent aseptic conditions. Twenty four hours prior to injection, mice will be irradiated in flat (sterile) irradiation-cages (2.4 Gy). Primary cells (1x10^6) or cell lines (KU812, K562, MCPV (a human MC progenitor cell line established in Vienna), HMC-1, each 1x10^5) will be injected into the tail vein or subcutaneously into adult female NSG mice. In case of primary cells, CD34+ MNC depleted of CD3^+ T cells will be injected. In typical experiments, mice will be treated with STAT5 inhibitors, AKT blockers, BCR/ABL blockers, KIT-blocking TKI, or other drugs, depending on results obtained from *in vitro* experiments. After injection, mice will be inspected daily and sacrificed as soon as they developed disease-symptoms or after a maximum observation period of 52 weeks. Then, mice will be sacrificed and BM cells (humeri, tibias, femurs, and pelvis) and spleens will be recovered. Pelvic and splenic samples will be prepared for immunohistochemistry, and long bones flushed to recover BM cells for flow cytometric and mRNA analyses. Multicolor flow cytometry will be performed using mAb against CD19, CD33, and CD45. Engraftment will be defined as a repopulation of at least 1% CD45^+ human cells in flushed mouse BM samples. Multilineage engraftment will be defined as engraftment with both CD19^+ and CD33^+ cells. CML engraftment is defined as BCR/ABL+ engraftment assessed by qPCR (at least 5% BCR/ABL). Total RNA will be isolated using RNeasy Micro-Kit (Qiagen, Hilden, Germany) and BCR/ABL mRNA will be quantified by qPCR. To define the effects of drugs and drug combinations on LSC-induced repopulation of neoplastic cells, 2 different protocols will be applied. In a first step, LSC will be pre-incubated with targeted drugs prior to injection. In another set of experiments, mice will be treated with drugs (combinations) after cells are injected. In protocol B, mice will be treated for 1-2 months and inspected for a total time period of 1 year (untreated post-therapy), in order to learn whether long term engraftment of LSC was affected by drugs. In case of cell lines, mice will be treated with drugs after injection of cells. In these experiments, the primary endpoint is disease-free survival and leukemic engraftment after a defined time period.

- **Deliverables:**
  a) Identification of individual drugs that block the *in vivo* growth of TKI-resistant CML cells or the *in vivo* growth of KIT D816V+ neoplastic mast cells
  b) Identification of most potent drug combinations that block the *in vivo* growth of TKI-resistant CML cells or the *in vivo* growth of KIT D816V+ neoplastic mast cells
  c) Confirmation that the most potent drug combinations have a visible effect on long-term engraftment of LSC in TKI-resistant CML and KIT D816V+ ASM/MCL (proof of curative potential of the drug combination).

  Coordination and logistics: P.V.; Preparation and selection of primary cells and cell lines: K.V.G.; Xenotransplantation experiments using cell lines and primary cells: Dr. X2.

- **Description of the methods and technical choices and the way in which solutions will be brought, the risks and the back-up solutions envisaged.**

  If no potent drug combination with clear synergistic effects can be detected, additional screens will be performed using new drug partners. In case that no visible engraftment of primary cells can be achieved, we will try to purify CML LSC and ASM/MCL LSC based on recently identified stem cells markers (CML: IL-1RAP^+, CD25^+; and ASM/MCL: CD123^+, CD52^+, KIT^+).
3.3 TASK SCHEDULE

Please see the Gantt diagram provided as well as the Table IV which describes all the project deliverables by each partner, the dates, the scientific/technical milestones and the contingencies.

Gantt diagram (M: planned consortium meeting)

Table IV: Summary of all the project deliverables (FP: French partner; AP: Austrian partner; ESP: External Service Provider)

<table>
<thead>
<tr>
<th>Task N° and Leader</th>
<th>Title</th>
<th>Scientific/technical milestones</th>
<th>Contingencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1/T2 Duration: 9 months FP</td>
<td>In silico modeling of AKT and STAT5 and identification of structures of interest</td>
<td>1) <em>In silico</em> modeling of the structures involved in the phosphorylation, dimerization or binding of STAT5 to DNA. 2) <em>In silico</em> modeling of binding sites of AKT to PI3-K, to PIP3, to PDK1 or to downstream partners. <em>In silico</em> modeling of phosphorylation sites of AKT.</td>
<td>1) No clear identification of the structures involved in the phosphorylation, dimerization or binding to DNA of STAT5. 2) No clear identification of the binding sites of AKT to PI3-K, to PIP3, to PDK1 or to downstream partners. No clear modeling of phosphorylation sites of AKT 3) No clear identification of the structures involved in the main biological functions of Tg1 and Tg2</td>
</tr>
<tr>
<td>T1'/T2' Duration: 9 months FP</td>
<td><em>In silico</em> modeling of one or two other critical signaling molecules (target 1: Tg1; target 2: Tg2) identified by the AP during his Task 3</td>
<td><em>In silico</em> modeling of the structures involved in the phosphorylation, dimerization or binding to DNA of STAT5.</td>
<td>1) No hits found specifically that specifically bind on the structures involved in the phosphorylation, dimerization or binding to DNA of STAT5. 2) No hits found that specifically bind binding sites of AKT to PI3-K, to PIP3, to PDK1 or to downstream partners or on phosphorylation sites of AKT 3) No hits found specifically that specifically bind on the structures involved in the biological functions of Tg1 and Tg2</td>
</tr>
<tr>
<td>T3 Duration: 2 months FP</td>
<td><em>In silico</em> screening of chemical libraries on potential docking sites of AKT and STAT5</td>
<td>1) <em>In silico</em> screening of chemical libraries on the structures involved in the phosphorylation, dimerization or binding to DNA of STAT5. 2) <em>In silico</em> screening of chemical libraries on binding sites of AKT to PI3-K, to PIP3, to PDK1 or to downstream partners or on phosphorylation sites of AKT.</td>
<td>1) No hits found specifically that specifically bind on the structures involved in the phosphorylation, dimerization or binding to DNA of STAT5. 2) No hits found that specifically bind binding sites of AKT to PI3-K, to PIP3, to PDK1 or to downstream partners or on phosphorylation sites of AKT 3) No hits found specifically that specifically bind on the structures involved in the biological functions of Tg1 and Tg2</td>
</tr>
<tr>
<td>Duration:</td>
<td>AP</td>
<td>FP</td>
<td>ESP</td>
</tr>
<tr>
<td>----------</td>
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</tr>
<tr>
<td>T3'</td>
<td>2 months</td>
<td>Synthesis (2 months) and optimization (2 months) of the various hits identified by <em>in silico</em> screening on AKT and STAT5</td>
<td></td>
</tr>
<tr>
<td>T4:</td>
<td>4 months</td>
<td>Synthesis (2 months) and optimization (2 months) of the various hits identified by <em>in silico</em> screening on potential docking sites of Tg1 and Tg2</td>
<td></td>
</tr>
</tbody>
</table>
| T4 and/or T4': | 4 months | 1) Drugability of the hits subject to caution  
2) Synthesis of the hits difficult  
3) Potential toxicity of the major hits identified |
| T5/T5'   | 2x5 months | *In vitro* testing of combinations of optimized hits on CML or mastocytosis cell lines and on normal hematopoietic progenitors in order to select a limited number of synergistic combinations with no or non-significant toxicity on normal cells |
| T1:      | 9 months | Recruitment and Sampling of TKIs-resistant CML or KIT D816V+ mastocytosis patients |
| T2:      | 10 months | Purification and characterization of malignant cells *ex vivo* |
| T3:      | 7 months | Analysis of the expression and functions of STAT5, AKT and of other signaling molecules in patients cells and in cell lines : identification of additional targets Tg1 and Tg2 |
| T4/T4'   | 2x4 months | Testing of optimized combinations of AKT and STAT5 (T4) and of AKT, STAT5, Tg1 and Tg2 inhibitors on patients cells *ex vivo* |
| T5/T5'   | 2x7 months | Engraftment of NSG mice with TK1-resistant CML cells or KIT D816V+ mast cells and treatment with optimized combinations of AKT, STAT5, Tg1 and/or Tg2 inhibitors *in vivo* |

| T4:      | 1) Synthesis of the various hits identified by *in silico* screening on AKT and STAT5.  
2) Optimization of selected hits identified by *in silico* screening on AKT and STAT5 whose chemical structure predicts for drugability and limited toxicity |
| T4':     | 1) Synthesis of the various hits identified by *in silico* screening on potential docking sites of Tg1 and Tg2.  
2) Optimization of selected hits identified by *in silico* on potential docking sites Tg1 and Tg2 whose chemical structure predicts for drugability and limited toxicity. |
| T5:      | 1) Large-scale dose-response testing of optimized drugs targeting AKT and STAT5 (T5), Tg1 and Tg2 (T5') *in vitro* in order to select the most potent drugs and determine their IC50 in malignant cells.  
2) Cross-testing of the combinations of most potent drugs used at their IC50 in order to evidence synergistic effects on malignant cells  
3) Testing on normal hematopoietic progenitors of the best combinations selected for synergistic effects , in order to evaluate toxicity |
| T5':     | 1) IC50 of AKT or STAT-5 inhibitors > 1 μM.  
2) No synergistic effects on malignant cells *in vitro*.  
3) Significant *in vitro* toxicity of the best combinations of drugs on normal hematopoietic progenitors. |
| T5'      | 1) IC50 of Tg 1 or Tg 2 inhibitors > 1 μM.  
2) No synergistic effects of Tg 1 or Tg 2 targeted drugs on malignant cells *in vitro*.  
3) Significant toxicity of the best combinations Tg 1 or Tg 2 targeted drugs on normal hematopoietic progenitors. |

| Recruitment of at least 20 TKI-resistant CML patients and 10 aggressive systemic mastocytosis (ASM) patients |
| Identification of a combination of cell surface antigens defining the stem cell fractions of the malignant cells *ex vivo* |
| Phenotypic characteristics of the malignant stem cells differing from one patient to one other |
| 1) demonstration that AKT and STAT5 are spontaneously activated in neoplastic cells in all patients tested  
2) identification of new targets (Tg1/Tg2 and demonstration of their functional role by shRNA |
| 1) Variable expression of activated AKT and/or STAT5 in the cohort of patients  
2) Failure in identifying other relevant targets |
| Lack of optimal and synergistic effects on patients cells |
| Demonstration that combined therapy induces optimal and synergistic effects including the eradication of neoplastic stem cells *in vivo* |
| 1) Failure of leukemic stem cells to engraft in NSG mice  
2) Failure to obtain complete and long-lasting remissions in engrafted mice |
**Planned consortium meetings**

- **Month 9:** 1 joint meeting in Cachan involving FP and AP to present the data obtained by *in silico* modeling of AKT and STAT5
- **Month 18:** 1 joint meeting in Cachan involving FP and AP to present the data obtained *in vitro* with combinations of specific inhibitors of AKT and STAT5
- **Month 23:** 1 joint meeting in Cachan involving FP and AP to present the data obtained *in silico* modeling of Tg1 and Tg2 molecules
- **Month 27:** 1 joint meeting in Vienna involving FP and AP to present the data obtained *in vitro* with combinations of specific inhibitors of Tg1, Tg2, AKT and STAT5
- **Month 33:** 1 joint meeting in Vienna involving FP and AP to confront the data obtained *in vitro, ex vivo* and *in vivo* with optimized combinations of AKT and STAT5 inhibitors
- **Month 36:** 1 joint meeting in Vienna involving FP and AP to confront the data obtained *in vitro, ex vivo* and *in vivo* with optimized combinations of AKT, STAT5, Tg1 and Tg2 inhibitors

The frequency of the meetings increases with time as expected data will become available *in vitro, ex vivo* then *in vivo* closely in time after a relatively long but necessary period of *in silico* modeling from the French side and of sampling and analysis of malignant cells from patients from the Austrian side.

4 **DISSEMINATION AND EXPLOITATION OF RESULTS AND INTELLECTUAL PROPERTY**

**Exploitation of results and protection of intellectual property**

The ultimate goal of this collaborative project is to identify critical signaling pathways in neoplastic cells in advanced SM and CML, to examine and validate these pathways, and based on these observations, to define most potent drug combinations capable of eliminating neoplastic stem and progenitor cells. Both partners will disseminate key results after having explored the possibility to file patents. Patent scouting, patent negotiations, and patent filing will be performed in collaboration with the National Funds and with the Hosting Institutions.

After having identify and patented several combinations of drugs targeting specifically and potently STAT5 and AKT or other key signaling molecules, we hope to license the drugs to a first rank pharmaceutical company interested in the treatment of such diseases (at that time, we can cite as potentially interested companies: Bristol-Myers Squibb, Novartis, Roche and Sanofi). Indeed, neither the FP nor the AP has access to all the facilities allowing them to substitute to a pharmaceutical company in order to perform pre-clinical and clinical development of such drugs.

An important aspect is to explore ways to translate key findings into clinical trials in future studies. However, obviously, the AP who takes care at the medical level of such patients will be certainly involved in the clinical phases of development of the drugs.

Before developing such drugs, a number of milestones have to be reached, from *in silico* modeling and screening to *in vitro* and *ex vivo* validation and finally exploring effects on neoplastic stem cells. In addition the program of research will need constant dialog between the two partners to ensure optimal selection of compounds.

As it is an interconnected joint program, in order to ensure its efficient protection and proper distribution of any intellectual property arising from its accomplishment, the coordinator will establish at the beginning of the program a confidentiality agreement to be signed by all members involved. It will focus on the results of each stage of the program and will prohibit any oral or written shared results to third people without the joint agreement of the coordinator and the partners.

**Dissemination of results**

The critical discoveries will be communicated in form of publications or as oral or written presentation at international congresses, only after patent filing has been completed. For presentation of data at congresses, financial support for registration fees, hotel, and travel costs is requested in the present program.

For the French partner’s tasks, only the results of the *in silico* modeling of AKT and STAT5, or of other key signaling molecule(s) if needed, will be presented, if it brings new data on the general structure of the molecules. Neither the structure of the potentially interesting pockets, nor the results of the *in silico* screening of chemical libraries or of the *in vitro* effects of hits selected by the previous steps will be presented before having secured IPR or having filed patents.

For the Austrian partner’s tasks, only the results of characterization and purification of malignant stem cells or the analysis of the cooperating signaling networks in CML and mastocytosis will be presented if it brings new data on the biology of these cells. Neither the effects of combinations of hits *ex vivo* on patient’s cells, nor the results of the testing of the combinations of drugs *in vivo* will be presented before having secured IPR or having filed patents.

The communication to other scientific communities, following the limitations fixed above, will take the form of the participation of the PIs of the two teams, as well as of the Post-Docs and the PhD students to, at least one international congress (such as the congress of the American Society of Hematology; ASH; which is held mid-December) twice during all the duration of the program. It is expected that the first participation will take
place in December 1014 (where data from in silico modeling will be presented) and the second one in December 2015 (where data from characterization of malignant stem cells and of the cooperating signaling networks in CML and mastocytosis will be presented).

PATENTS AND ECONOMIC OUTCOMES

We expect to file patent applications for at least the following subjects:
- The new molecules that will bind specifically to AKT, STAT5, or any other critical signaling intermediates or that will inhibit the binding of these signaling intermediates to their molecular partners.
- The use of these molecules, in combination, to overcome resistance in TKIs-resistant CML, or in KIT D816V+ mastocytosis.
- The potential use of these combinations to treat any other kind of hematologic malignancies or solid tumors where they will show activity in additional studies out of the frame of the present program.

Patents deposit will be made conjointly by our supervisory authorities (CNRS and ENS-Cachan for the FP and Medical University for the AP). The inventors will include at least the PIs of each partner and, following their respective merits, the other members of the two teams. The relative percentage of each contributor, as well as all the processes of patenting then of licensing of the drugs will be jointly managed by the departments of valorization, patents and industrial contracts of the CNRS, the ENS-Cachan and the Medical University of Vienna.

Only after patenting, the structure of the targeted drugs, as well as their effects in vitro, ex vivo and in vivo will be published, according to the policy of the license given to pharmaceutical company.

In terms of economic impact in case of success of the research program, it should be noted that, although it does not address the most common pathologies, the type of targeted therapies that we offer is extremely expensive and the potential market important in terms of return on investment. For example, the current treatment of CML with imatinib in France costs about 28 KEuros/year/patient, knowing that this is life-long. Given that the number of new cases of CML patients is estimated around 600/year in France, this represents a mean market nearing 100 million Euros/year for the country. Obviously, a treatment that will show any superiority in terms of efficacy or even stem cell elimination (so that all drugs can be discontinued safely) or/and will overcome TKI-resistance in CML or ASM must be expected to get a significant position on the market. Economic benefits expected from this program, if successful, can be considerable also in terms of jobs created in the pharmaceutical industry. In addition, we hope to be able to target other more common tumors which currently have a deceiving treatment and for which the involvement of STAT5 and AKT in cell proliferation has been clearly demonstrated, such as AML, breast cancer, prostate or colon cancer, and melanoma or hepatocellular carcinoma. In this case, the economic benefits arising from this program will be even much more significant.

5 CONSORTIUM DESCRIPTION

5.1. PARTNERS DESCRIPTION, RELEVANCE AND COMPLEMENTARITY

1- The French partner:

The French partner (FP-P1) is a group of research headed by M. Arock, which belongs to the team “Molecular Oncology and Pharmacology” (MOP) of the LBPA unit. P1 has developed over many years researches on the molecular mechanisms leading to allergic/inflammatory reactions focused primarily on the physiology of mast cells (MCs). More recently the team has approached the study of the involvement of these cells in innate immunity. Finally, since 1999, P1 conducts studies on the pathophysiology of mastocytosis. P1 has published over 100 research articles mostly focused on MCs and, more recently on mastocytosis, as well as over 30 peer-reviewed articles on MCs and/or on mastocytosis. His h-index arrived at 39 and his articles have been cited over 5000 times. He is co-inventor of 7 national or international patents, all about MCs.

Regarding mastocytosis, P1 is particularly interested in the biological consequences of activating mutations of KIT (especially mutations in the kinase domain, such as KIT D816V) on proliferation and differentiation of MCs as well as on intracellular signaling, particularly AKT and STAT5. In addition, P1 studies the effect of new kinase inhibitors on these phenomena. In this context, the French partner team works closely with the Bioinformatics, Molecular Dynamics and Modeling (BiMoDyM) group (headed by Dr Luba Tchertanov), which belongs also to the MOP team. Besides, the French partner works in close collaboration with the team of the Austrian partner since 2002. Since then they have published 16 co-authored original articles or reviews and the French partner is the French correspondent for the European Competence Network on Mastocytosis that has been established by the Austrian partner, and the Vice-President of the French Association of mastocytosis patients (AFIRMM). Finally, the French and the Austrian partners co-share as inventors an international patent on a new human KIT D816V+ MC line, have on-going common research projects and are co-supervisors of a PhD student who has been granted a Marie Curie Fellowship in Cancer Stem Cell (Euro Cancer Stem Cell training network) from 2010 to 2014. Both P1 and P2 have common working meetings once or twice a year since 2009 in Vienna or in Paris.

2- The Austrian partner:
The Vienna partner (AP-P2), P. Valent, is a leading authority in the field of MCs and mastocytosis research. He has established a European Competence Network on Mastocytosis (ECNM) and runs a Center of Excellence of the ECNM in Vienna. The partner published over 500 articles, over 200 peer-reviewed articles on mast cells and over 100 peer-reviewed articles on mastocytosis. His h-index arrived at 71 and his articles have been cited over 27000 times. He is co-inventor of 5 international patents. The partner P2 and his group have dedicated their work to the evaluation of signaling molecules and pathways contributing to abnormal growth and function of neoplastic mast cells and the evaluation of specific molecules as potential targets of drug therapy. The labs of the partner provide optimal conditions for the current project, because they are located at the Vienna General Hospital (Medical University of Vienna) providing access to patients with mastocytosis or CML, high speed sorters, xeno-transplantation facilities, and other core facilities required for the current project. Moreover, the staff exhibits substantial experience in the isolation, culture and functional characterization of normal and neoplastic mast cells as well as of malignant stem cells, the evaluation of signaling nodes and pathways, and the effects of various targeted drugs on such cells. Altogether, the labs of the research group in Vienna provide an optimal environment for the conduct of the current study and will nicely complement research performed in the lab of the partner in Paris.

3- Complementarity and added value of the collaboration:

Both French and Austrian partners are involved for long times into the study of the biology of MCs and into studies concerning the physiopathology, classification, diagnostic and treatment of mastocytosis. In addition, the Austrian partner, who has easy access to CML and ASM patients, is also involved for years in the study of malignant stem cells for both diseases.

The French partner (FP) has a long-term expertise in the study of the biology and ontogeny of MCs derived from hematopoietic progenitors of various animals and of humans. As an example he co-shares a patent on pig mast cell lines derived from hematopoietic progenitors (Pig mast cell cultures and uses thereof. WO 2003/035853), as well as a patent on mouse peritoneal mast cell lines (The use of peritoneal mast cells as a heparin source. WO 2008/116887). Besides, in the 80’s and in the 90’s, he has published a number of research articles focusing on various properties of human MCs derived from hematopoietic progenitors. Since 1999, the French partner has focused most of his research topics on the pathophysiology, classification, prognostic and treatment of mastocytosis. Being since then Vice-President of the French Association of mastocytosis patients (AFIRMM: http://www.afirmm.com), he has been involved in the constitution of the national network of research on the disease as well as of the national registry of patients. Since 2002, he is the French representative at the European Competence Network on Mastocytosis (ECNM: http://ecnm.net/homepage/index.php) established and headed by Professor Peter Valent (The Austrian Partner). Regarding his involvement in the field of the physiopathology of mastocytosis, the FP has participated in the national screening of KIT structure in French children and adults suffering from mastocytosis and has shown on a cohort of more than 600 adult patients that the KIT D816V mutation could be evidenced in more >85% of them, whereas on a cohort of 110 children, the KIT D816V mutation was found in 35% of the patients and that 40% of the other patients were positive for new mutations mainly located in the extracellular domain of KIT. Since 1999, the FP has been granted more than 1250 K Euros from ANR, ARC, MRT, or other funding sources for his researches on MCs and mastocytosis. In 2007, the French and Austrian Partners have begun a close collaboration on the topic with common research projects and meetings. In 2009, the French partner has established a new human MC line called ROSA KIT WT which, unlike other human MC lines described to date, grows rapidly, exhibits the morphological and biological properties of normal MCs and has a normal KIT structure. The FP has immediately shared this cell line with the Austrian partner and joint experiments have been achieved or are still ongoing on the cell line. In addition, by lentiviral transfection, the French partner has stably introduced the D816V mutant form of KIT in the ROSA cells, giving rise to a new human factor independent MC line with all the characteristics of malignant MCs from patients with systemic mastocytosis. This ROSA KIT D816V cell line has been also immediately shared with the AP and joint experiments are still ongoing on the cell line. Interestingly, these joint researches conducted on ROSA KIT D816V cell have already shown that AKT and STAT5 are constitutively activated in these cells, a phenomenon not observed in ROSA KIT WT cells. These 2 ROSA cell lines have been patented with as co-inventors M. Arock and P. Valent and will be used in vitro by the FP and in vivo by the AP for experiments proposed in the present program of research. The Austrian partner has established the MCPV cell line, an immature mast cell leukemia cell line that is rapidly expanding in vitro and in vivo in NSG mice and thus another useful tool to test various antineoplastic drugs.

With regards to CML the FP has established a new human BCR/ABL+ leukemic cell line, termed ERY-1 since it express erythroblastic markers, from the peripheral blood of a CML patient in blastic phase. ERY-1 is imatinib-sensitive but can be driven to resistance by long-term treatment (ERY-1R) with the ITK. Furthermore, the French partner has also in hands the imatinib-resistant BCR/ABL T315I+ CML cell line (KBM5-T315I) where it appears that AKT and STAT5 are constitutively phosphorylated (Lu et al, Mol Cancer. 2010 May 19;9:112).

In addition, the FP works in collaboration with the Bioinformatics, Molecular Dynamics and Modeling (BiMoDyM) group (Dr L. Tchertanov), which belongs to the same team in Cachan. This group is specialized in

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computational biology and use methods of bioinformatics, modeling and molecular dynamics, normal modes, free energy calculations of reaction path and docking, and is highly interested by (i) the mechanisms of activation/deactivation of protein kinases (PKs), (ii) the role of oncogenic mutations, (iii) the structural changes associated with differential signaling of PKs and, (iv) the mechanisms of inhibition and of resistance to inhibitors. This group will collaborate closely with the FP to establish in silico modeling of AKT and STAT5 and screening of potential inhibitors.

To sum up, the FP has access (i) to in silico modeling of AKT and STAT5 and screening of potential inhibitors, (ii) to chemical synthesis by collaboration with other members of the LabEx LERMIT (which brings together biologists, physicians, chemists and in order to design and produce medicines of the future, and for which cancer is one of the three major topics) and, (iii) to the relevant cell line models of mastocytosis and of CML, where STAT5 and AKT are constitutively phosphorylated, that will be used to confirm in vitro the inhibitory activity of selected hits and to decipher the mechanisms of action of these molecules on cell proliferation and apoptosis.

The Austrian partner (AP) heads a team which is world-wide known for its long-time expertise in the field of mast cell- and mastocytosis research. His labs are located at the Vienna General Hospital (Medical University of Vienna) providing access to patients with mastocytosis or CML, high speed sorters, xenotransplantation facilities, and other core facilities. He has established a European Competence Network on Mastocytosis (ECNM). Specific aims in the ECNM are to provide the best available Information for patients and doctors, provide access to important diagnostic tests for all patients, to establish standards for the diagnosis and treatment of mastocytosis, to establish Reference Centers and Centers of Excellence in Europe, and to facilitate referrals to specialists in these centers for all patients, either through doctor-doctor tele-net-contact, or direct referral if required. The network also cooperates with authorities in the field of mastocytosis in the US and other non-European countries (External Consultants) in order to establish worldwide standards. Besides, the AP runs a Center of Excellence of the ECNM in Vienna, where most of the Austrian SM patients (> 200) but also CML patients are diagnosed, investigated and followed. The AP has thus access to and follows patients with SM (including aggressive subtype, ASM) or TKI-resistant CML, which is a prerequisite for the successful performance of the present program of research. The team has dedicated its work to the evaluation of signaling molecules and pathways contributing to abnormal growth and function of neoplastic MC and CML cells and the evaluation of specific molecules as potential targets of drug therapy. In addition, for about 10 years, the AP is working on neoplastic stem cells, particularly in the frame of CML and mastocytosis and has already characterized the phenotype of such cells as evidenced by several publications. Moreover, the AP has been the first to demonstrate that (i) AKT and STAT5 are constitutively phosphorylated in neoplastic MC in SM (ii), that this activation is linked to the presence of the constitutively activated mutant KIT D816V, and (iii) that knock-down of either STAT5 or AKT activity results in growth inhibition of neoplastic MCs. These crucial data suggest that a downstream STAT5-PI3K-AKT signaling cascade is essential for KIT D816V-mediated growth and survival of neoplastic MCs and thus provides major arguments supporting the proposed program of research. With regard to CML, the Austrian partner has conducted a number of research projects, mostly focusing on the discovery of new mechanisms and novel targets, as well as to the characterization and targeting of leukemic stem cells. As an example, the Austrian partner has identified several new targets that could overcome imatinib resistance in CML, such as polo-like kinase 1 (PLK1), heat shock protein 32 (HSP32)/heme oxygenase-1 (HO-1), or BTK.

To sum up, the AP has access (i) to ex vivo samples of patients suffering from ASM or imatinib-resistant CML, (ii) to high speed sorters allowing to purify different populations of malignant stem cells, to analyze their phenotypes, their expression of various signaling molecules (such as STAT5 and AKT) and their response in vitro to treatment by the various chemical combinations identified by the FP and (iii) to xeno-transplantation facilities which will allow the AP to engraft NSG or other highly immuno-deficient mice with purified malignant stem cells then to treat these animals with the best combinations of STAT5 and AKT inhibitors as evidenced above.

All in all, the long-term close collaborations and constant sharing of materials and concepts on mastocytosis and other hematological malignancies that exist between the two partners make them perfect allies to carry out this joint research program. In addition, the complementary methods used and their access to all necessary platforms and biological materials will allow synergy between the two teams to optimize the combinations of molecules of therapeutic interest across all screens required, from in silico to in vivo through in vitro then ex vivo.

5.2 Qualification and Contribution of Each Partner

Features documenting the ability of the French partner:

The French partner, Michel Arock, PharmD, PhD, has a long-term expertise in the study of the ontogeny of MCs and on their biological properties. Beginning in the 80’s, he has published a number of research articles focusing on various properties of normal MCs. Since 1999, he has focused most of his researches on the pathophysiology, classification and treatment of mastocytosis, allowing him to publish 27 research articles or reviews on the topic. Being since then Vice-President of the French Association of mastocytosis patients (AFIRM MM), he has been involved in the constitution of the national network of research on the disease as well as
of the national registry of patients. Since 2002, he is the French representative at the ECNM. After the obtention of his PhD in 1988, and all along his career, first as associate Professor, then as Full Professor, he has headed research groups composed at least of one technician and of two or three PhD students, often plus a post-doc. He is presently heading a research group composed by one full time research technician and four PhD students (among them one is in co-direction with the AP and one is in co-direction with Dr Luba Tchertanov, Research Director and head of the research group “BiMoDyM”, which is also involved in the present project for the in silico tasks). Besides, throughout his career, Michel Arock oversaw many successful PhD students and has consistently obtained financial support from various public organizations or associations allowing him to carry out his researches (as an example, since 1999, he has been granted more than 1250 KEuros from ANR, ARC, MRT, Fondation de France, or from other funding sources). He is considered as one of the best French expert in mast cell biology and in mastocytosis and has ongoing collaboration with different national and international teams on various aspects of these topics. He is regularly invited to give conferences on these topics. Besides, he is the President of the section “Biological sciences” of the French National Academy of Pharmacy and a member of the board of the SFBC (French Society of Clinical Biology). However, for five years, Michel Arock has established very close collaborative links with Pr. Valent in Vienna, which led to the publication of numerous articles in common and to frequent joint meetings. Thus the common program of research, although supported by the coordinator in France, is a logical consequence of this active transnational collaboration between the two teams interested to put together their strengths and complementarities.

**Short CV of the members of the French partner:**

**Arock Michel; 54 years old PharmD, PhD, Professor**

**Career Path:**

Doctor in Pharmacy: June 1982; PhD: December 1988
Assistant Professor in Hematology: June 1983 to October 1989
Associate Professor in Hematology: October 1989 to September 1995
Full Professor in Hematology: September 1995 to September 2005

**Current situation (from 2005)**

Full Professor First Class in Physiology; Laboratoire de Biologie et Pharmacologie Appliquée (LBPA) CNRS UMR 8113 - Ecole Normale Supérieure de Cachan.

**Synopsis in Science:**

129 publications in international reviews (18 in the last 5 years); 7 national or international patents; 56 communications in international congresses; Cites/year: 157.77; Cites/paper: 22.54; Cites/author: 1062.25; h-index: 39.

**List of five most significant publications (and/or patents) over the last 5 years:**


**Tchertanov Luba; 54 years old, PhD, Research Director**

**Career Path:**

Physicist (Master's degree, 1983, Kazan State University, USSR), Doctor of Science in crystallography (summa cum laude,1988, Nesmeyanov Institute of the Academy of Sciences of the USSR, Moscow)
Qualified to Supervise Research Life Sciences (HDR, 1998 at Université Paris XI, Orsay).

**Current situation**

Research Director (DR2) of the CNRS, head and founder of the team BiMoDyM (Http://tinyurl.com/tchertanov) at the LBPA and member of the Steering Committee of LabEx LERMIT.

**Global scientific production:**
L. Tchertanov has published 85 original articles, 3 magazines (h index =20, 1141 citations for research with chertanova or Tchertanov *) and 3 Russian patents.

**List of five most significant publications (and/or patents) over the last 5 years:**


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**Bibi Siham; 24 years old, PhD student**

**PhD student - Combined inhibition of PI3K/AKT and other signaling pathways: a new therapeutic approach to acute myeloid leukemia and KIT D816V + systemic mastocytosis** Molecular Oncology and Pharmacology Team, LBPA, ENS Cachan.

**Education**

2012-Current: PhD in Cancerology. Paris Sud 11 University

2012: University Diploma in Hematology. Paris Sud 11 University

2011: Master 2 degree in Molecular and Integrative Biology (Research path). Versailles Saint Quentin en Yvelines University – France.

2010: Master 2 degree in Experimental Pharmacology (Research path). Jijel University-Algeria.

2008: Bachelor in Natural Sciences and Life. Jijel University-Algeria.

**Other professional experiences**


Mar-Jun 2011: 4 months internship. ENVA-Laboratoire de Génétique Fonctionnelle et Médicale – UMR 955 INRA - ENVA. Characterization of transgenic mouse lines for overexpression of RACK1 protein in melanocyte lineage

Nov-Jan 2011: 3 months internship. ENS de Cachan – Laboratory of Applied Biology and Pharmacology – CNRS UMR 8113. In vitro study of the effect of doxorubicin nanoparticles on human mast cells

Jan-Jun 2010: 6 months internship. Jijel University – Laboratory of Cellular and Molecular Toxicology. Evaluation of haematotoxicty of doxorubicin encapsulated in liposomes in rats

Feb-Apr 2008: 3 months internship. Jijel University – Laboratory of Cellular and Molecular Toxicology. Induction of liver cancer by aflatoxin B1 in rats wistar albino.

**Scientific communication**


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**Langenfeld Florent; 26 years old, PharmD, PhD student**

**Education**


2004 – 2011 **Pharm.D.** Faculty of Pharmacy, Henri Poincaré-Nancy 1 University, France. Degree conferred: October, 2011

**Employment Experience**


Advisors: Pr. M. Arock and Dr. Luba Tchertanov. Laboratory of Biology and Applied Pharmacology, CNRS-ENS Cachan, Cachan, France.


April 2009 – August 2009: Internship. Setting up of a clinical multicentre study on telemedicine. Advisors: Dr. N. Therin, PharmD and Dr. D. Laplaud. Auxilia Medical, Maxéville, France.

Jeanningros Sylvie; 27 years old, research technician
Research Technician - Molecular Oncology and Pharmacology Team, LBPA, ENS Cachan

Education
Oniris, Nantes, France
2012: French Level-1 authorization for animal experimentation
University of Bourgogne - Franche-Comté, Besançon & Dijon, France
2009: Master of Science (Professional path) in Management and Innovation in Biotechnology with honours
2008: Master of Science (Research path) in Biochemistry, Cell and Molecular Biology with honours
2006: Bachelor of Science in Biochemistry, Cell Biology and Physiology with honours

Other Professional Experiences
Feb 2009 - Sep 2011 (2 years and 7 months): Biopredic International, Rennes, France
Training period then Junior Engineer job – Production of human and animal primary cells (Hepatocytes, PBMC, Huvecs…) or cell lines (HepaRG®) for Research, Drug discovery, Drug development, Pharmacology and Toxicology
Jan - Jul 2008 (7 months): Inserm U866 « Lipides, Nutrition, Cancer » - LBMN, Dijon, France
Training period of Master of Science Degree level 2 in Biochemistry, Cell and Molecular Biology. Subject: study of lipid membrane pathway involved in sensitisation of tumour cells by resveratrol.
Feb - Mar 2007 (2 months): Inserm UMR U645 – EFS Bourgogne Franche-Comté, Besançon, France
Training period of Master of Science Degree level 1 in Biochemistry, Cellular and Molecular Biology. Subject: Impact of culture conditions on alloreactivity of gene modified T cells.

Publications

Short CV of the members of the Austrian partner (P2):

Valent Peter; 50 years old MD, Professor

Career Path:
1987 M.D.
1990 Group Leader at the Medical University of Vienna
1992 Associate Professor Experimental Hematology
1995 Associate Professor Internal Medicine
2008 Director Ludwig Boltzmann Cluster Oncology

Current Situation:
Research Group: 5 Senior Researchers, 5 Postdocs, 3 PhD Student, 2 Technicians, 3 Diploma Students

Synopsis in Science:
Invited Lectures =140; Funded Grants (PI) =40; Clinical Trials (PI) =35; Major Scientific Awards =12; Member in Societies =09; Reviewer, peer-R-Journals =24; Editor, peer-R-Journals =07; Original Articles =400; Review Articles =100; Impact factor Total =3000; h-index =71; Citation Index First Author =3500; Patents n=05

Network Coordination:
European Competence Network on Mastocytosis = ECNM: www.ecnm.net
CML platform and MDS platform of ÖGHO

List of five most significant publications (and/or patents) over the last 5 years:


Sperr Wolfgang; 47 years old, MD, Professor

Career Path:
1991 M.D. at the Medical University of Vienna
1991-1999 Training in Internal Medicine
2001 Specialist Degree – Hematology & Oncology
2001 Associate Professor Internal Medicine
2004 Program Director for AML and MDS
2009 Specialist Degree – Intensive Care Medicine

Current situation:
Coordinator of Clinical Trials and Data Set in AML, CML, MDS and SM at the Department of Medicine I, Division of Hematology, Medical University of Vienna

Synopsis in Science:
Invited Lectures=36; Funded Grants (PI)=3; Clinical Trials (PI)=15; Original Articles n=226; Impact Factor Total n=1100; Citation Index First Author=1200; h-index=52.

List of five most significant publications (and/or patents) over the last 5 years:

Gleixner Karoline; 30 years old, MD, Staff Hematologist

Career Path:
2000 General French Baccalaureate, scientific section
2006 M.D. and Thesis
2012 Applied for Associate Professorship

Current Situation:
Current Position: Staff Hematologist and Researcher at the Medical University of Vienna (MUV)

Synopsis in Science:
Invited Lectures n=6; Scientific Awards n=2; Original Articles n=26; Impact Factor Total n=160; Citation Index First Author n=150; Patents n=1

List of five most significant publications (and/or patents) over the last 5 years:


Other on-going projects in close relation to the present program:

<table>
<thead>
<tr>
<th>Partner Nº and name of involved people</th>
<th>Project name</th>
<th>Grant allocated</th>
<th>Funding institution</th>
<th>Funding period</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. Arock (P1)</td>
<td>Resistance to targeted therapies in oncology: Strategy of identification of new mechanisms of inhibition of validated therapeutic targets</td>
<td>50 k€</td>
<td>Fondation de France</td>
<td>2012-2013</td>
</tr>
<tr>
<td>P. Valent (P2)</td>
<td>Euro Stem Cell Training</td>
<td>250 k€</td>
<td>EU MC</td>
<td>2011-2013</td>
</tr>
<tr>
<td>P. Valent (P2)</td>
<td>Pathogenesis in Mastocytosis</td>
<td>40 k€</td>
<td>TMS</td>
<td>2011-2013</td>
</tr>
</tbody>
</table>

- The project “Resistance to targeted therapies in oncology: Strategy of identification of new mechanisms of inhibition of validated therapeutic targets” of the French partner (P1) has as main aim to combine the methods of molecular modeling and in silico screening with experimental biology to identify the molecular mechanisms of activated tyrosine kinase receptors, and particularly KIT, presenting with drug-resistant mutations and to discover allosteric inhibitors of these mutants. Thus, this project is clearly related to the present program.

- The project “Euro Stem Cell Training” of the Austrian partner (P2) has the aim to validate recently identified novel leukemic stem cell markers by screening sample-series and by functional assays using siRNA or shRNA, natural ligands, and various targeted drugs. One of the PhD student co-directed by P1 and P2, Mrs Ghaiith Wedeh, is a recipient of the Marie Curie Fellowship in Cancer Stem Cell from 2010 to 2014. Mrs Wedeh works on the following topic: “pharmacological differentiation of neoplastic stem cells in mastocytosis as a new therapeutic strategy”, which is highly relevant to the present program since it involves characterization of malignant stem cells in mastocytosis.

- The project “Pathogenesis in Mastocytosis” of the Austrian partner (P2) has the major aim to identify novel drug targets in neoplastic mast cells, and to screen for drug effects. Again the project is highly relevant to the present program since it involves identification of new drug targets in mastocytosis.

6. SCIENTIFIC JUSTIFICATION OF REQUESTED RESOURCES

Detailed financial support requested for each partner:

**The French partner (P1)** requests a total aid from ANR of 376 168 Euros for 36 months, which is subdivided into the following items:

- Post-doc salary for 36 months: 151 200 Euros, all charges included; External Services: 80 000 Euros; Consortium missions to Vienna and participation to international meetings: 25 500 Euros; Other external expenses (consumables, small equipment, ...), including publication costs: 105 000 Euros; Management fees (public organizations or foundations funded research in marginal cost) (4%): 14 468 Euros.

The total cost of the project for the French partner is estimated at 1 019 729.20 Euros, the environmental cost being 353 227.20 Euros with an environmental rate of 80%.

**The Austrian partner (P2)** will request a total aid to FWF (Fonds zur Förderung der wissenschaftlichen Forschung) of 352 122.75 Euros for 36 months which is subdivided into the following items: Post-doc salary for 36 months: 181 830 Euros, all charges included - Technician salary for 18 months: 60525 Euros, all charges included - Consortium missions to Paris and participation to international meetings: 21 000 Euros - Other external expenses (consumables, small equipment): 72 000 Euros - General costs (management, public organizations or foundations funded research in marginal cost, congress attendance) (5%): 16767.75 Euros

6.1 Partner 1: French partner (please see enclosed detailed financial request above).

- No additional equipment will be required

Staff: the non permanent staff involved in the project for the French partner includes:

- One second year PhD student (Florent Langenfeld) who has already a PhD grant from La ligue contre le Cancer, who is co-directed by M. Arock and L. Tchertanov, and who belongs to the Doctoral School of Cancerology (Paris XI-ENS Cachan). His task in the program is to achieve the large scale in silico modeling of
STAT5 to find docking sites of interest on the protein (non-phosphorylated or phosphorylated, monomeric or dimeric, free or linked to DNA) and the in silico screening of chemical libraries in search of new molecules specifically docking on these sites. He will devote 100% of his time to this research program for the years 2013 and 2014. He will participate in the consortium missions to Vienna during the first year of the program.

- One first year PhD student (Siham Bibi) who has already a PhD grant from The French Ministry of Research, who is under the supervision of M. Arock, and who belongs to the Doctoral School of Cancerology (Paris XI-ENS Cachan). Her task in the program will be the in vitro testing of selected combinations of AKT, STAT5, Tg1 and Tg2 targeted drugs on TKIs-resistant CML and KIT D816V+ SM cell lines and the analysis of the effects of these drugs on cell signaling, cell cycle and apoptosis. She will devote 100% of his time to this research program for the years 2013, 2014 and 2015. She will participate in the consortium missions to Vienna during the two first years of the program.

- One Post-doctoral researcher, to be recruited at the beginning of the present program. The candidate will have a strong background in molecular modeling and in drug screening in silico, but also capabilities for in vitro researches on cell lines. He/she will be under the joint supervision of M. Arock and of L. Tchertanov and his/her major task in the present program will be in silico modeling of AKT and screening of virtual chemical library on the docking sites of interest identified. As he/she will be engaged for 3 years (years 2014-2015-2016), he/she will be also involved in the final optimization of the targeted drugs following the analysis of the data generated ex vivo by the AP as well as in the analysis of other signaling pathways recruited by mutant BCR/ABL or KIT, in the continuation of the work begun by Miss Bibi. He/she will devote 100% of his time to the present research program. He/she will be in charge of the reporting in congresses, will be involved in the writing of publications, and will participate in all the consortium missions to Vienna, together with the coordinator.

. Operating costs

In order to be able to achieve in silico modeling and screening, particularly for AKT, we will need a permanent access to PDB and Cambridge - CSD, version 2010 - Database, Vista, IsoStar, IsoGen (annual ), as well as to the Meso-Informatics Center at the ENS Cachan (cluster of 112 Intel Xeon cores running CentOS). In addition we will need to buy additional computers and additional software for modeling and molecular dynamics. Cost for consortium symposiums and meetings for the French partner are indicated above.

. No additional equipment will be required.

. Additional financing obtained are indicated in the Table “Other on-going projects”. The French partner will also answer to grant opportunities from ARC and from la Ligue Nationale contre le Cancer.

6.2 Partner 2: Austrian partner (please see enclosed detailed financial request above).

. No additional equipment will be required

. Staff: the non permanent staff involved in the project for the Austrian partner includes:

- One Post-doc, to be recruited at the beginning of the present program. She/he will perform target-screen assays (proteomics, genomics, synthetic lethality screens), target validation assays using siRNA and shRNA approaches, and mouse xeno-transplant experiments. He/she will be engaged for three years (years 2014-2015-2016). He/she will devote 100% of his/her time to the present research program. He/she will be in charge of reporting in congresses, will be involved in the writing of publications, and will participate in all the consortium missions to Paris, together with the PI of the Austrian partner.

- One Research Technician: she/he will perform cell isolation, cell storage, cell culture experiments, and flow cytometry experiments. In addition the technician will perform immunostainings, and drug incubation experiments. He/she will be engaged for three years (years 2014-2015-2016). He/she will devote 50% of his/her time to the present research program.

. Operating costs

Cost for consortium symposiums and meetings for the Austrian partner are indicated above.

. No internal invoicing procedures will be requested.

. Additional financings obtained are indicated in the Table “Other on-going projects”.

7. REFERENCES


