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# SMO inhibitor specifically targets the Hedgehog Pathway and reverts the drug-resistance of Leukemic Stem Cells

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# 1. INTRODUCTION

## 1.1 STEM CELL

Stem cells are immature cells that have not yet developed into specialised cells. Found throughout the body from just after conception right through to adulthood, stem cells are able to generate all the different types of cells in our bodies. The stem cells possess two properties:

- *Self-renewal*: the ability to go through numerous cycles of cell division while maintaining the undifferentiated state.
- *Potency*: the capacity to differentiate into specialized cell types. In the strictest sense, this requires stem cells to be either totipotent or pluripotent to be able to give rise to any mature cell type, although multipotent or unipotent progenitor cells are sometimes referred to as stem cells. Apart from this it is said that stem cell function is regulated in a feed back mechanism.

### 1.1.1 SELF RENEWAL

Self-renewal is the process by which a stem cell divides asymmetrically or symmetrically to generate one or two daughter stem cells that have a developmental potential similar to the mother cell. The ability to self-renew is essential for stem cells to expand their numbers during development, to be maintained within adult tissues, and to restore the stem cell pool after injury. Defects in self-renewal mechanisms can lead to developmental defects, premature aging phenotypes, and cancer. The elucidation of self-renewal mechanisms offers the potential for fundamental insights into development, cancer, and aging. Self-renewal is not the same as proliferation, although both processes depend on cell division. Proliferation is a more general term that incorporates all types of stem and progenitor cell divisions, self-renewing and otherwise. Self-renewal requires that at least one of the daughter cells has a developmental potential similar to the mother cell [1-2] (Figure 1).

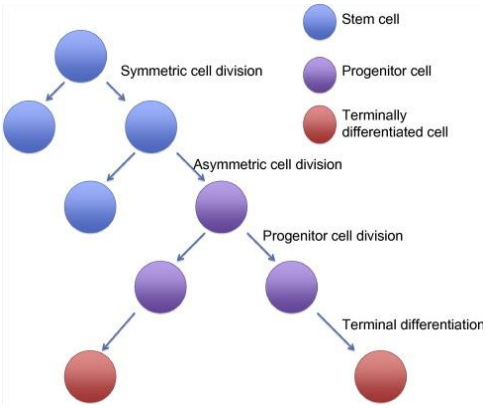


Fig. 1. Stem cell division and differentiation. In order to maintain self-renewal, stem cells undergo two types of division. During symmetrical division, each stem cell give rise to two identical daughter cells. Asymmetric division gives rise to one identical daughter stem cell and a partially defined progenitor cell..

Genes and pathways involved in the molecular control of self-renewal include the Wnt /β-catenina , Notch and Sonic Hedgehog Pathway.

### 1.1.2 POTENCY

Potency specifies the differentiation potential (the potential to differentiate into different cell types):

- Totipotent stem cells can differentiate into embryonic and extraembryonic cell types. Such cells can construct a complete, viable organism. These cells are produced from the fusion of an egg and sperm cell. Cells produced by the first few divisions of the fertilized egg are also totipotent [3].
- Pluripotent stem cells are the descendants of totipotent cells and can differentiate into nearly all cells, i.e. cells derived from any of the three germ layers [4].
- Multipotent stem cells can differentiate into a number of cells, but only those of a closely related family of cells.
- Oligopotent stem cells can differentiate into only a few cells, such as lymphoid or myeloid stem cells.
- Unipotent cells can produce only one cell type, their own, but have the property of self-renewal, which distinguishes them from non-stem cells (e.g., muscle stem cells).

## 1.2 HAEMATOPOIETIC STEM CELL

In animals, we can identify two broad classes of stem cell: embryonic stem cells (*ESCs*), and adult (somatic) stem cells (*ASCs*). *ESCs* are present in the inner of the embryo and can give rise to the three embryonic germ layers (endoderm, mesoderm and ectoderm) while *ASCs* are responsible for normal tissue maintenance. Each organ system of the body has a hierarchical cellular structure. At the head of hierarchy there is a somatic or ASCs which can self-renew within a specific tissue for indefinite periods; ASCs have also have the unique ability to retain the potential to asymmetrically divide into daughter cells, as well specialised cell types (hematopoietic, neural and mesenchymal stem cells) depending on instructions from the surrounding environment (Fig. 2).

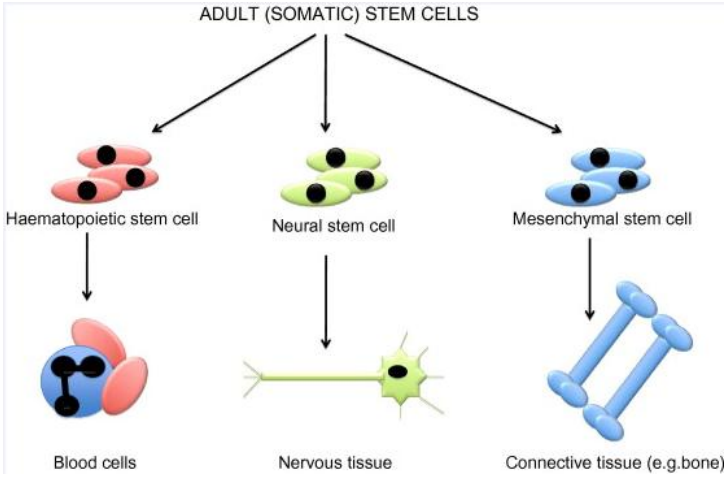


Fig. 2. The developmental potential of adult stem cell..

The hematopoietic system is thought to originate from pluripotent hematopoietic stem cells (HSCs) capable of producing a hierarchy of downstream multilineage and unilineage progenitor cells that differentiate into mature cells [5-6]. The first experimental evidence to indicate the existence of HSCs was the discovery in 1961 by Till and McCulloch of a population of clonogenic bone marrow cells capable of generating myelo-erythroid colonies in the spleen of lethally irradiated hosts [7]. HSCs have self-renewal and clonogenic abilities and can differentiate into multiple lineages. HSCs with the capacity for both long-term (LT-HSC) and short-term (ST-HSC) repopulation of the mouse hematopoietic system have been characterized. These rare cells give rise to progenitor cells, which are destined to generate fully differentiated cells [8].

Stem cells reside in highly regulated microenvironments called niches, which allow them to maintain a balance of self-renewal and differentiation. The concept of the stem cell niche was proposed by R. Schofield in 1978 for the hematopoietic stem cell (HSC) in bone marrow (Schofield 1978). Schofield stated "*The cellular environment which retains the stem cell I shall call a stem cell 'niche'* [9]. Although Schofield is generally credited with the concept of a stem cell niche, it was Trentin who proposed the idea of an inductive microenvironment. In these classic "bone-in-spleen" experiments, the inductive effect of the immediate local environment was demonstrated and the term hematopoietic inductive microenvironment was coined. The microenvironment is defined as the cells adjacent to or surrounding a stem cell. These cells, together with stem cells, constitute the stem cell niche. The inductive microenvironment provides the mechanical support and extrinsic molecular mechanisms that maintain stem cell fate and inhibit differentiation [10]. These microenvironments are maintained by a constant dialogue between the stem cells and the surrounding niche cells. The niche provides the stem cells shelter from differentiation stimuli, apoptotic stimuli and any other stimuli that might challenge the stem cell stores [11]. The niche must also protect the stem cells from overproduction, which if not properly controlled may lead to cancer [12]. The bone marrow contains multiple stem cell types, including the hematopoietic stem cells (HSCs) and the mesenchymal stem cells (MSCs). The HSCs are the best-characterized adult stem cells and have been purified close to homogeneity. Yet, only recent studies have begun to shed some light on their niche(s). In the adult bone marrow, the HSCs are known to reside in two different niches, an "endosteal" niches, an "endosteal" niche and a "perivascular" niche [11] (Figure 3).

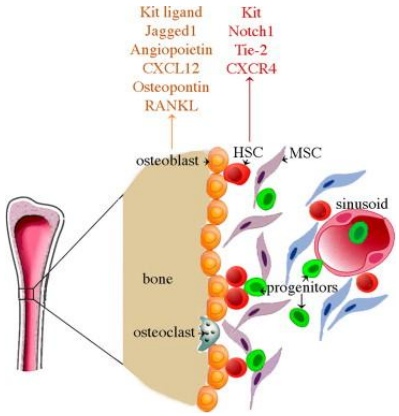


Fig. 3. Schematic representation of the endosteal and pericascular niches of hematopoietic stem cells (HSC) within the bone marrow, and signaling molecules involved in their regulation. Stromal cells, possibly including mesenchymal stem cells (MSCs), surround hematopoietic stem and progenitor cells.

- In the endosteal niche, the hematopoietic stem cells interacts with osteoclasts, osteoblasts and mesenchymal cells, involving transduction pathways of WNT, NOTCH and PTEN. The function of this niche is to ensure quiescence kinetics and the maintenance of self-renewal of HSCs. Furthermore, the osteoclasts are able to maintain the HSC in the niche or to determine its mobilization, under appropriate stimulation.

-In the vascular niche the HSCs interact with sinusoidal endothelial cells and pericytes (perisinusoidal monocytes), via the axis of communication APO1-Tie2; the niche performs its function in the regulation of the maturation process and in the mobilization of circulating HSC. It was in fact observed the ability of the latter to migrate from a niche to another, through the sinusoids.

So there is a compartmentalization of the hematopoietic tissue, which is achieved due to the different distribution of bone marrow cells and growth factors (GFs) secreted by them, within the scaffold by the elements of the extracellular matrix (collagen, fibronectin, glycosaminoglycans).

The interaction of HSCs with the different cell types and with the ECM is through the recognition of specific adhesion molecules that mediate signal transduction, thereby regulating the processes of adhesion, proliferation and differentiation of stem cells.

The haematopoietic growth factors, soluble or bound to the matrix, are instead recognized by specific receptors. called hemopoietin. They are in fact cytokine produced mainly by bone marrow cells, and lymphocytes and macrophages, mostly to paracrine action, which regulate via a system of cooperation differentiation and proliferation of stem cells.

One can distinguish hemopoietin that act by stimulating the cells more immature, from HSCs to progenitors, defined early acting GFs, and growth factors that act instead of precursors already commissioned, said late acting GFs or CSF (Colony Stimulating Factors). SCF (Stem Cell Factor) and Flt3-ligand are the two GFs early acting par excellence, while among the most important CSF were detected multi-CSF, which stimulates the differentiation of different precursors of the myeloid lineage, and the main lineage-CSF specific:

- TPO or thrombopoietin for platelet lineage;
- EPO or erythropoietin for erythroid lineage;
- GM-CSF for the granulo-monocytic lineage;
- IL-5 and IL-4 respectively for eosinophil and basophil lineage;

Other cytokines play instead a less specific, acting on different cell types (pleiotropic effect).

No less important is also the function of the negative regulators of hematopoiesis, including TGF-β, TNF-α, interferon and prostaglandins.

It is therefore clear how the different spatial distribution of these growth factors in the environment medullary stimuli differentially maturation of HSCs.

A schematic representation of the maturational process and GFs involved can be given from the tree differentiation hematopoiesis (Fig. 4), which shows how from one cell.

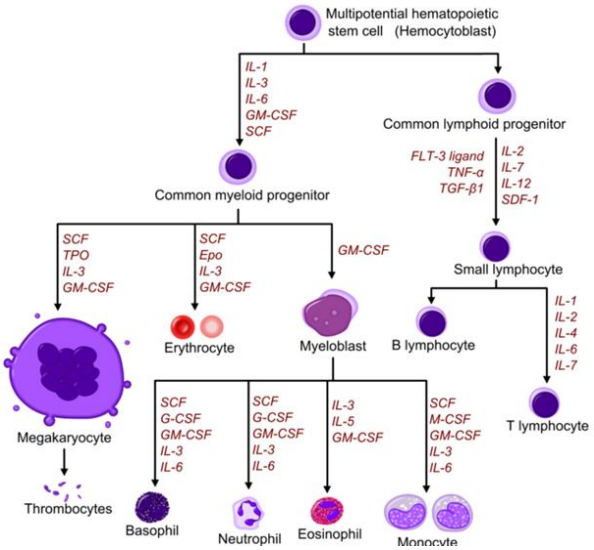


Fig. 4: Diagram showing the development of different blood cells from haematopoietic stem cell to mature cells.

### 1.2.1 CD34+ HAEMATOPOIETIC STEM CELL

In 1985 were discovered monoclonal antibodies that bind to a portion of the cell surface present on all hematopoietic progenitors, indicated as CD34. This molecule is expressed by a heterogeneous population of human hematopoietic stem cells, cells endothelial progenitor cells, vascular endothelial cells, embryonic fibroblasts and some cells of the fetal nervous tissue and not with a large capacity for proliferation and differentiation. This cellular compartment is in fact very heterogeneous, including primitive stem cells, progenitors and precursors involved in the differentiation morphologically differentiated. The CD34 molecule is an integral membrane glycoprotein. The sequence of 373 aa protein (40 kDa) is heavily glycosylated and not presents homologies identified with any known protein. The gene coding for the CD34 antigen is localized on chromosomal region 1q32, in a region containing a group of genes that encode for the adhesion molecules. The mucin-like structure of CD34 suggests its possible role in cell adhesion, perhaps in the accession of progenitor and stem cells to cells stromal cells. CD34 antibodies are widely used for applications in the field of

research as immunological studies of hematologic malignancies. Particularly significant are the studies to determine the phenotype of the compartment more immature, $CD34^+$ cells, which constitutes only a small fraction of hematopoietic tissue, those stem cells quiescent capable of self-renewal used in experiments with cell cultures and in vivo transplantation. $CD34^+$ cells are pluripotent stem cells that give rise to all cell types in blood. These stem cells are typically found in the bone marrow; fewer than 1% of nucleated cells in the blood are $CD34^+$. $CD34^+$ cells be also be found in umbilical cord blood where they typically make up 1% of the mononuclear cells.

## 1.3 LEUKEMIC STEM CELL

Some form of normal stem or progenitor cell undergoes a mutation giving rise to an entity that is functionally defined as a leukemic stem cell. The normal stem cells continue to differentiate into the hematopoietic lineage giving rise to erythrocytes, platelets, leukocytes, and granulocytes. The mutated stem cells have properties similar to the normal stem cells and can also differentiate into the hematopoietic lineage carrying the defect/s or can remain and accumulate as immature progenitor cells, also known as blast cells [13-14] (Figure 5).

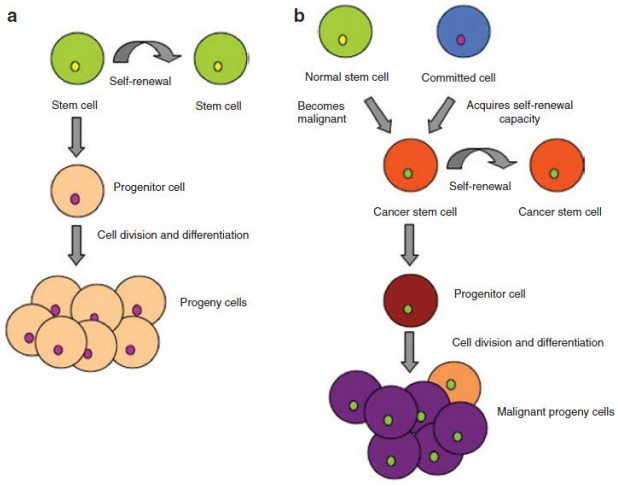


Figure 5: Asymmetric division in stem cells. (**a**) Asymmetric division in a normal stem cell. A stem cell can self-renew to give rise to another stem cell (green) but can also divide to form a progenitor cell (pink). (**b**) Asymmetric division in a cancer stem cell. A cancer stem cell (orange) can also asymmetrically divide to form another cancer stem cell (orange) or give rise to a progenitor cell (brown).

The chemotherapeutic agents used today effectively eradicate the blast cells in many patients. However, those same agents have very little if any activity at the level of the blast progenitor

cell, the leukemic stem cell (LSC), which is biologically distinct from most of the cells that are found in a typical patient. An LSC is a functionally defined entity not necessarily named because it arises from a normal stem cell, but because it fulfills the same criteria used to define normal stem cells. These cells can undergo self renewal, are multipotent, and highly proliferative. The origin of the LSC has been the subject of considerable research in recent years [15]. During normal developmental progression from stem cell to progenitor cell to mature cell, mutations may potentially occur at any point during this evolution, giving rise to a malignant entity. However, there is experimental evidence that suggests that mutations in a progenitor cell that no longer has all the characteristics of a stem cell can also give rise to an entity that can initiate and maintain leukemic disease [16]. For most leukemia, as for most cancers, the target cell of transforming mutations is still unknown. Because normal stem cells and LSCs share the ability to self-renew, as well as various developmental pathways, it has been postulated that LSCs are HSCs that have become leukemic as the result of accumulated mutations. HSCs have the machinery for self-renewal already activated and therefore may require fewer mutations to maintain it than more differentiated cells would require to activate it ectopically. HSCs also persist throughout life and therefore have much greater opportunities to accumulate mutations than more mature cells, which persist only for a short period. Conversely, LSCs could also be a more restricted progenitor or even a differentiated mature cell, which would have first to reacquire the stem cell capability for self-renewal before becoming tumorigenic to accumulate additional mutations [17]. The development of cancer is a stepwise process in which increasing numbers of somatic mutations give rise to an increasingly transformed clonal population of cells [18]. The multistep model of carcinogenesis was originally postulated to require a clonal event causing increased proliferation, which, together with mutations blocking cellular differentiation, synergizes to cause transformation.

## 1.4 LEUKEMIA

Leukemia is a monoclonal diseases of the hemopoietic system arising from the mutation of a single stem cell. The neoplastic transformation can affect cell differentiation into different stages: if it hits a multipotent HSC originates a hybrid leukemia, with population biphenotypic or biclonal (both myeloid lymphoid elements), and if the mutation occurs in a stem cell already commissioned for myelopoiesis or lymphopoiesis, originate leukemias with phenotypic myeloid or lymphoid characteristics respectively.

The neoplastic population is characterized by a high polymorphism, linked firstly to the stage of differentiation of the transformed cell and secondly to the maintenance of the same capacity maturation and differentiation.

Can be distinguished two types of leukemia: acute and chronic. In acute leukemia, the mutated cell loses the ability to differentiate and causes the proliferation and accumulation of immature cells in the bone marrow or blast cells. In contrast, the chronic form is characterized by the proliferation of cells with phenotypic characteristics of mature, of one or more cell lines depending on the potential of the cell originally mutated.

The onset of this disease is related to the acquisition of a proliferative advantage of transformed cells than normal cells. Mutations leading to activation of a oncogene or inactivation of a tumor suppressor gene are the main responsible for the survival and proliferation of cancer cells. Etiological factors behind these changes are not fully known but have been identified some risk agents that can contribute in varying degrees to the onset of the disease:

- exposure to ionizing radiation, particularly at high doses;
- the Smoke, for the presence of leukemogenic agents in cigarettes;
- exposure to chemicals such as benzene, urethane, and others, for their ability to bind and permanently damage the DNA;
- treatment with conventional chemotherapy;
- the infection by specific viruses such as retroviruses leukemogenic acute, chronic leukemogenic retroviruses (including HTLV and HIV), and Epstein-Barr virus.

Ongoing studies are also evaluating the possible correlation between acute leukemia and prolonged exposure to electromagnetic fields.

The course of the disease can be very variable depending on the type of leukemia that affects the patient, of molecular and cytogenetic abnormalities that characterize it, and therapeutic approaches must always be more specific for subtype identified. To increase the probability of success is desirable to move towards the study and planning of treatment programs customized.

### 1.4.1 ACUTE MYELOID LEUKEMIA

Acute myeloid leukaemia (AML) is defined as a clonal disorder caused by malignant transformation of a bone marrow-derived, self-renewing stem or progenitor cell, which demonstrates an enhanced proliferation as well as aberrant differentiation resulting in

haematopoietic insufficiency (i.e. granulocytopenia, thrombocytopenia or anaemia). The clinical signs and symptoms of AML are diverse and nonspecific, but they are usually directly caused by the leukaemic infiltration of the bone marrow, with resultant cytopenia. AML is considered to be a heterogeneous group of disorders with variable underlying abnormalities and clinical behaviour, including responses to treatment. Therefore, classification of the disease is important and several classification systems exist to subdivide AML [19]. Historically, AMLs were divided into subtypes based on the type of cell from which the leukaemia developed and the level of maturation, French-American-British (FAB) classification (Table 1) [20]:

| **FAB subtype** | **Name** | **Adult AML patients (%)** |
|---|---|---|
| M0 | Undifferentiated acute myeloblastic leukemia | 5% |
| M1 | Acute myeloblastic leukemia with minimal maturation | 15% |
| M2 | Acute myeloblastic leukemia with maturation | 25% |
| M3 | Acute promyelocytic leukemia | 10% |
| M4 | Acute myelomonocytic leukemia | 20% |
| M4eos | Acute myelomonocytic leukemia with eosinophilia | 5% |
| M5 | Acute monocytic leukemia | 10% |
| M6 | Acute erythroid leukemia | 5% |
| M7 | Acute megakaryocytic leukemia | 5% |

Table 1: French-American-British (FAB) classification.

Nowadays, the World Health Organization (WHO) provides a classification system in which morphology, cytogenetics, molecular genetics, and immunological markers are incorporated and interrelated (Table 2) [21]:

| |
|---|
| AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1* |
| AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBEB-MYH11* |
| Acute promyelocytic leukemia (APL) with t(15;17)(q22;q12); *PML-RARA* |
| AML with t(9;11)(p22;q23); *MLLT3-MLL* |
| AML with t(6;9)(p23;q34); *DEK-NUP214* |
| AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1* |
| AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1* |
| Provisional entity: AML with mutated NPM1 |
| Provisional entity: AML with mutated CEBPA |

Table 2: World Health Organization (WHO).

Recently, for the first time, specific gene mutations (i.e. mutations in CEBPA and NPM1) have been included as 'provisional entities' in the revised WHO 2008 classification for AML [22]. There is growing evidence that these two gene mutations represent primary genetic lesions (so-called class II mutations) that impair haematopoietic differentiation [23]. Mutations in the fms-related tyrosine kinase 3 (FLT3) gene (e.g. FLT3-ITD or FLT3 kinase domain mutations) are considered class I mutations conferring a proliferation and/or survival advantage. AML with FLT3 mutations is not considered a distinct entity, although determining the presence of such mutations is recommended because they have prognostic significance [24].

A number of clinical and biological features that reflect the heterogeneity of AML are used to predict the likelihood that a patient will have a response to treatment or relapse. Adverse prognostic factors in AML include increasing age, a poor performance before treatment, unfavourable cytogenetic abnormalities and a high white blood cell count.

Furthermore, therapy-related AML or AML arising after a myelodysplastic or myeloproliferative syndrome is usually more resistant to standard treatment than de novo AML. Important predictors of disease outcome are the pre-treatment cytogenetic and molecular findings in AML blasts. To date, in AML approximately 200 different structural and numerical aberrations have been described [24-25]. Cytogenetic findings permit patient risk to be categorised as favourable, intermediate or unfavourable, with very different cure rates. Although there may be (subtle) differences in the criteria used to define these risk groups among different study groups, the presence of for instance t(8;21)(q22;q22), t(15;17)(q22;q21) and inv16(p13q22)/t(16;16)(p13;q22) is generally classified as favourable-risk AML (with leucocytes <20 x 109). On the other end of the spectrum is the unfavourable-risk group, which includes blasts showing e.g. monosomies of chromosome 5 or 7, deletion of the long arm of chromosomes 3, 5 and 7 and complex karyotypes. Of note, the monosomal karyotype, defined as non-core-binding factor (CBF) leukaemias with a karyotype with at least two autosomal monosomies or one single autosomal monosomy in the presence of one or more structural cytogenetic abnormalities, is considered to be a better predictor of (very) poor outcome than the traditionally defined complex karyotype [26]. The intermediate-risk group includes AMLs with a normal karyotype and AMLs which are not classified in the other two risk groups. In recent years, the discovery of mutations in e.g. genesencoding *FLT3*, *NPM1* and *CEBPA* has shown to be of major importance. Nowadays, it is increasingly possible to distinguish subsets of patients with differing outcomes from the large cohort with a normal karyotype AML or miscellaneous cytogenetic abnormalities considered as

intermediate-risk cytogenetics. The majority of *FLT3* receptor tyrosine kinase gene mutations are internal tandem duplications (ITD); less frequent are mutations involving the tyrosine kinase domain (TKD). Several groups have consistently reported that *FLT3*-ITD is a major independent adverse risk factor in AML.27-31 The prognostic relevance of *FLT3*-TKD mutations, however, remains controversial [24]. *FLT3*-ITD has a prevalence of 20 to 25% in young adults and nearly 35% in the older adult population. The ratio of the *FLT3*-ITD and the wild-type *FLT3* (measured by polymerase chain reaction, PCR) varies from patient to patient, and this difference may have clinical implications. Thiede *et al.* found that patients with an allelic ratio (AR) above the median (0.78) had significantly shorter overall and disease-free survival, whereas survival in patients with ratios below 0.78 did not differ from those without *FLT3* aberrations [27]. *CEBPA*, a transcription factor involved in normal myelopoiesis, is mutated in ~10% of AML cases and predicts a relatively favourable outcome in paediatric and adult AML, however, only when *CEBPA* is mutated on both alleles [28-29].

Approximately 50% of adult normal karyotype AMLs harbour an *NPM1* mutation, which leads to delocalisation of the NPM1 protein to the cytoplasm.38 *NPM1* and *FLT3*-ITD commonly co-exist in normal karyotype AML suggesting that they may cooperate in generating the leukaemic phenotype. The presence of an *NPM1* mutation (in the absence of an *FLT3*-ITD mutation) is associated with better outcome in terms of higher complete response rates and increased long-term survival compared with patients lacking the mutation [30-33]. Consequently, it has been suggested that cytogenetically normal AML involving the genotype of mutant *NPM1* without *FLT3*-ITD should no longer be classified as intermediate-risk leukaemia but rather should be classified as favourable-risk leukaemia [32]. Furthermore, patients with mutant *NPM1* without *FLT3*-ITD may not benefit from related-donor transplantation as first-line treatment [32]. Mutations in the Wilms' tumour gene (*WT1*), present in ~10% of patients with normal karyotype AML, have been found to be associated with poor outcome, especially in combination with an *FLT3*-ITD [34-37]. RAS mutations, occurring in ~15% of cases, are suggested to be prognostically neutral [30].

Recently, mutations in genes involved in metabolism have been discovered [38-39]. In AML, but also in low-grade gliomas and secondary glioblastoma multiforme (GBM), mutations in cytosolic isocitrate dehydrogenase 1 (*IDH1*) and its mitochondrial homolog *IDH2* have been identified. Both IDH1 and IDH2 are important enzymes in the citrate cycle (Krebs cycle). Two distinct alterations are caused by the tumour-derived mutations in *IDH1* or *IDH2*: loss of its normal catalytic activity in the production of α-ketoglutarate (α-KG) and gain of the

catalytic activity to produce 2-hydroxygulatrate (2-HG). Consequently, less α-ketoglutarate is available for biological processes in which it functions as a co-factor. Remarkably, *IDH1*/*2* mutations, occurring in ~10 to 25% of AML cases, were mutually exclusive with mutations in gene encoding the a-ketoglutarate-dependent enzyme tet oncogene family member 2 (*TET2*) (occurring in 12 to 20% of AML cases) [40-42]. Loss-of-function mutations in *TET2* were associated with similar epigenetic defects as *IDH1*/*2* mutants. Interestingly, a shared proleukaemogenic effect between *TET2* mutations and mutations in *IDH1* and *IDH2* was suggested since α-ketoglutarate is a co-factor for *TET2* in the hydroxylation of 5-methylcytosine and thus effects the methylation process [43].

In cytogenetically favourable core binding factor (CBF AML (i.e. AML with t(8;21) or inv(16)/t(16;16)), the presence of a mutation in the *KIT* receptor tyrosine kinase has been shown to have an unfavourable influence on outcome in retrospective studies. Recently, highly recurrent mutations in the DNA methyltransferase gene DNMT3A have been discovered and were found to be independently associated with poor outcome in AML [44]. Other mutations as those involving protein tyrosine phosphatase, non-receptor type 11 (*PTPN11*) and runt-related transcription factor 1 (*RUNX1*) are relatively rare (i.e. <5% of cases), making their relevance to risk-stratified treatment approaches uncertain at the present time [45]. The panel of known molecular markers is continuosly increasing, for example, considering the recently described EZH2, DNMT3A, ASXL1 and IDH1/2 mutations. Mutations such as ASXL1, RUNX1, EZH2, ETV6/TEL and TP53 have an adverse impact on patient overall survival. Early evidence suggests that some mutations might influence treatment response, necessitating reassessment of the prognostic effect of genetic alterations in the light of every new treatment. The introduction of next generation sequencing will certainly catalyze the molecular characterization of AML.

### 1.4.2 CRONIC MYELOID LEUKEMIA

Cronic Myeloid Leukemia (CML) arises as the result of a mutation in a pluripotent stem cell and is characterized by progressive granulocytosis, marrow hypercellularity, and splenomegaly [46-49]. The diagnostic hallmark is the Ph chromosome (Figure 6), [50] which is present in all dividing cells of hematopoietic lineage, as well as in B and T cells in some patients, but is absent in all other cells. Although hematopoiesis is overwhelmed by the Ph-chromosome–positive clone, a normal Ph-chromosome–negative pool of stem cells persists [47, 51]. The goal of treatment is the suppression or elimination of the Ph-chromosome–

positive clone and the restoration of Ph-chromosome–negative hematopoiesis. The diagnosis of the disease is based on the blood count to assess the level of leukocytosis, usually recorded around 20-30 billion cells / L for the massive mobilization of tumor cells from the bone related to the alteration of adhesion. The number of platelets can instead be normal, increased or reduced. The morphological analysis of peripheral blood shows that the cell types responsible for the leukocytosis are mainly neutrophils and immature granulocyte series, from myeloblast to metamyelocyte. Finally, the bone marrow aspiration is essential to confirm the diagnosis: there was a marked hyperplasia of the granulocytic and megakaryocytic series at the expense of the erythroid compartment and the fatty component.
CML has a biphasic or triphasic course but is usually diagnosed during the initial, or chronic, phase, in which the granulocytic population expands but remains able to differentiate. The chronic phase in which there are clinical symptoms that allow the diagnosis may occur from 2 to 10 years after the beginning of the biological disease. At this stage, the uncontrolled proliferation of the granulocytic line ultimately leads to suppression of hematopoietic stem cell pool of healthy (Ph-). The chronic phase is relatively stable and responds to therapy, but it eventually evolves into an intermediate, accelerated phase characterized by blastic elements in the peripheral blood, anemia and thrombocytopenia, related to the accumulation of mutations in stem cells neoplastic with progressive loss of the cell maturation. The accelerated phase in which increasing doses of hydroxyurea are needed to control disease, followed by a blast phase. Blast-phase disease resembles acute leukemia. Its phenotype is myeloblastic in 70 to 80% of patients and lymphoblastic in 20 to 30%. The clinical features becomes very similar to that of a LAM and symptoms such as fever, muscle and bone pain, fatigue and weight loss. Typically this stage is of short duration (< 6 months) and leads very quickly to the patient's death due to resistance to conventional treatments gained in the inactivation of tumor suppressor genes such as p53. With conventional treatment the median survival among patients with CML is about five years, but the range is very broad. Blast-phase cells may be more dependent on secondary oncogenic aberrations than on the tyrosine kinase activity of BCR-ABL. As the leukemic clone becomes unable to differentiate, blast cells accumulate, leading inexorably to a blast crisis. Some patients with an aggressive form of chronic-phase disease survive only months, whereas others, who have indolent, chemoresponsive CML, live 10 years or longer. The Ph chromosome is a truncated chromosome 22 that results from a reciprocal exchange of genetic material between the long arms of chromosomes 9 and 22 (Fig. 6). The translocation t(9;22) results in the juxtaposition of 3' DNA sequences derived from the Abelson (ABL) proto-oncogene normally located on

chromosome 9 with 5' sequences of the breakpoint cluster region (BCR) gene on chromosome 22 [52-54]. The ABL proto-oncogene is homologous with the transforming gene present in the Abelson leukemia virus, which causes leukemia in mice [46, 55] ABL encodes a tyrosine kinase that is tightly regulated, whereas the activity of BCR-ABL is autonomous and markedly increased relative to that of normal ABL. The Ph chromosome is present in approximately 95% of patients with classic CML. About half of the remaining 5% of patients have been found to have the BCR-ABL gene when the polymerase chain reaction (PCR) is used for identification and are classified as being Ph-chromosome–negative, BCR-ABL–positive. Although the precise oncogenic mechanism of BCR-ABL is unknown [46, 56], its tyrosine kinase activity leads to the chronic phase of CML [57]. Transplantation of hematopoietic stem cells containing a BCR-ABL gene construct into mice results in a disease resembling CML [58-59]. The Ph chromosome is also detected in about 25% of adults and 5% of children with acute lymphoblastic leukemia (ALL) and is associated with an aggressive course and poor survival [60-62]. Only about one third of patients with Ph-chromosome–positive ALL have the 210-kD BCR-ABL protein characteristic of CML; approximately two thirds have a smaller chimeric BCR-ABL protein of 185 to 190 kD that has more potent tyrosine kinase and oncogenic activity [47, 57, 63] continuously (or constitutively) active BCR-ABL oncoprotein phosphorylates substrates of remarkable diversity, including RAS, that activate multiple signaling pathways [46, 55, 64]. Because RAS serves as a critical control point for signal transduction from cell membrane to nucleus [64-65], the BCR-ABL–mediated overexpression of RAS appears to alter signal transduction in a target stem cell, leading to abnormal mitosis and neoplastic expansion. In addition, BCR-ABL reduces cellular adhesion to stromal matrix [66-69], which may disrupt the interaction between hematopoietic cells and stromal cells and membrane signaling mediated by cytoadhesion molecules, allowing myeloid progenitor cells to remain longer in the proliferative phase before undergoing differentiation [70]. BCR-ABL also diminishes cellular responsiveness to apoptotic stimuli, providing a survival advantage to the leukemic clone [71-74]. In theory, since chronic-phase CML is dependent on the tyrosine kinase activity of BCR-ABL, a potent BCR-ABL inhibitor might eliminate the leukemic clone and restore normal Ph-chromosome–negative hematopoiesis. Although the mechanism for blastic transformation is unknown, possible scenarios have been considered. For example, BCR-ABL promotes genomic instability in the leukemic clone [75], which may lead to secondary mutations (e.g., trisomy 8).

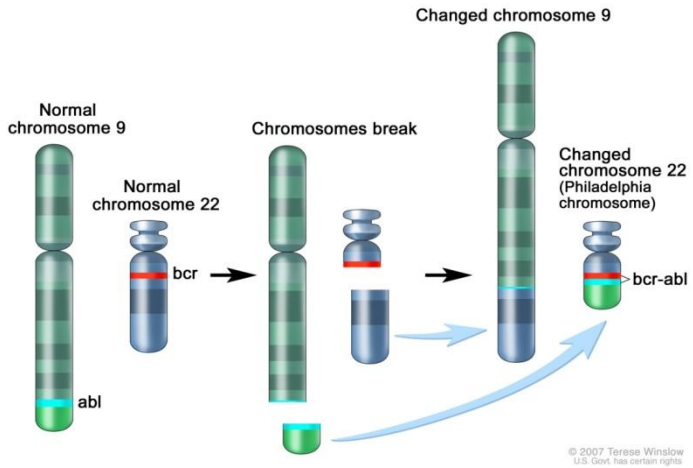


Fig. 6: Translocation leading to the Philadelphia (Ph) Chromosome and the role of BCR-ABL in the pathogenesis of CML.

During the chronic phase it is possible to obtain hematologic remission of 95% of cases thanks to the use of drugs recently discovered that inhibit the activity of tyrosine kinases involved in oncogenesis, such as c-Abl, c-kit and PDGFR. Imatinib mesylate, the Nilotinib, Dasatinib and the Ponatinib are an example. These inhibitors are able to bind the inactive forms of the tyrosine kinase targets (eg. BCR-ABL), preventing binding of ATP and the conversion to the active form. This shuts down the signaling pathways iperattivated and the loss of the leukemic phenotype: although cytogenetic remission is only achieved in 75% of cases, at the molecular level the action of BCR-ABL is however neutralized. An alternative to treatment with current chemotherapy remains the allogeneic HSC after chemotherapy conditioning. In 40% of cases, the transplant can result in complete cure, which is due to the phenomenon of graft-vs-leukemia in which the immune system of healthy donor is directed against leukemia cells are not eliminated by chemotherapy. The low percentage of success is tied to complications during or post-transplant, as for example the graft-vs-host disease in which there is the rejection of donor cells.

### 1.4.3 ACUTE LYMPHOBLASTIC LEUKEMIA

ALL represents a biologically and clinically heterogeneous group of B/T-precursor-stage lymphoid cell malignancies arising from genetic insults that block lymphoid differentiation and drive aberrant cell proliferation and survival. Incidence and cure rates differ among children and adults. In children, ALL is the commonest malignancy accounting for approximately 25 % of childhood cancer and it has 5-year event-free survival rates ranging between 76 % and 86 % in patients receiving protocol-based therapy. In adults, ALL

is less common and generally carries a worse prognosis with a long-term survival probability less than 35–40 % [76-77]. Although there is remarkable progress made in the treatment of ALL in children and, with less efficacy, in adults, several ALL subtypes continue to have a poor prognosis and in a proportion of longterm surviving patients, treatment is responsible for short and long-term toxicities. Consequently, there is a need in improving the molecular dissection of subtypes, identifying genetic alterations that predict the risk of treatment failure, and developing novel and targeted therapies. Cytogenetics has long been used for diagnosis, risk stratification, and therapeutic implications, however experimental models [78] [79] have established that primary cytogenetic abnormalities alone are insufficient to induce leukemia and that cooperative mutations are required.

The symptoms and signs of the disease depend on the following pathophysiologic mechanisms:

a) reduction of normal hematopoiesis, with consequent anemia, granulocytopenia, and thrombocytopenia;

b) expansion of leukemic cells in bone marrow (normally the blast infiltration in the bone marrow is subtotal or total). Only 4 % of cases have an infiltration < 40% in the peripheral blood and, secondarily, in other extramedullary sites;

c) release of cytokines and inflammatory mediators, by both the leukemia cells that of normal cells (symptoms relatively more frequent in children).

The early simpto anemia, fatigue, pallor, fever, infection, and bleeding (bruising, petechiae, epistaxis, mucosal bleeding). Lymphadenomegaly and splenoepatomegalia occur in approximately half of the cases; mediastinal lymph nodes and abdominal have a volume increased in most of the ALL-T and can cause cardiorespiratory problems. The central nervous system is commonly involved in diagnosis, but his interest can cause neurological disorders, such as headache or cranial nerve palsies, complicating the course of the disease. Poliartromialgie are frequent, often very intense and resistant to anti-inflammatory therapy. The involvement of other organs in a clinically relevant manner occurs in less than 5% of cases and affects the kidneys, skin, eye and retina, lungs, pleura, heart, pericardium, testicles, ovaries, lymph nodes, abdominal or retroperitoneal [76, 78-79].

The development of microarray technologies to profile gene expression and structural genetic alterations in a genome-wide and high-resolution fashion have revolutionized our ability to identify genetic abnormalities providing important insights into the pathways deregulated in ALL [80]. Moreover, recently the development of next-generation sequencing (NGS) technologies has provided researchers with completely new and effective tools for the

discovery of novel alterations, depicting an exhaustive picture of the leukemia genome complexity.

Although the eziogenesis of the disease is unknown, molecular genetic studies have allowed us to identify different genomic alterations at the base of leukemic transformation in more than 60% of ALL. Most of these alterations leads to activation of proto-oncogenes, or through the deregulation of the same or, more frequently, through the formation of hybrid genes of fusion with aberrant activity. In the LAL with the alteration of the adult phenotype B more frequent (25-30% of cases) is the t (9; 22) that leads to the formation of the fusion gene BCR / ABL on the Philadelphia chromosome. As in CML, constitutive activation of Abl promotes cell proliferation, inhibits apoptosis and alters cell adhesion to bone marrow microenvironment. Unlike CML, however, in the ALL is not formed a fusion product of 210 kDa but a shorter protein, p190, whose transforming power is higher. Furthermore, according to the morphology of blast cells in the circulation at the time of diagnosis, it is possible to distinguish three classes of ALL, according to the FAB classification:

L1: ALL small blasts homogeneous prevalent in children;

L2: ALL blasts heterogeneous in size and cell characteristics, prevalent in the adult;

L3: ALL large blasts with basophilic cytoplasm and ipervacuolato.

At the morphological diagnosis is always well associate a diagnosis and immunophenotypic analysis cytogenetic-molecular, so as to be able to program a targeted therapy, and thus more effective, particularly against the subtype of ALL.

The standard treatment of ALL involves three steps:

- Induction of remission by chemotherapy with agents such as corticosteroids, anthracyclines, vincristine, and asparaginase;
- Prophylaxis of leukemia localization in the CNS by intrathecal administration of anticancer drugs or radiation farmci on the skull;
- Intensification and maintenance of remission with chemotherapy.

In more severe cases may need to be autologous or allogeneic bone marrow. Specific therapies are available for the subtypes of ALL with a known genetic alteration at the base. The most representative example is that of Philadelphia positive ALL, so we adopt a targeted therapy against BCR-ABL with tyrosine kinase inhibitor drugs, such as Imatinib mesylate. The advent of high-resolution genome-wide analyses of gene expression, DNA copy number alterations (CNA), and loss of heterozygosity have led to the detection of many novel genetic abnormalities refining the prognostic models for ALL-B (Table 3) and ALL-T (Table 4) [81].

| Gene name | Genetic alteration | Frequency | Prognosis |
|---|---|---|---|
| IKZF1 | Deletions or sequence mutations | ~80 % of BCR-ABL1 positive ALL; 15 % of pediatric B-ALL cases | Associated with poor outcome |
| PAX5 | Deletions, sequence mutations or translocations | ~30 % of pediatric and adult B-ALL | No association with outcome |
| CDKN2A/B | Deletions | ~30 % of pediatric and adult B-ALL; 47 % of relapsed BCR-ABL1-ALL | Associated with poor outcome in adult BCR-ABL1 positive ALL; controversial prognosis in other B-ALL subtypes |
| CRLF2 | Overexpression due to IGH@-CRLF2 or P2RY8-CRLF2 rearrangement or F232C mutation | Up to 16 % of pediatric and adult B-ALL; >50 % Down syndrome ALL | Associated with poor outcome |
| JAK1/2 | Sequence mutations | ~10 % of high-risk BCRABL1-like ALL and 18–35 % of Down syndrome ALL | Associated with poor outcome |
| CREBBP | Deletions and sequence mutation | 19 % of relapsed B-ALL | Associated with glucocorticoid resistance |
| iAMP21 | Intrachromosomal amplification | Up to 2 % in older children with B- ALL | Associated with poor outcome when patients are treated with standard therapy |
| TP53 | Deletions and sequence mutations | 12.4 % of B-ALL | Associated with non-response to chemotherapy and poor event-free survival and overall survival rates |

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Table 3: Novel recurring genetic alterations occurring in B-progenitor ALL and their correlation with outcome.

| Gene name | Genetic alteration | Frequency | Prognosis |
|---|---|---|---|
| NOTCH1 | Sequence mutations | ~50 % of T-ALL | Associated with favorable outcome in children |
| FBXW7 | Sequence mutations | ~20 % of T-ALL | Associated with favorable outcome in children |
| PTEN | Deletions or sequence mutations | 6–8 % of T-ALL | Associated with poor response to chemotherapy and resistance to pharmacological inhibition of NOTCH1 |
| CDKN2A/B | Deletions | 30–70 % of T-ALL | Associated with poor outcome in adult and children T-ALL |
| CDKN1A | Deletions or sequence mutations | 12 % of T-ALL | To be investigated |
| 6q15-16.1 | Deletion | 12 % of T-ALL | Associated with poor outcome |
| PHF6 | Deletions or sequence mutations | 16 % of pediatric T-ALL cases; 38 % of adult T-ALL cases | Associated with reduced overall survival |
| WT1 | Frameshift mutations | 13 % of pediatric T-ALL cases; 12 % of adult T-ALL cases | No association with outcome |
| LEF1 | Focal deletions or sequence mutations | 15 % of pediatric T-ALL cases | Associated with younger age and a trend toward a better overall survival |
| JAK1 | Sequence mutations | 18 % of adult T-ALL cases | Associated with reduced disease-free survival and overall survival |
| FLT3 | Internal tandem duplication | 4 % of adult T-ALL cases; 3 % of pediatric T-ALL cases | No association with outcome |
| PTPN2 | Deletion | 6 % of T-ALL | Down-regulation of PTPN2 expression results in prolonged survival of ALL-SIL cells after Imatinib treatment |

Table 4: Novel recurring genetic alterations occurring in T-progenitor ALL and their correlation with outcome.

## 1.5 BCR-ABL TYROSINE KINASE INHIBITORS

The fusion protein BCR-ABL is necessary and sufficient to induce the phenotype transformant of leukemic cells. If the tyrosine kinase activity of BCR-ABL is turned off, the cell pH is suppressed. Therefore, the leukemic cells Ph+ constitute an ideal target for therapeutic targeting of inhibitors of tyrosine kinase. The treatment of CML and ALL Ph+ patients was revolutionized by the introduction of Imatinib mesylate (IM, Gleevec®), a BCR-ABL tyrosine kinase inhibitor (TKI). The clinical use of specific BCR-ABL inhibitors has resulted in a significantly improved prognosis, response rate, overall survival, and patient outcome in CML patients compared to previous therapeutic regimens. However, the complete eradication of CML in patients receiving Imatinib was limited by the emergence of resistance mostly due to mutations in the ABL kinase domain and to a lesser extent by molecular residual disease after treatment. The second-generation BCR-ABL TKIs Nilotinib (Tasigna®) and Dasatinib (Sprycel®), showed significant activity in clinical trials in patients intolerant or resistant to Imatinib therapy, except in those patients with the T315I BCR-ABL mutation. Bosutinib is a third generation tyrosine kinase inhibitor. It is being tested in clinical trials and looks very promising. It has been useful in patients whose leukemia is resistant to both first and second generation tyrosine kinase inhibitors.

### 1.5.1 IMATINIB MESYLATE

In 1996 was developed by the collaboration of Druker and Novartis Pharmaceuticals (Ciba-Geigy, Basel, Switzerland), an investigational drug, now known as Imatinib mesylate (CGP57148B; STI571, Gleevec®) (Fig. 5A), which is a derivative of 2-phenyl-amino-pyrimidine and acts specifically by inhibiting the tyrosine Abl kinase. In 2001 Imatinib receives FDA approval (Food and Drug Administration) and EMEA (European Medicines Evaluation Agency) as second-line treatment of CML, but in the following year Imatinib is approved as a drug of first-choice treatment for CML [82].

Initially it was thought that the specific inhibitory action of Imatinib was based on the ability to compete with ATP in the catalytic domain of the kinase, inhibiting, thus, the cascade of signal transduction. Thereafter, the crystallographic studies of the kinase domain of Abl complexed with a homologue of Imatinib, showed that the inhibitor binds to an inactive conformation of Abl that is, thus, unable to bind ATP and activate the signals that cause leukemogenesis. Imatinib has inhibitory activity against other members of the family of

tyrosine kinases, including PDGFRα / β and c-kit, involved in other syndromes (eg, hypereosinophilic syndrome or systemic mastocitsi) [83].

The domain of Abl tyrosine kinase is characterized by highly conserved residues that form the binding site, the catalytic loop and the activation loop. The orientation and the state of phosphorylation of residues of the DFG motif (Asp-Phe-Gly) present in the activation loop, determine the transition from the inactive to the active conformation of the kinase. In the inactive form of the activation loop preclude access of ATP to its binding site, and with the transition to the active form of the ATP binding is made possible. The activation loop in the inactive conformation orients the N-terminal of the residue Phe382, Asp381 instead, thereby preventing the formation of the salt bridge between this last residue and the complex Mg2 + - ATP: the Tyr412 interacting through a hydrogen bond with the residue of Asp381, it mimics the substrate binding by blocking the activity of the protein.

The oncoprotein in leukemia cells is in dynamic equilibrium between its active and inactive form. This balance depends on the recruitment of phosphotyrosine ligands for disassembly constraints inhibitors, and phosphatase that can transiently dephosphorylate the protein, allowing the binding of Imatinib. Imatinib seems then bind monomeric and inactive conformation of BCR-ABL [83-84] (Fig. 5B). Imatinib clearly differs from traditional therapies as regards the toxic effects: the most common effect is a moderate nausea; edema, myalgia, arthralgia, diarrhea and skin rash are found in 10% of patients; rarely have periorbital edema and more in general syndrome retention of fluids. Myelosuppression is more common in blast phase than in cronic phase [85] (Figure 7).

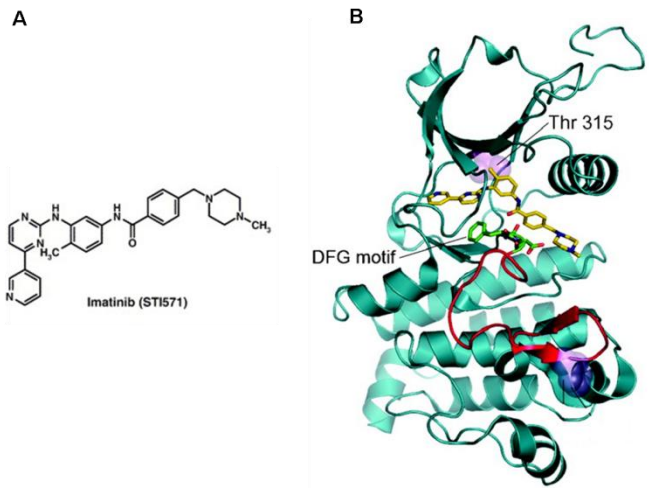



Fig. 7: A: structure of the Imatinib molecule; B. structure of the kinase domain of Abl complexed with Imatinib (in yellow) in which are highlighted the DGF motif of the activation loop and the residue Thr315 necessary for the formation of a hydrogen bond with Imatinib.

### 1.5.2 NILOTINIB

Nilotinib is an oral ATP-competitive inhibitor of BCR-ABL in clinical development with a modified aminopyrimidine backbone comparable to Imatinib. Similar to Imatinib, Nilotinib can bind only the inactive conformation of the ABL kinase domain but with a 25-fold greater affinity than Imatinib [86] (Figure 8).

In 2007 Nilotinib has been approved by the FDA as an second generation tyrosine kinase Inhibitor for the treatment of patients resistant or intolerant to Imatinib [87-88]. Nilotinib has demonstrated activity in Imatinib-resistant mutations, with the exception of T315I [89-90]. Nilotinib also inhibits the activity of Arg, Kit, and platelet-derived growth factor receptor (PDGFR), but not Src-family kinases (SFK) [90]. Nilotinib is 10 to 50 times more potent than Imatinib in inhibiting the proliferation and autophosphorylation of wild-type BCR-ABL cell lines and most of the BCR-ABL mutants, except the T315I mutant [90]. It is superior to Imatinib in reducing leukemic burden and prolonging the survival of mice transplanted with wild-type BCR-ABL, the M351T and E255V mutants [90].

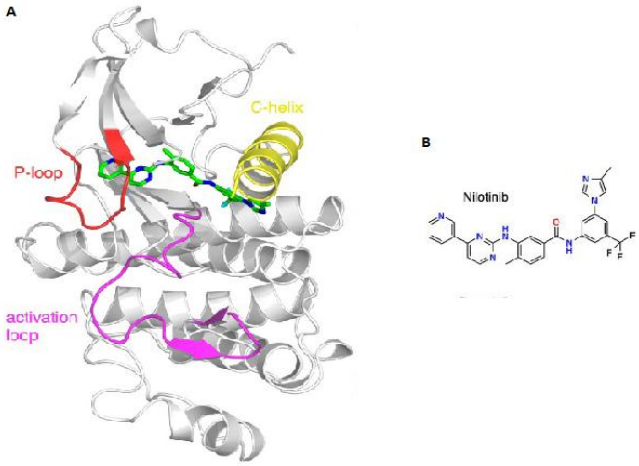



Fig. 8: A. Ribbon diagram showing the Abl kinase (gray) with linked Nilotinib (green), showing the P-loop (red), the activation loop (magenta) and the C helix (yellow). B. Chemistry of Nilotinib.

However, Nilotinib and Imatinib produced equivalent reduction in CrkL phosphorylation in primary CD34+ CML cells, suggesting that they were equipotent for inhibiting BCR-ABL activity [91]. Furthermore, Nilotinib did not induce apoptosis in the primitive quiescent population. Nilotinib is from 10 to 50 times more potent in inhibiting the proliferation of Imatinib and the autophosphorylation of the cell lines BCR-ABL wild-type and the majority of those mutanti [92]. And superior to Imatinib in leukemic reduce the charge and prolong the survival of mice transfected with BCR-ABL wild-type, the mutant M351T and E255V.

However, Nilotinib and Imatinib induced a dephosphorylation equivalent Crkl lines in primary CD34 + chronic myeloid leukemia, suggesting that the two drugs are equipotent in inhibiting the activity of BCR-ABL [93]. Nilotinib is well tolerated, and common ad verse events included grade 3-4 myelosuppression, elevated bilirubin and lipase levels.

### 1.5.3 DASATINIB

Dasatinib (BMS-354825, (Sprycel®); Bristol Myers Squibb) is a multi-target kinase inhibitor of BCR-ABL, SFK, ephrin receptor kinases, PDGFR and Kit. In addition, Dasatinib binds to other tyrosine and serine/threonine kinases, such as the TEC family kinases, the mitogen-activated protein kinases and the receptor tyrosine kinase, discoidin domain receptor 1[94]. Dasatinib is more potent than Imatinib and is effective against the Imatinib-resistant active conformation of the kinase domain (Figure 9).

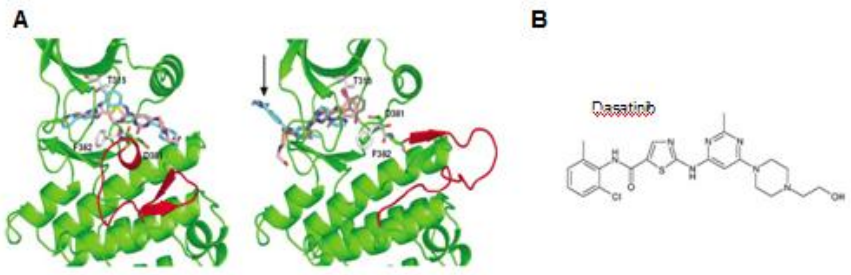



Fig. 9: A Inactive conformation of the protein on the left and on the right active conformation, with the drug that in both cases we go to place within the pocket. B. Chemistry of Dasatinib.

It is capable of inhibiting the proliferation and kinase activity of wild-type and most BCR-ABL mutant cell lines except the T315I mutant. In vivo studies in murine models demonstrated the activity of Dasatinib in inhibiting the leukemic cell growth and prolonging the survival of mice harbouring wild-type BCR-ABL and the M351T, but not the T315I mutant.15 Dasatinib is well tolerated but grade 3-4 myelosuppression is common, especially in the advanced phases. Non-hematological side effects include diarrhea, nausea, headache, peripheral edema and pleural effusion. However, resistance to Dasatinib is also an emerging problem. Not surprisingly, the pre-existence or selection of the T315I mutant is the most frequent mechanism of resistance.20 The emergence of the F317L mutant has also been commonly observed in Dasatinib-resistant patients [95]. In addition, although Dasatinib significantly inhibited CrkL phosphorylation and caused a reduction in the total number of

CD34+ CD38– CML cells compared to Imatinib, it did not eliminate the most primitive, quiescent fraction [96].

### 1.5.4 BOSUTINIB

Bosutinib (SKI-606; Wyeth) has potent antiproliferative activity against Imatinib-sensitive and -resistant BCR-ABL– positive cell lines, including the Y253F, E255K and D276G mutants, but not the T315I mutant [97-98] (Figure 10).

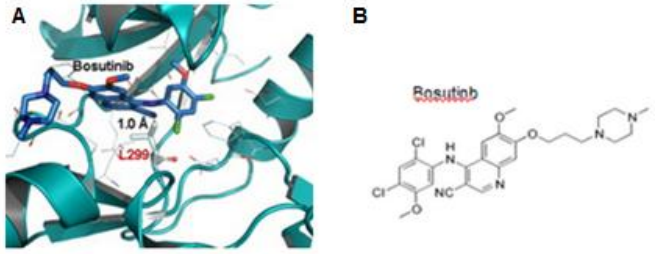



Fig. 10: A Bosutinib-Abl complex. B. Chemistry of Bosutinib.

Bosutinib is also classified as a histone deacetylase (HDAC) inhibitor. It is able to bind to both inactive and intermediate conformations of BCR-ABL [97]. Bosutinib inhibited the proliferation of CML progenitors but was moderately effective in inducing apoptosis and was not able to eliminate the primitive, quiescent population [99]. Bosutinib was also effective in patients previously treated with Dasatinib or Nilotinib. Unlike Dasatinib, Bosutinib does not significantly inhibit Kit or PDGFR and has a more favorable toxicity profile [97]. Adverse events are commonly gastrointestinal in nature and grade 3-4 myelosuppression usually occurs only in the advanced phases.

## 1.6 CYTOSINE β-D-ARABINOFURANOSIDE (ARA-C)

Acute myelogenous leukemia describes a group of related hematologic malignancies that are being approached therapeutically from several perspectives. Conventional chemotherapeutic agents, such as anthracyclines and cytosine β-D-arabinofuranoside (Ara-C), are useful in treating AML. Ara-C (Figure 11) is a selective inhibitor of DNA synthesis that

does not affect RNA synthesis in mammalian cells. This deoxycytidine analog is incorporated into the C sites of the DNA strand in primer assays and causes a cessation of DNA strand elongation at the incorporation site. It is used as an antineoplastic and antiviral agent. The pharmacologic basis for Ara-C cytotoxicity rests on its intracellular conversion to the active metabolite, Ara-C 5'-triphosphate (Ara-CTP), which acts as a competitive inhibitor of DNA polymerase'3 and is directly incorporated into DNA, with further deleterious effects on DNA structure and function. Ara-C is a cell cycle-dependent antimetabolite with its greatest toxicity directed against cells in S phase [100]. As such, the overall mechanism of action and net toxic effects should depend on the fraction of proliferating cells in the treated population at the time of drug exposure. In this light, growth kinetic heterogeneity, a characteristic of pretreatment leukemic cell populations, may in part account for the apparent failure of measurements of intracellular Ara-C biochemical pharmacology alone to accurately predict net drug effect and clinical response [101].

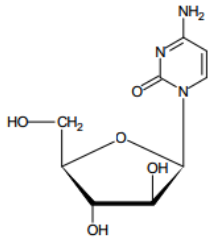



Fig. 11: A. Chemistry of Cytosine β-D-Arabinofuranoside.

## 1.7 CHEMORESISTANCE MECHANISMS IN LEUKEMIC CELLS

Intrinsic and acquired resistance against chemotherapy remains a major challenge in the management of cancer in general and of leukemia in particular. Several potential molecular or cellular mechanisms responsible for chemo-resitance have been elucidated.

### 1.7.1 MECHANISMS OF RESISTANCE TO IMATINIB MESYLATE

Resistance to Imatinib can be defined as the lack of complete hematological response in patients with chronic phase disease or as a lack of return to chronic phase for patients in acute phase, in blast crisis CML, or with Ph positive ALL. Depending on the time of onset,

two categories of resistance can be distinguished: If there is no response after initial treatment, resistance is described as primary or extrinsic. In contrast, secondary or intrinsic resistance is present if resistance develops after achieving an objective response [83].
In general the mechanisms that are involved in resistance are divided into:

BCR-ABL dependent mechanisms:

1. Point mutations in BCR–ABL decrease Imatinib sensitivity. Potentially the most frequent clinically relevant mechanisms that change Imatinib sensitivity in BCR–ABL transformed cells are mutations within the ABL kinase, affecting several of its properties. Point mutations can directly influence the proper binding of Imatinib to the target molecule, as well as the binding of ATP. Furthermore, mutations can lead to conformational changes of the protein, affecting binding of either Imatinib or ATP in an indirect way. Imatinib-resistant mutations are likely to be induced by Imatinib itself, due to selection of BCR–ABL expressing clones that harbor the point mutation. In these particular cells, Imatinib is unable to efficiently bind and thus permits a growth advantage due to lack of ABL kinase inhibition. This is consistent with the finding that resistance-mediating mutations can be found at very low levels in patients prior to clinical Imatinib resistance.
2. BCR–ABL gene amplification. The over-expression of BCR-ABL protein due to gene amplification, can cause resistance to Imatinib.
3. Overexpression of the P-glycoprotein, encoded by the gene MDR-1 (multidrug resistance) is often implicated in resistance to many chemotherapeutic drugs. Its physiological function is to bind soluble components in the cytoplasm, and transport them to the outside of the cell: this mechanism would alter the uptake of Imatinib. The MDR-1 gene is commonly over-expressed in blast cells.
4. Overexpression of A1-acid glycoprotein (AGP), a plasma protein, which would be able to bind the Imatinib reducing its intracellular concentration. In studies in mice treated with erythromycin (which binds AGP) have shown this hypothesis, since these animals were able to lose the resistance. However, the significance of this mechanism and the correlation with the resistance are still the subject of study.

BCR-ABL indipendent mechanisms:

The expression of BCR-ABL is associated with genomic instability. Indeed, it is well documented the association of the t (9; 22) with additional mutations. The accumulation of these secondary mutations may be sufficient to ensure the neoplastic transformation, in a manner independent of BCR-ABL itself. These alterations include aneuploidy, reciprocal translocations, aberrations of chromosome 17 (on which is located the gene coding for the tumor suppressor p53). Finally in hematopoietic cells transformed BCR-ABL, it is observed an increase in the concentration of reactive oxygen species: this mechanism may induce, in turn, a transformed phenotype [83].

### 1.7.2 MECHANISMS OF RESISTANCE TO NILOTINIB

Nilotinib has shown effect against most mutations that are associated with Imatinib resistance but the T315I mutant remains resistant to Nilotinib [102-103]. Its ineffectiveness against the T315I mutant seems to be a consequence of the loss of an H-bond interaction between threonine-O and aniline-NH on Nilotinib and a steric clash between the isoleucine-methyl group and 2-methylphenyl phenyl group of Nilotinib. On the other hand, resistance to Nilotinib is associated with a limited spectrum of BCR-ABL kinase mutations that mostly affect the P-loop and T315I. However all mutations except T315I were effectively suppressed by increasing Nilotinib concentration. Although Nilotinib is more potent than Imatinib it is possible that its specific mode of binding to Abl may make other sites vulnerable to new kinds of drug resistance.

### 1.7.3 MECHANISMS OF RESISTANCE TO DASATINIB

Since Dasatinib is an inhibitor of Src family kinases, it can overcome resistance due to Src family kinase activation. Because it does not bind to BCR-ABL with the same stringent conformational requirements as Imatinib, it can inhibit all BCR-ABL kinase domain mutants except for T315I. Dasatinib is also not a substrate of multidrug P-glycoprotein efflux pumps like Imatinib. Because of this Dasatinib may be active in some patients after failure with both Imatinib and Nilotinib [104]. Although Dasatinib is much more potent than Imatinib it is possible, like with Nilotinib, that its specific mode of binding to Abl may lead to new vulnerable sites that could confer new kinds of drug resistance. Mutations have been found on Phe317 so that is a potential vulnerable site for this drug.

### 1.7.4 MECHANISMS OF RESISTANCE TO BOSUTINIB

Bosutinib inhibited cells expressing a variety of mutations, some of which led to Imatinib resistance, but the T315 mutation was completely resistant to Bosutinib [102, 105]. In contrast to Imatinib, Nilotinib and Dasatinib, Bosutinib is not an efficient substrate for multidrug resistance (MDR) transporters that promotes efflux of foreign molecules from cells. Bosutinib even inhibits these transporter proteins in higher concentrations [105].

### 1.7.4 ADENOSINE TRIPHOSPHATE BINDING CASSETTE (ABC) TRASPORTERs

Adenosine triphosphate The family of (ATP) binding cassette, or ABC, transporters includes over 50 members [106]. These transmembrane transporters mediate the transfer of a diverse array of substrates across cellular membranes. The ABC transporter family is characterized by a high level of sequence homology between family members, and also a high level of conservation among species. Several ABC transporters have been associated with antineoplastic drug efflux from tumour cells. Thus, tremendous interest was generated as these transporters were recognized as a source of drug resistance in the treatment of malignancies.

The first such transporter was termed P-glycoprotein (P-gp), encoded by the gene MDR1 (ABC), and belonging to the ABCB subfamily (Figure 1a). P-gp upregulation has been associated with clinical resistance to antineoplastic agents (including anthracyclines, taxanes, epipodophyllotoxins, and vinca alkaloids) and worsened outcomes in a number of human malignancies. Subsequent studies revealed another ABC subfamily, designated the C subfamily, consisting of the multidrug resistance proteins (MRP1-7), as also producing a phenotype of multidrug resistance. In general, anthracyclines, epipodophyllotoxins, and vinca alkaloids are MRP substrates. MRP expression has been found at a fairly high frequency in human malignancies [107]. P-gp and several MRP subfamily members have been detected in a variety of hematopoietic cells, although not in the very primitive subpopulation that expresses ABCG2 [108]. ABCG2 differs from these other transporters because it functions as a homodimer composed of two identical subunits, also referred to as a half-transporter structure (Figure 1b) [109].

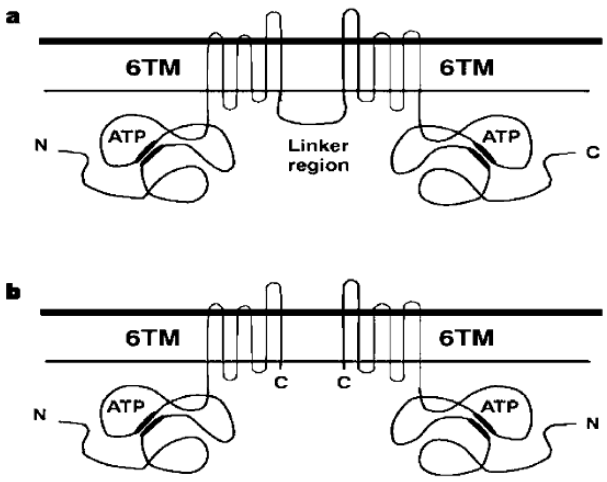



Fig. 12: A. Schematic representations of ABC transporters. (a) MDR1 is a full transporter, containing two ATPbinding domains and two transmembrane domains, connected by a linker region. (b) ABCG2 is a half-transporter, requiring homodimerization for functionality. Each molecule contains a single ATP-binding domain and transmembrane domain.

The ABCG2 gene is located on chromosomal locus 4q22 and has extensive homology with the Drosophila White gene. Unlike other ABC half-transporters which are localized on intracellular membranes, ABCG2 is expressed exclusively on the plasma membrane [110]. Consistent with other ABC half-transporters, the ABCG2 protein contains a single transmembrane domain (TMD) involved in drug binding and efflux and a single cytosolic nucleotide-binding domain (NBD) which is involved in ATP binding and hydrolysis. Most studies of ABCG2 have focused on its potential role in producing the multidrug resistance phenotype in cancer cells and its expression in primitive cell populations. ABCG2 expression is associated with side population phenotype.

P-glycoprotein (Pgp), the product of the MDR1 (ABCB1) gene, has been studied extensively and is known to transport a wide range of chemotherapy drugs such as the anthracyclines, vinca alkaloids, taxanes, etoposide, mitoxantrone, bisantrene and the histone deacetylase inhibitor depsipeptide. [111]. ABCB1 is a major negative prognostic factor in adult patients with AMLs [112].

## 1.8 HEDGEHOG SIGNALING PATHWAY

The Hedgehog pathway is a critical mediator of embryonic patterning and organ development, including hematopoiesis. It influences stem cell fate, differentiation, proliferation and apoptosis in responsive tissues. In adult organisms, Hedgehog pathway

activity is required for aspects of tissue maintenance and regeneration, however, there is increasing awareness that abnormal Hedgehog signaling is associated with malignancy. Hedgehog signaling is critical for early hematopoietic development, but there is controversy over its role in normal hematopoiesis in adult organisms where it may be dispensable. Conversely, Hedgehog signaling appears to be an important survival and proliferation signal for a spectrum of hematological malignancies. Furthermore, Hedgehog signaling may be critical for the maintenance and expansion of leukemic stem cells and therefore provides a possible mechanism to selectively target these primitive cell subpopulations which are resistant to conventional chemotherapy [113].

### 1.8.1 HEDGEHOG SIGNALING PATHWAY IN DROSOPHILA

The Hedgehog (Hh) signaling pathway was initially discovered by Nusslein – Wolhard and Weischaus in 1980 through a genetic screen for factors influencing drosophila embryonic patterning [114]. Characterization of the hh gene revealed that it encodes a secreted protein expressed in a repeated pattern across the drosophila embryo. Absence of this HH protein prevented normal segmentation and gave the drosophila embryo a characteristic "prickly" appearance reminiscent of a curled Hedgehog. As studies progressed, it became clear that Hh signaling is a conserved process between species and is critically important in vertebrate embryogenesis where it is required for development of internal organ, midline and neurological structures, limb patterning and development of the hematopoietic system. It has been well established that absence of functional Hh signaling causes dySmorphisms such as holoprosencephaly (cyclopia), limb abnormalities and improper biological system and organ development [115-116].

The multiple-pass, transmembrane proteins Patched (Ptc) and Smoothened (Smo) interact with one another at the cell surface and Ptc inhibits the positive signaling activity of Smo. The Costal2 (Cos2), Fused (Fu), and Cubitus interruptus (Ci) proteins are bound together in a high molecular weight protein complex which is attached to microtubules. Cos2 is a kinesin-like microtubule motor protein with a N-terminal motor domain; Fused is a serine/threonine kinase; and Ci is a zinc-finger transcription factor. In the absence of Hh stimulation, the complex is bound to microtubules and Ci is cleaved into a smaller N-terminal fragment called Ci75 which moves to the nucleus and represses Hh target genes. Upon secretion, Hh binds to Ptc and relieves the inhibitory effect that Ptc normally has on Smo. Once Smo is freed of the inhibitory effects of Ptc, Smo then signals through unknown mechanisms to the

Fu/Cos2/Ci complex, causing hyperphosphorylation of Fu and Cos2 and causing the complex to loosen its hold on microtubules. This leads to the stabilization of full length Ci, which can then travel to the nucleus and function as a transcriptional activator, upregulating transcription of Hh target genes. Also involved in Hh signaling are Suppressor of Fused (Su[Fu]), a protein which binds Fu and Ci and which appears to be involved in retaining Ci in the cytoplasm, and Supernumerary limbs (Slmb, an F-box protein), an F-box protein involved in proteasomal targetting of Ci. Finally, Drosophila Protein Kinase A phosphorylates Ci on several sites and these phosphorylation events are required for the cleavage of Ci into Ci75 [117] (Figure 13).

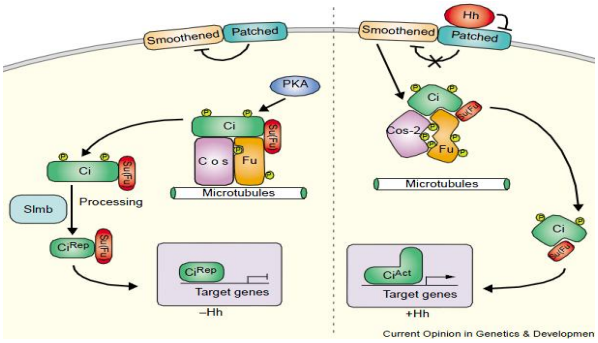



Fig. 13: A 'standard' model of the accepted mechanism of Hh signal transduction. Phosphates are represented by yellow circles with a 'P'.

### 1.8.2 HEDGEHOG SIGNALING PATHWAY IN MAMMALIAN CELLS

There are three isoforms of mammalian ligand Hedgehog, Sonic Hedgehog, Indian and Desert. Sonic is the most represented.

Hedgehog interacts with the twelve-alpha helices trans-membrane receptor Patched (Ptch1). In the absence of ligand Hedgehog, Ptch1 inhibits the catalytic activity of the protein Smoothened (Smo), which possesses a similar structure to a receptor with seven alpha helices trans-membrane and keeps it in a state dephosphorylated. Unphosphorylated Smo is removed from the cell surface by endocytosis and is degraded by lysosomes. The C-terminal portion of the protein Smo binds directly kinesin-like COS2, which acts as a scaffold protein, maintaining together all the components of the pathway, which include the transcriptional activator Gli (full-lenght 155 kDa) and multiple serine-threonine as the Pka, Gsk3β and CK1. In the absence of Hedgehog, Gli is hyperphosphorylated by Pka, which acts as a priming kinase, and by GSK3 and casein 1 (CK1), which go to phosphorylate Gli. The hyperphosphorylation promotes the Gli recognition by ubiquitin, leading to the generation

truncated form of the transcriptional repressor of Gli, GliR (75 kDa), which is thus brought to the proteasome and can not translocate into the nucleus.

The binding of Hedgehod on Ptch results in a loss of activity of the latter, and a consequent activation of Smo. Smo will then be free to transduce the Hedgehog signaling in the cytoplasm. Following the stimulation of Hedgehog, Smo is hyperphosphorylated and its endocytosis and degradation is blocked. The full-length form of Gli1 is no longer phosphorylated by complex PKA, GSK3 and CK1, and is therefore free to enter the nucleus and transcribes target genes.

Gli genes coding for a zinc-finger proteins (Gli1, Gli2, Gli3) belonging to sequences specific DNA binding family.

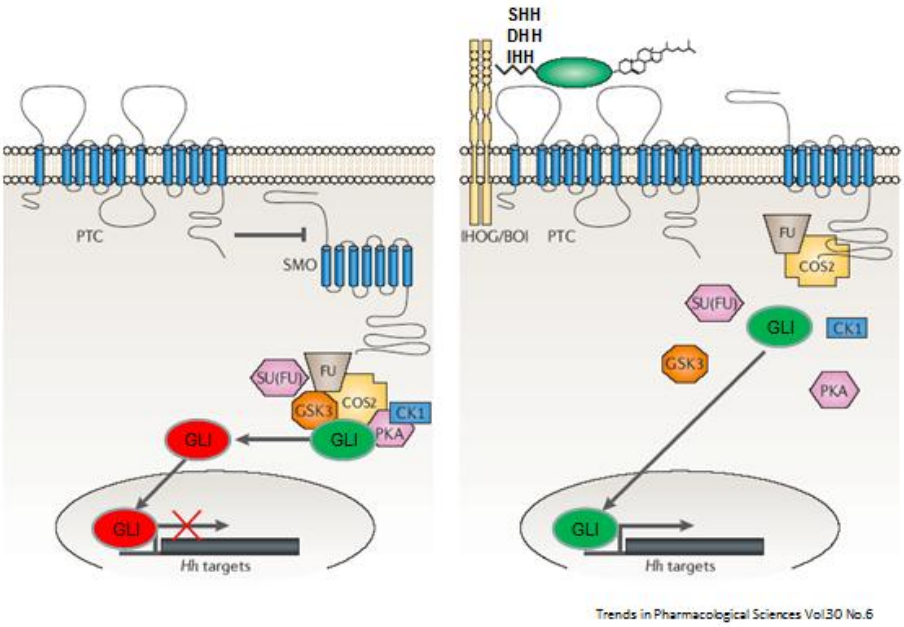



Fig. 14: Hedgehog signaling pathway in mammalian cells.

### 1.8.3 HEDGEHOG SIGNALING PATHWAY AND CANCER

Abnormal Hh signaling is associated with diverse human malignancies [118]. Its oncogenic properties were first identified through the realization that the genetic abnormality causing Gorlin's syndrome (associated with an excess risk of rhabdomyosarcoma, medulloblastoma and basal cell carcinoma [BCC]) is an inherited inactivating PTCH mutation [119]. Sporadic cases of the same malignancies are frequently associated with either inactivating mutations of PTCH or activating mutations of SMO indicating that PTCH

and SMO act as tumor suppressor and oncogene, respectively [118]. Further work confirmed that GLI transcription factors were responsible for driving tumor formation [120]. The association of Hh signaling with oncogenesis is not surprising given the critical role that Hh signaling performs in regulating cell proliferation, cell cycle machinery, apoptosis, chromatin modeling, self-renewal and epithelial-to-mesenchymal transition in responsive cells [121].

Recent evidence suggests that Hh signaling may have distinct mechanisms of action in different tumor environments. For example, in BCC and medulloblastoma, the most common lesions in Hh signaling are gain-of-function SMO mutations or loss of-function PTCH mutations [122-123]. These are rare in glioblastoma and lymphoma; however Hh signaling remains critically important as, in these tumors, malignant cells respond to HH ligand secreted from the surrounding microenvironment [124-125]. Furthermore, while Hh signaling is important in pancreatic cancer tumorigenesis, this is not through direct signaling to tumor cells but rather due to HH ligand production by tumor cells interacting with the local stroma [126].

Thus Hh signaling appears to be associated with development and maintenance of malignancy through (i) ligand independent signaling due to cell intrinsic mutations or (ii) autocrine / paracrine signaling between tumor cells and stroma or vice versa. Lastly, there is accumulating evidence that CSCs are dependent on Hh signaling for population maintenance and expansion.

Three basic models have been proposed for Hh pathway activity in cancer [127] (Figure 15). The first discovered were type I cancers, which harbor pathway-activating mutations and are, thus, Hh ligand independent, such as basal cell carcinomas.

Type II cancers are ligand dependent and autocrine (or juxtracrine), meaning that Hh is both produced and responded to by the same (or neighboring) tumor cells.

Type III cancers are also ligand dependent but paracrine, in that Hh produced by the tumor epithelium is received by cells in the stroma (analogous to the epithelial-to-mesenchymal signaling during development), which feed other signals back to the tumor to promote its growth or survival. A new variation is ‘reverse paracrine’ signaling, whereby Hh is secreted from stromal cells to receiving cells in a tumor (type IIIb in Figure 15c). In addition to the extracellular matrix, the stroma contains many cell types including fibroblasts, endothelial cells and immune cells, the complex interactions of which with the tumor constitute a microenvironment favorable for its growth [128].

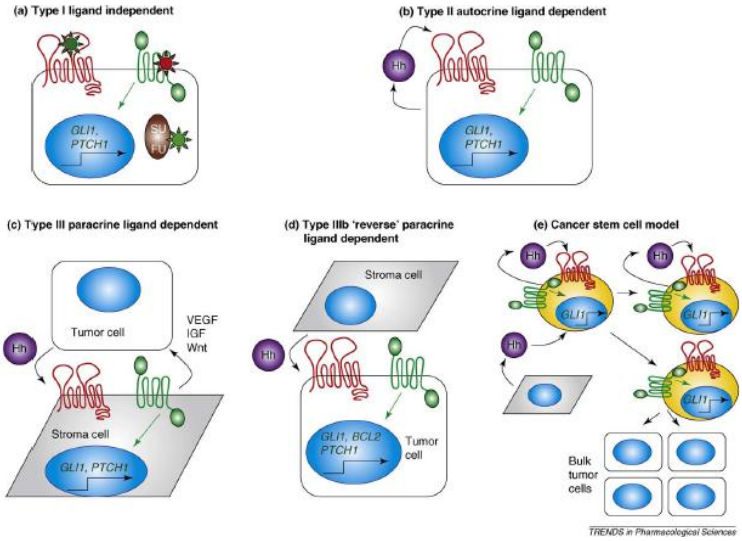



Fig. 15: Illustrations of the different models of Hh pathway activation in cancer. (a) Type I ligand-independent cancers harbor inactivating (green star) mutations in the negative regulators PTCH1 or SUFU, or activatingmutations (red star) in SMO, leading to pathway activation in a cell-autonomousmanner even in the absence of ligand and expression of target genes such as GLI1 and PTCH1. (b) Type II ligand-dependent autocrine cancers both secrete Hh and respond to it, leading to cell-autonomous pathway activation. (c) Type III ligand-dependent paracrine cancers secrete Hh, which binds to PTCH1 on stromal cells, leading to pathway activation in that cell type and feedback of other growth or survival signals (such as IGF, VEGF and Wnt) to the tumor. (d) Type IIIb 'reverse paracrine' tumors receive Hh secreted from stromal cells such as the bone marrow or lymph nodes, which leads to pathway activation in the tumor, upregulating survival genes such as BCL2 in addition to GLI1 and PTCH1. (e) The cancer stem cell model postulates that Hh signaling occurs only in self-renewing putative cancer stem cells (yellow), which either produce their own Hh ligand or receive it from stromal cells, including the vasculature. A subset of the cancer stem cells will then differentiate into Hh pathway-negative tumor cells that comprise the bulk of the tumor. Key: PTCH1, red; SMO, green; SUFU, brown; Hh, purple; nucleus, light blue; receiving cell, white rectangle; stromal cells, grey diamonds; cancer stem cells, yellow circles.

### 1.8.4 HEDGEHOG SIGNALING PATHWAY AND CHEMORESISTANCE MECHANISMS

Loss of Smoothened (Smo), an essential component of the Hh pathway [129], impairs haematopoietic stem cell renewal and decreases induction of chronic myelogenous leukaemia (CML) by the BCR– ABL1 oncoprotein [130]. Loss of Smo causes depletion of CML stem cells—the cells that propagate the leukaemia—whereas constitutively active Smo augments CML stem cell number and accelerates disease. As a possible mechanism for Smo action, we show that the cell fate determinant Numb, which depletes CML stem cells, is increased in the absence of Smo activity. Furthermore, pharmacological inhibition of Hh signaling impairs not only the propagation of CML driven by wild-type BCR–ABL1, but also the growth of Imatinib-resistant mouse and human CML. These data indicate that Hh pathway activity is required for maintenance of normal and neoplastic stem cells of the haematopoietic system

and raise the possibility that the drug resistance and disease recurrence associated with Imatinib treatment of CML [131-132] might be avoided by targeting this essential stem cell maintenance pathway.

Imatinib mesylate, which binds to the ABL1 kinase domain and inhibits phosphorylation of substrates, has been used to treat CML, but it is not curative because the cancer stem cells that propagate the leukaemia are resistant to therapy and are not eradicated [91, 132-133]. Furthermore, Imatinib resistance due to mutations in the drug-binding site can occur, especially in advanced stage disease, leading to disease relapse and progression [131]. The design of effective new therapies thus critically depends on the identification of signals that are required for CML propagation and in particular signals required for CML cancer stem cell maintenance.

The pharmacological inhibition of Hh signaling can impair growth of CML driven by wild-type and Imatinib-resistant BCR–ABL1, as well as human CML, raise the possibility that Hh antagonists may be useful as a therapy for normal and drug resistant CML [134].

Activation of the Hh pathway is both sufficient and essential for resistance to classical chemotherapy in myeloid leukemia. The mechanism of action of Hh inhibitors is probably dependent on its effect on P-gp expression levels [135].

Hh signaling to modulate the expression of two ABC transporters, multi-drug resistance protein 1 (MDR1; p-Glycoprotein, ABCB1) and breast cancer resistance protein (BCRP; ABCG2), whose expression has been correlated with clinical chemoresistance [136-137]. Hh activation increases chemotherapeutic resistance by regulation intracellular drug uptake in an ABC transporter-dependent manner. The discovery that Hh signaling regulates ABC transporter expression in tumors could provide potentially a novel therapeutic strategy because inhibiting Hh signaling may not only affect tumor proliferation but may also increase chemotherapeutic effect, and result in improved treatment responses [138].

It has been demonstrated that Hh signaling promotes up-regulation of Bcl-2. Bcl-2 is relevant in response to Hh signaling since the expression of Bcl-2 was proven to be up-regulated via direct binding of Hh-activated transcription factor GLI1 or GLI2 in solid cancer cells (Figure 16) [124, 139-140]. The pharmacological inhibition of Hh signaling reduced expression levels of Bcl-2 in Hh receptor-positive CD34+ leukemic cells. It has been reported that Bcl-2 expression could be correlated with resistance of cancer therapy. inhibition of Hh signaling augments the therapeutic efficacy of Ara-C in AML [141]. In conclusion, Hh signaling could be involved in the survival and drug resistance of CD34+

leukemic cells. Inhibition of Hh signaling can be a therapeutic option to directly induce apoptosis and reduce drug resistance of AML.

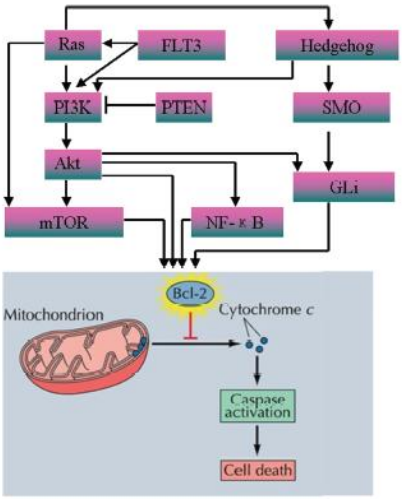



Fig. 16: Signal transduction pathways important in leukemic stem cells.

### 1.8.5 MOLECULE INHIBITORS OF THE HEDGEHOG SIGNALING PATHWAY

Over the past decade, the Hedgehog signaling pathway has attracted considerable interest because it plays important roles in the tumorigenesis of several types of cancer as well as developmental processes. It has also been observed that Hedgehog signaling regulates the proliferation and self-renewal of cancer stem cells and may promote tumor repopulation after chemotherapy and contribute to chemotherapy resistance in cancers. A great number of Hedgehog pathway inhibitors have been discovered through small molecule screens and subsequent medicinal chemistry efforts. Among the inhibitors, several Smo antagonists have reached the clinical trial phase. It has been proved that the inhibition of Hedgehog signaling with Smo antagonists is beneficial to cancer patients with basal cell carcinoma and medulloblastoma. We provide an overview of Hedgehog pathway inhibitors with focusing on the preclinical and/or clinical efficacy and molecular mechanisms of these inhibitors [142].

The first Smo inhibitors (SMOi), cyclopamine and jervine, were isolated from corn lilies as compounds causing teratogenic effects (including cyclopia) in lambs whose mothers had ingested this plant [143] and were subsequently shown to inhibit the Hh pathway [144] by binding to SMO [145], the GPCR-like structure of which makes it an ideal drug target [146]. However cyclopamine has low affinity and poor oral bioavailability for this suboptimal

pharmacokinetics [50], more potent and/or acid-soluble derivatives of it have been synthesized [147-149] (Table 5, 6).

| Compound | Organization | Target | Cancer type |
|---|---|---|---|
| GDC0449 | Roche/Genentech/Curis | SMOH | Medulloblastoma |
| GDC0449 | Roche/Genentech/Curis | SMOH | Recurrent glioblastoma multiforme |
| GDC0449 | Roche/Genentech/Curis | SMOH | Basal-cell nevoid syndrome |
| GDC0449 | Roche/Genentech/Curis | SMOH | Advanced basal-cell carcinoma |
| GDC0449 | Roche/Genentech/Curis | SMOH | Stomach or gastroesophageal junction |
| GDC0449 | Roche/Genentech/Curis | SMOH | Metastatic colorectal |
| GDC0449 | Roche/Genentech/Curis | SMOH | Small cell lung |
| GDC0449 | Roche/Genentech/Curis | SMOH | Ovarian |
| GDC0449 | Roche/Genentech/Curis | SMOH | Metastatic pancreatic |
| +Gemcitabine | Roche/Genentech/Curis | +DNA replication | Metastatic pancreatic |
| BMS-833923 | BMS/Exelixis | SMOH | Basal-cell carcinoma |
| BMS-833923 | BMS/Exelixis | SMOH | Basal-cell nevoid syndrome |
| BMS-833923 | BMS/Exelixis | SMOH | Small cell lung |
| +Carboplatin | BMS/Exelixis | +DNA alkylation | Small cell lung |
| +Etoposide | BMS/Exelixis | +Topo II | Small cell lung |
| BMS-833923 | BMS/Exelixis | SMOH | Met. Gastric and esophageal |
| +Cisplatine | BMS/Exelixis | +Topo II | Met. Gastric and esophageal |
| +Capecitabine | BMS/Exelixis | +DNA replication | Met. Gastric and esophageal |
| BMS-833923 | BMS/Exelixis | SMOH | Multiple myeloma |
| +Lenalidomide | BMS/Exelixis | +Not known | Multiple myeloma |
| +Bortezomib | BMS/Exelixis | +Proteosome inh. | Multiple myeloma |
| LDE225 | Novartis | SMOH | Medulloblastoma |
| LDE225 | Novartis | SMOH | Skin basal-cell carcinoma |
| PF0449913 | Pfizer | ? | Chronic myeloid leukemia |
| +Dasatinib | Pfizer | +Src kinase inh. | Chronic myeloid leukemia |
| IPI-926 | Infinity | SMOH | Metastatic solid tumors |

Table 5: Small molecule Smo inhibitors and indicative targets in current clinical trials.

| | Target | Origin | Original assay | Cells | IC50 (μM)[a] |
|---|---|---|---|---|---|
| (a) HH-GLI antagonists | | | | | |
| Natural and derivatives | | | | | |
| Jervine | Smo | *Veratrum californicum* | HNF3β mRNA level | Chick neural plate | 0.5 |
| Cyclopamine | Smo | *Veratrum californicum* | Gli luc reporter | NIH3T3 | 0.3 |
| KAAD-Cyclopamine | Smo | Cyclopamine | Gli luc reporter | NIH3T3 | 0.02 |
| Cyclopamine tartrate | Smo | Cyclopamine | ? | ? | ? |
| Cabohydrate–Cyclopamine (5f) | Smo | Cyclopamine | MTS assay | A549 | 33 |
| Antigen peptide–Cyclopamine | Smo | Cyclopamine | MTS assay | DU145 | ? |
| IPI-269609 | Smo | Cyclopamine | Gli luc reporter | NIH3T3 | ? |
| IPI-926 | Smo | Cyclopamine | Alkaline phophatase induction | C3H10T1/2 | 0.007 |
| Vitamine D3 | ? | Activated 7-Dehydrocholesterol | ? | ? | ? |
| Curcumin | ? | *Curcuma longa* | *GLI1* and *PTCH1* mRNA levels | DAOY | ? |
| Zerumbone | Gli | *Zingiber zerumbet* | Gli luc reporter | HaCaT | 7.1 |
| Staurosporinone | Gli | *Nocardiopsis* sp. | Gli luc reporter | HaCaT | 1.8 |
| Arcyriaflavin C | Gli | *Arcyria ferruginea* | Gli luc reporter | HaCaT | 11.3 |
| Physalin B | Gli | *Physalis minima* | Gli luc reporter | HaCaT | 0.66 |
| Physalin F | Gli | *Physalis minima* | Gli luc reporter | HaCaT | 0.62 |
| Triazole itraconazole | ? | (FDA approved) | ? | ? | ? |
| Synthetic | | | | | |
| Robotnikinin | Shh | Macrocycle | Gli luc reporter | NIH3T3 | 4 |
| Estrone Cyclopamine | Smo | Steroidal precursors | Gli luc reporter | HaCaT | ? |
| SANT1 | Smo | ? | Gli luc reporter | HaCaT | 0.02 |
| SANT2 | Smo | Benzimidazole | Gli luc reporter | HaCaT | 0.03 |
| SANT3 | Smo | ? | Gli luc reporter | HaCaT | 0.1 |
| SANT4 | Smo | ? | Gli luc reporter | HaCaT | 0.2 |
| TC132 | Smo | SANT2 derivative | Gli luc reporter | HaCaT | 0.08 |
| SANT74 | Smo | Chlorobenzothiophene deriv. | Gli luc reporter | HaCaT | 0.07 |
| SANT75 | Smo | Chlorobenzothiophene deriv. | Gli luc reporter | HaCaT | 0.02 |
| Cur-61414 | Smo | Aminoproline | *GLI1* mRNA levels | HaCaT | 0.2 |
| HhAntag691 | Smo | Benzimidazole deriv. | *GLI1* mRNA levels | HaCaT | 0.04 |
| GDC-0449 | Smo | Benzimidazole deriv. | *GLI1* mRNA levels | C3H10T1/2 | 0.013 |
| BMS-833923/XL139 | Smo | ? | ? | ? | 0.006–0.035 |
| LAB687 | Smo | Ortho-biphenyl carboxamide | Gli luc reporter | TM3 | 0.01 |
| 1-Amino-4-benzylphtalazine | Smo | Benzylphtalazine | Gli luc reporter | TM3 | 0.003 |
| LDE225 | Smo | ? | ? | ? | ? |
| Heterocyclic antag. | Smo | Heterocycles | Alkaline phophatase induction | C3H10T1/2 | 0.000026–42 |
| PF-04449913 | ? | ? | ? | ? | |
| JK184 | Gli | Imidazopyridine deriv. | Gli luc reporter | C3H10T1/2 | 0.03 |
| GANT58 | Gli | Thiophene with pyridine rings | Gli luc reporter | NIH3T3 | 5 |
| GANT61 | Gli | Hexahydropyrimidine | Gli luc reporter | NIH3T3 | 5 |
| HIP1 | Gli | ? | Gli luc reporter | NIH3T3 | 15 |
| HIP2 | Gli | ? | Gli luc reporter | NIH3T3 | 20 |
| HIP3 | Gli | ? | Gli luc reporter | NIH3T3 | 30 |
| HIP4 | Gli | ? | Gli luc reporter | NIH3T3 | 30 |
| (b) HH-GLI agonists | | | | | |
| Natural and derivatives | | | | | |
| 20α-Hydroxycholesterol | ? | Cholesterol deriv. | *Ptc1-LacZ* expression | Ptc1$^{-/-}$;p53$^{-/-}$ | 0.1 |
| 22(S)-hydroxycholesterol | ? | Cholesterol deriv. | *Ptc1-LacZ* expression | Ptc1$^{-/-}$;p53$^{-/-}$ | 0.2 |
| 24-Hydroxycholesterol | ? | Cholesterol deriv. | *Ptc1-LacZ* expression | Ptc1$^{-/-}$;p53$^{-/-}$ | 3 |
| 25-Hydroxycholesterol | ? | Cholesterol deriv. | *Ptc1-LacZ* expression | Ptc1$^{-/-}$;p53$^{-/-}$ | 1 |
| Synthetic | | | | | |
| Hh-Ag1.1 and deriv. | Smo | Chlorobenzothiophene | Gli luc reporter | C3H10T1/2 | 3 |
| SAG | Smo | Chlorobenzothiophene | Gli luc reporter | NIH3T3 | 0.003 |
| Purmorphamine | Smo | Purine deriv. | Gli luc reporter | NIH3T3 | 1 |

Table 6: (a) Smo antagonists and (b) Smo agonists.

# 2. AIM

Hedgehog (Hh) signalling is a fundamental pathway for cells survival, moreover it rappresents proliferation signal for the hematological malignancies. Furthermore, Hedgehog signaling may plays a critical role in the maintenance and expansion of leukemic stem cells. For these reasons, this pathway provides a possible mechanism to selectively target the primitive cell subpopulations which are resistant to conventional chemotherapy [113].
Pharmacological inhibition of Hh signaling impairs the essential stem cell maintenance pathway. Loss of Smoothened (Smo), an essential component of the Hh pathway [129], damages haematopoietic stem cell self-renewal and causes depletion of leukemic stem cells.
Since the chemotherapy-resistant phenotype of leukemia cells correlates with the activation of Hh signaling, the overexpression of Hh pathway components induces chemoprotection and the inhibition of the pathway reverts chemoresistance of leukemia cells. For this, we have identified Hh pathway as an essential component of chemoresistance in human leukemia. According to this view we suggest that targeting this pathway could be an interesting therapeutic approach to overcome chemoresistance in leukemia cells. Smo, a key player of Hedgehog signaling, is the most suitable target to inhibit this pathway. To this aim several molecules, antagonists of Smo, have been synthesized, and some of them have started the phase I in clinical trials.

In order:

1. To investigate whether Smo Inhibitor (SMOi) treatment targets specifically the Hedgehog pathway and identify 'ex vivo' new potential clinical biomarkers of responsivness and/or resistant to this treatment, we analyzed gene expression profiling in leukemic stem cell-enriched $CD34^+$ fraction collected before and after 28 days start of treatment from patients enrolling in a phase I Clinical Trial active in our hospital.

2. To confirm the 'ex vivo' gene expression data, we performed real-time PCR.

3. To analyze Hedgehog target genes expression in primary AML and ALL cells, we used real-time PCR.

4. To explore, by WST-1 and Annexin V/Propidium Iodide staining analysis, the '*in vitro*' efficacy on cell viability and toxicity of SMOi, we treated AML, ALL, CML

cell lines and primary cells from healthy donor using increasing concentration of this drug.

5. To confirm, by real-time PCR and Western Blot assay, that '*in vitro*' SMOi treatment inhibits Hh pathway, we treated AML, ALL and CML cell lines with this drug.

6. To investigate whether SMOi treatment in combination with conventional chemoterapy could be a therapeutic option to induce apoptosis and reduce drug resistance of leukemic cells, we performed WST1 Annexin V/Propidium Iodide staining analysis on CML cell line using SMOi in combination with Nilotinib, Imatinib, and Bosutinib.

# 3. MATERIALS AND METHODS

## 3.1 $CD34^+$ IMMUNOMAGNETIC SEPARATION

### 3.1.1 PRINCIPLE OF THE MACS® SEPARATION

First, the CD34+ cells are magnetically labeled with CD34 MicroBeads. Then, the cell suspension is loaded onto a MACS® Column which is placed in the magnetic field of a MACS Separator. The magnetically labeled CD34+ cells are retained within the column. The unlabeled cells run through; this cell fraction is thus depleted of CD34+ cells. After removing the column from the magnetic field, the magnetically retained CD34+ cells can be eluted as the positively selected cell fraction.

### 3.1.2 BACKGROUND INFORMATION

The CD34 antigen is a single chain transmembrane glycoprotein expressed on human hematopoietic progenitor cells, endothelial progenitor cells, vascular endothelial cells, embryonic fibroblasts, and some cells in fetal and adult nervous tissue. The CD34 MicroBead Kit contains MicroBeads directly conjugated to CD34 antibodies for magnetic labeling of CD34-expressing cells from peripheral blood, cord blood, bone marrow, apheresis harvest, or differentiated ES and iPS cells. Hematopoietic progenitor cells, present at a frequency of about 0.05−0.2% in peripheral blood, 0.1−0.5% in cord blood, and 0.5−3% in bone marrow, can be rapidly and efficiently enriched.

Protocol:

- Sample preparation

When working with anticoagulated peripheral blood or buffy coat, peripheral blood mononuclear cells (PBMCs) should be isolated by density gradient centrifugation, for example, using Ficoll-Paque™

- Magnetic labeling

Work fast, keep cells cold, and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labeling.

Volumes for magnetic labeling given below are for up to $10^7$ total cells. When working with fewer than $10^8$ cells, use the same volumes as indicated. When working with

higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for $2\times10^8$ total cells, use twice the volume of all indicated reagent volumes and total volumes).

For optimal performance it is important to obtain a single-cell suspension before magnetic labeling. Pass cells through 30 µm nylon mesh (Pre-Separation Filters, 30 µm # 130-041-407) to remove cell clumps which may clog the column. Moisten filter with buffer before use.

The recommended incubation temperature is 2–8 °C. Higher temperatures and/or longer incubation times may lead to nonspecific cell labeling. Working on ice may require increased incubation times.

1. Determine cell number.
2. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend cell pellet in 300 µL of buffer for up to $10^8$ total cells.
4. Add 100 µL of FcR Blocking Reagent for up to $10^8$ total cells.
5. Add 100 µL of CD34 MicroBeads for up to $10^8$ total cells.
6. Mix well and incubate for 30 minutes in the refrigerator (2−8 °C).
7. (Optional) Add fluorochrome-conjugated CD34 antibody recognizing another epitope than QBEND/10 or fluorochrome-conjugated CD45 antibody, and incubate for 5 minutes in the dark in the refrigerator (2−8 °C).
8. Wash cells by adding 5−10 mL of buffer for up to $10^8$ cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
9. Resuspend up to $10^8$ cells in 500 µL of buffer.
10. Proceed to magnetic separation.

### 3.1.3 MAGNETIC SEPARATION

Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of $CD34^+$ cells.

| Column | Max. number of labeled cells | Max. number of total cells | Separator |
|---|---|---|---|
| **Positive selection** | | | |
| MS | $10^7$ | $2\times10^8$ | MiniMACS, OctoMACS, VarioMACS, SuperMACS II |
| LS | $10^8$ | $2\times10^9$ | MidiMACS, QuadroMACS, VarioMACS, SuperMACS II |
| XS | $10^9$ | $2\times10^{10}$ | SuperMACS II |

Protocol:

1. Place column in the magnetic field of a suitable MACS Separator.
2. Prepare column by rinsing with the appropriate amount of buffer:
MS: 500 µL LS: 3 mL
3. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
4. Wash column with the appropriate amount of buffer. Collect unlabeled cells that pass through and combine with the flowthrough from step 3.
MS: 3×500 µL LS: 3×3 mL
5. Remove column from the separator and place it on a suitable collection tube.
6. Pipette the appropriate amount of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
MS: 1 mL LS: 5 mL
7. (Optional) To increase the purity of $CD34^+$ cells, the eluted fraction can be enriched over a second MS or LS Column. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new column.

### 3.1.4 EVALUTATION OF HEMATOPOIETIC PROGENITOR CELL PURITY

The purity of the isolated hematopoietic progenitor cells can be evaluated by flow cytometry or fluorescence microscopy. Analysis of $CD34^+$cells can be accomplished by direct immunofluorescent staining using an antibody recognizing an epitope different from that recognized by the CD34 monoclonal antibody.

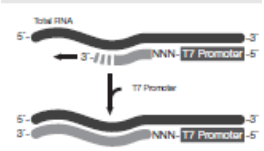
## 3.2 GENE-CHIP HUMAN GENOME U133 PLUS 2.0 ARRAY

The sequences from which these probe sets were derived were selected from GenBank®, dbEST, and RefSeq. The sequence clusters were created from the UniGene database (Build 133, April 20, 2001) and then refined by analysis and comparison with a number of other publicly available databases, including the Washington University EST trace repository and the University of California, Santa Cruz Golden-Path human genome database (April 2001 release).

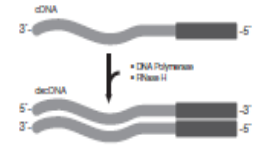
In addition, there are 9,921 new probe sets representing approximately 6,500 new genes. These gene sequences were selected from GenBank, dbEST, and RefSeq. Sequence clusters were created from the UniGene database (Build 159, January 25, 2003) and refined by analysis and comparison with a number of other publicly available databases, including the Washington University EST trace repository and the NCBI human genome assembly

Protocol:

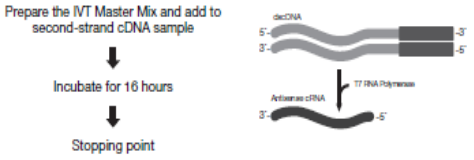
1. Synthesize first-strand cDNA



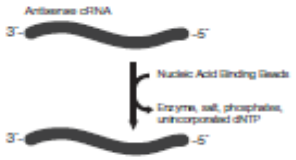

2. Synthesize second-strand cDNA



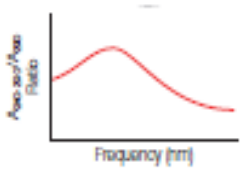

3. Synthesize cRNA using in vitro transcription



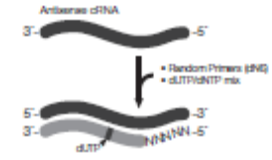

4. Purify cRNA



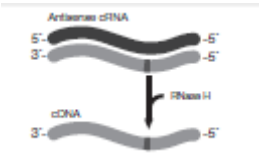
5. Assess cRNA yield and size distribution



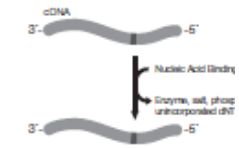

6. Synthesize 2nd-cycle cDNA



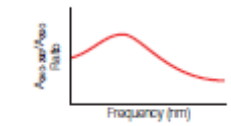

7. Hydrolyze using RNase H



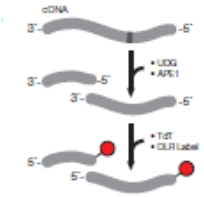

8. Purify 2nd-cycle cDNA




9. Assess cDNA yield and size distribution




10. Fragment and label the single-stranded cDNA



## 3.3 DRUGS

• Smo inhibitor (SMOi): The powder was dissolved in DMSO so as to create a stock solution 10 mM to store at – 80 °C. By dilution of this solution with RPMI 1640 medium to 10% of FBS were prepared the successive diluitions needed for the experiments:1 mM, 100 uM, 10 µM.

• Imatinib: tyrosine kinase inhibitor provided by Novartis. The powder was dissolved in DMSO to reach a concentration of 10 mM and the solution was stored at – 80 °C. From the stock solution were prepared by successive dilutions by adding RPMI 1640 10% FBS in order to obtain the concentrations of drug used during the experiments: 1 mM, 100 uM, 10 µM, 100 nM.

## 3.4 CELL LINES

All cell lines used were purchased by the bank of cell lines DMSZ (Deutsche Sammlung von und Mikroorganismen Zellkulturen GmbH), http://www.dsmz.de/.

### 3.4.1 BV173

BV-173 are a precursor leukemic human B lymphocyte line, isolated in 1980 from the peripheral blood of a 45 year old man affected by chronic myeloid leukemia (CML) in blast crisis. Cells are characterized by the translocation t(9; 22) in which the expression of the fusion gene BCR / ABL was confirmed by RT-PCR.
Their shape varies from round to elongated and grow as single cells in suspension.
The immunophenotype is characterized by: CD3-, CD10 +, CD13 +, CD19 + CD37-, CD80-, CD138-, HLA-, DR +, sm/cyIgM-, sm/cykappa-, sm/cylambda-.
Culture conditions: The BV173 were seeded at concentration of 1 x $10^{\wedge 6}$ cells / ml and maintained in the range between 0.5 to 1.5 x $10^{\wedge 6}$ cells / ml in a medium consisting of 80% RPMI 1640 and 20% of FBS. They were incubated at 37 °C with 5% CO2. Their doubling time is about 48 hours and are splitted 1:2 or 1:3 every 3-4 days.

### 3.4.2 SUP-B15

SUP-B15, are a precursor of human leukemic lymphocyte line B. These cells were derived in 1984 from the bone marrow of a 9 year old boy affected by Philadelphia-chromosome positive acute lymphoblastic leukemia in second relapse.

Morphologically appear as small round cells growing singly in suspension.

The immunophenotype is characterized by CD3-, CD10 +, CD19 +, CD20 +, CD34 +, CD37-, CD38 +, CD138-, HLA-DR +, sm/cyIgG-, sm / IgM +, sm/cykappa-, sm/cylambda-.

Culture conditions: SUP-B15 were seeded at concentration of 1 x $10^{\wedge 6}$ cells / ml in a medium consisting of 80% RPMI 1640 and 20% FBS and were incubated at 37 °C with 5% CO2. Their doubling time is about 60 hours and are splitted 1:2 or 1:3 once a week.

### 3.4.3 K562

The K562 cells are human chronic myelogenous leukemia in blast crisis isolated from a woman of 53 years old.

The cells are characterized by the chimeric gene BCR / ABL and morphologically appear as cells large, round which grow individually in suspension.

The immunophenotype is characterized by CD3-, CD13 +, CD15 +, CD19-, CD33 +, CD71 +, CD235a +.

Culture conditions: The K562 were maintained at a concentration of 0.1-0.5 x $10^{\wedge 6}$ cells / ml in a medium consisting of 90% RPMI 1640 and 10% FBS. They were incubated at 37 °C with 5% CO2. Their doubling time is about 30-40 hours and are splitted 1:3 every 3 days.

### 3.4.4 HL60

The HL60 cells are human acute myeloid leukemia, isolated in 1976 from the peripheral blood of a woman suffering from AML.

They are large and round cells growing singly in suspension.

The immunophenotype is characterized by: CD3 -, CD4 +, CD13 +, CD14 -, CD15 +, CD19 -, CD33 +, CD34 -, HLA-DR -.

Culture conditions: The HL60 were maintained at a concentration of 0.1-0.5 x $10^{\wedge 6}$ cells / ml in a medium consisting of 90% RPMI 1640 and 10% FBS. They were incubated at 37 °C

with 5% CO2. Their doubling time is about 40 hours and are splitted from 1:2 to 1:5 every 1-2 days.

### 3.4.5 KG1

The KG1 cells are human acute myeloid leukemia, isolated in 1977 from the bone marrow of a 59 year old man affected by erythro- leukemia evolved into AML.
These cells form colonies in soft-agar in response to the factor that stimulates the formation of colonies and can become dendritic cells in the presence of factors such as GM-CSF, IL-4.
The KG1 have a slightly irregular shape and grow individually in suspension.
The immunophenotype is characterized by: CD3 -, CD13 +, CD14 -, CD15 +, CD19 -, CD33 +, CD34 +, HLA-DR +.
Culture conditions: The KG1 were maintained at a concentration of 0.2 to 1 x 10^6 cells / ml in a medium consisting of 90% RPMI 1640 and 10% FBS. They were incubated at 37 °C with 5% CO2. Their doubling time is about 38 hours and are splitted from 1:2 to 1:4 every 2-3 days.

### 3.4.6 MOLM13

The MOLM13 are human cells of monocytic acute myeloid leukemia (FAB class M5a), isolated in 1995 from the peripheral blood of a man of 20 years affected by myelodysplastic syndrome evolved in AML type M5a.
This line has a particular genetic alteration: the internal tandem duplication of the Flt3 gene.
The MOLM13 are round cells that grow individually in suspension.
The immunophenotype is characterized by: CD3 -, CD4 +, CD13 +, CD14 -, CD15 +, CD16 +, CD19 -, CD33 +, CD34 -, HLA-DR -.
Culture conditions: The MOLM13 were seeded at a concentration of 1 x 10^6 cells / ml and maintained in the range between 0.4 to 2 x 10^6 cells / ml in a medium consisting of 90% RPMI 1640 and 10% FBS. They were incubated at 37 °C with 5% CO2. Their doubling time is about 50 hours and are splitted from 1:2 to 1:3 every 2-3 days.

## 3.5 PREPARATION OF CULTURE MEDIUM

RPMI 1640 10% FBS L-Gln P / S (Final volume 500 ml)

• 450 ml of RPMI 1640 (EuroClone, CELBIO);
• 50 ml decomplemented fetal bovine serum (FBS heat-inactivated fetal calf serum);
• 5 ml Glutamine 200 nM final concentration of 1 %
• 2.5 ml Penicillin / Streptomycin (P / S) to a final concentration of 0.5%.

RPMI 1640 20% FBS L-Gln P / S

• 400 ml of RPMI 1640 (EuroClone, CELBIO);
• 100 ml of FBS (FBS heat-inactivated fetal calf serum);
• 5 ml Glutamine 200 nM final concentration of 1%;
• 2.5 ml Penicillin / Streptomycin (P / S) to a final concentration of 0.5%.

The preparations were then filtered through Vacuum Driven (Millipore).

## 3.6 COUNTING – TRYPAN BLUE ASSAY –

The Trypan Blue is a dye that can penetrate only in damaged or death cells, in which the integrity of cellular membrane permeability is compromised.
Before proceeding with the counts is necessary to make a 1:5 dilution of the sample cell: in a well of a 96-well plate wells (Nunc) (which may be reused for the subsequent count) are rated 25 µL of PBS, 15 µL of Trypan Blue and 10 µL of cell suspension.
From the solution thus prepared is taken a volume of 10 µL which is loaded in the Neubauer chamber.

## 3.7 EVALUATION OF CELL VITALITY

### 3.7.1 CELL VITALITY

The cell viability has been evaluated using "Cell Proliferation Reagent WST-1" (Roche, Germany), a colorimetric assay that uses the conversion, by the metabolically active cells, of tetraziolium salts in formazan, a detectable product using a spectrophotometer (Thermo Electron Corporation Multiskan EX).

Protocol:

The cells have been seeded in a 96-well plate at a concentration of 500,000 cells / µL to a final volume of 100 µL / well. The plate has been incubated for 2 hours at 37 °C so after treatment with various concentrations of drug. A sample of cells untreated and one treated with DMSO at 0.1% have been adopted as control, while only culture medium was used as negative control. All samples are sown in the plate in triplicate, to ensure a greater reliability of the results.
The signal was read at 24h, 48h and 72h after treatment, adding 10 µL of WST1 reagent in each well before the read in darkness.
The values obtained are representative of the fraction of living cells, expressed as a percentage and normalized subtracting the value of the negative control.

### 3.7.2 APOPTOSIS

The cytotoxicity effects, of the tested drugs on different cell lines was conducted using the Annexin-V-FLUOS Staining Kit (Roche). The assay involves the use of two reactive fluorochromes, the Annexin-V-FITC, with the emission of green fluorescence, and Propidium Iodide (PI), in red fluorescence, to marker apoptotic and necrotic cells respectively. Such discrimination is possible thanks to the ability to bind annexin V phosphatidylserine (PS), a phospholipid membrane that is expressed on the external of cell membrane only by apoptotic cells. Since the PS is normally located on the inner surface of the membrane, the Annexin V could bind it even in case of necrosis, in which the membrane

integrity is lost. In order not to confuse the two signals necrotic cells are stained with PI, a DNA intercalating able to penetrate the plasma membrane if it is damaged.

The analysis by flow cytometry, which detects the fluorescence emitted by individual cells, to identify living cells negative for both PI and Annexin V to cells in early apoptosis, positive only for annexin V-positive cells only the IP definitely necrotic, and cells positive for both fluorochromes, necrotic or apoptotic advanced.

Protocol:

The cells were been seeded in 6-well plates at a density of 500,000 cells / µL and a final volume of 2 ml. The plate has been incubated for 2 hours at 37 °C after treatment with various concentrations of drug. It has been used a sample treated with DMSO at 0.1% as a control.
At the time point, determined at 48 hours, the cells have been centrifuged and the pellet was resuspended in the labeling mix containing Annexin V and propidium iodide. The quantities for 10 samples are: 1 µL of incubation buffer, 20 µL of Annexin V-FITC and 20 µL of PI.
The samples have been left for 30' at 4 °C in darkness and then each sample was read by flow cytometer, measuring at 530 nm for FITC and 620 nm for PI.

## 3.8 EVALUATION OF TRASNSCRIPT LEVELS

The differential expression of genes of interest has been evaluated by the quantification of the corresponding transcripts using the technique of Real-time PCR. For this purpose from all the cell lines the total RNA has been extracted and than using reverse transcription converted into complementary DNA(cDNA).

### 3.8.1 MANUAL EXTRACTION RNA

For RNA extraction was used the 'RNeasy ® Mini Kit (Qiagen, Valencia, CA).
The cell samples, stored in GITC (guanidinium isothiocyanate), were resuspended with a tuberculin syringe, in order to completely destroy the membranes.

For each sample 350 µL of 70% ethanol have been added and the final volume has been loaded on an extraction QIAamp spin column, equipped with a acetate membrane which binds RNA. All the columns have been centrifuged for 15'' at 13000 rpm, and the eluete collected has been eliminated. Subsequently for each column 350 µL of RW1 buffer has been added and after a second centrifugation the eluete has been discarded. Then 80 µL of a solution composed by 10 µL of DNAsi and 70 µL of RDD buffer have been added for an incubation time of 15 minutes. After this incubation time a second wash with 350 µL of RW1 buffer have been done and after a third centrifugation the eluete has been discarded again. Then the acetate membranes have been washed twice by the addiction of 500 µL of RPE buffer. At the end of this process after a final centrifugation for 1' at 13000 rpm the total RNA has been eluted using 30 µL of RNAsi free water.

The quantification of the concentration of RNA has been based on the spectrophotometric reading of the absorbance of 1 µL of sample to 260 λ through the instrument Nanodrop (ThermoScientific). The instrument can measure directly the concentration of total RNA in ng / µL and also provides information regarding the level of purity of the sample.

### 3.8.2 REVERSE TRANSCRIPTASE PCR (RT-PCR)

The Reverse Transcriptase PCR (RT-PCR) is based on the reaction of reverse transcription of messenger RNA into a molecule of complementary DNA (cDNA) by the enzyme reverse transcriptase, an RNA-dependent DNA polymerase.

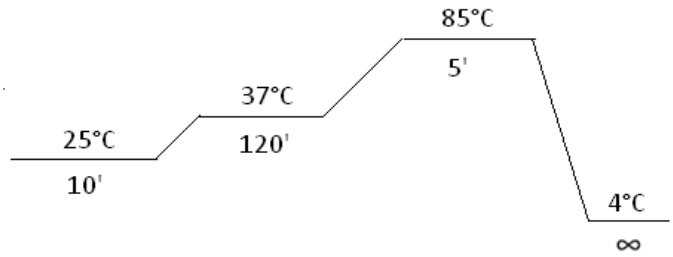
This reaction has been exploited the High Capacity cDNA Reverse Transcription Kits Applied Biosystems. The protocol provides for the preparation of 2 × RT Master Mix Whereas for each sample a final volume of Master Mix equal to 10 µl:

| Reagenti | Vol |
|---|---|
| **mq$H_2O$** | 4,2µl |
| **10x RT *Buffer*** | 2 µl |
| **25x dNTPs** | 0,8 µl |
| **10x RT random** | 2 µl |
| **RT enzima** | 1 µl |
| **V tot** | 10 µl |

For each sample have been added 10 µL of Master Mix, 1 µg of RNA (the necessary volume is calculated from the measured concentration to the Nanodrop) and MilliQ water to reach a final volume of 20 µL.

At the end of the reaction all the samples have been eluted in 30 µL of water coming to a final volume of 50 µL.

Amplification conditions:




### 3.8.3 REAL-TIME PCR

The gene expression profile has been estimated through the use of Real-Time PCR, an amplification technique that allows the quantify the number cDNA molecules of interest. To follow in real time the amplification reaction was used TaqMan ® Probe-Based Gene Expression Analysis (Applied Biosystems), that consist of a pair of unlabeled PCR primers and a TaqMan ® probe with a FAM ™ or VIC ® dye label on the 5 'end and minor groove binder (MGB) nonfluorescent quencher (NFQ) on the 3 'end, and was used a specific software to detect the number of cDNA copies as a function of increased fluorescence.

The gene most suitable to be used as a standard is that coding for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), whose level of expression is steady in the cells of interest.

The TaqMan probes used were specific for the following genes: Smo, Gli1, Gli2, Gli3, ABCB1, ABCG2 (Applied Biosystems).

The reaction mix for each sample have been prepared as follows:

| | |
|---|---|
| Taqman Universal Master Mix | 12.5 µL |
| Taqman probe | 1.25 µL |
| $H_2O$ | 6.25 µL |
| cDNA | 5 µL |

The amplification reaction has been conducted by loading each sample on a 96-well plate (MicroAmp Optical 96-well reaction plate, Applied Biosystems) with total reaction volume of 25 µL. The Real-Time PCR was performed on an ABI Prism 7300 SDS instrument (Applied Biosystems), setting the following amplification conditions: 50 ℃ for 2 ', 95 °C for 10', followed by 40 cycles at 95 ℃ for 15" and 60 °C for 1 '. The quantitative analysis has been performed using the ΔΔCt method. All samples have been loaded in duplicate.

## 3.9 WESTERN BLOT ANALYSIS

### 3.9.1 PROTEIN EXTRACTION

In order to extract proteins from cell samples treated, it has been necessary to lyse cellular membrane. To avoid proteins from degradation the lysis protocol has been conducted entirely in ice. To obtain cell pellet, all the samples have been centrifugated at 1700 rpm for 7 '; then the supernatant has been discarded and after two washes with 1 mL of cold PBS and a second centrifugation at 7000 rpm for 2 'in refrigerated centrifuge at 4 °C, providing to carefully remove the supernatant.

The dry pellets thus obtained may be stored at - 80 °C or be immediately subjected to cell lysis. The latter involves the use of a Lysis Buffer consisting of 0.1 M KH2PO4 (pH 7.5), 1% Igepal (NP-40), β-glycerophosphate and 0.1 mM PMSF 1X complete Protease Inhibitor Cocktail (Roche Diagnostics ).

For each sample, containing approximately $4x10^{\wedge 6}$ cells / mL, are added to 100 µL of Lysis Buffer and proceed with vigorous resuspension, favoring the mechanical disruption of cell membranes. It vortex then the sample and left to incubate for 30 'on ice, vortexing every 10'. Following is centrifuged the lysed samples at 14000 g for 30 'at 4 ℃, allowing the cell debris to settle to the bottom. The supernatant, containing the proteins in solution, is collected into 1.5 mL eppendorf that are immediately placed on ice. The lysates thus obtained can be stored at - 80 ℃ for not more than one month.

### 3.9.2 PROTEIN QUANTIFICATION

Protein samples are quantified by Bradford colorimetric assay (BIO-RAD), which provides for the reading of the optical density of the samples in a spectrophotometer.
In duplicate for each sample plus one for the white, is rate 1 mL of BIO-RAD Protein Assay (BIO-RAD), previously diluted 1:4 in $H_2O$. Is taken then 1 µL of lysate from each sample and is added in the corresponding tube. It is important whenever the vortex tube before removing a portion, to ensure that the concentration is homogeneous. Once you have set the spectrophotometer, constructing the calibration curve using known concentrations of BSA (Bovine Serum Albumin), we proceed to the transfer of the prepared samples into the tubes in the appropriate quote cuvette. Is read first the white, consisting only of Bradford reagent, and continue with the reading of all samples prepared in duplicate. The instrument is able to track, by interpolating the absorbance values on the calibration curve, the protein concentration in the sample in mg / µL and performs an average of the values measured in duplicate. The concentration is considered reliable if the two values obtained separately do not differ more than 1 mg / µL.

### 3.9.3 WESTERN BLOT

- SDS-PAGE

For each samples 80 µg of protein were separated on a NuPAGE Tris-Acetate Gel 12 wells, denaturing gradient 3-8% (Invitrogen) .

- Transfer to nitrocellulose membrane

At the end of the run, the gel was transferred on a nitrocellulose membrane by the use of a kit Trans-blot transfer turbo pack (Bio-Rad).
The instrument Turbo (Bio-Rad) allow to transfer by semi-dry method.
The membranes were incubated for 2 ' with Ponceau Red reagent, which makes visible the protein bands on the membrane, a sign that the race has been done successful.

- Marking with antibodies

The membranes were incubated with BSA or milk (5% milk powder in food not fat), for 1h at room temperature under stirring, in order to saturate not specific binding sites. After this

period of incubation the membranes were incubated with primary antibodies, diluted in BSA or milk as indicated in the data sheet of each antibody, in agitation at 4 °C overnight.
Primary antibodies used were directed against the major components of the Hh pathway, such as Smo, Gli1, Gli2, Gli3, ABCG1, Abcb2 (Origen, Cell Signaling Technology).
The next day the primary antibodies were recovered and the membranes were washed three times using PBS 0.1% Tween to remove the excess of antibody. The secondary antibodies, anti-goat, anti-rabbit or anti-mouse depending on the primary antibody used, were diluted 1:8000 or 1:10000 in milk or BSA (following the directions on the data sheet), and were incubated in stirring for 1 hours at room temperature.
Finally we proceed with the last three washes with PBS Tween 0.1%, each by 10 ', and to the development of the membrane.

- Development

For the development of the membrane was used ChemiDoc-IT, a tool that allows you to detect the activity of horseradish peroxidase conjugated secondary antibody, using the Enhanced ChemiLuminescent ECL kit (GE). The presence in the kit of the peroxidase substrate induces a reaction that leads to the formation of a chemiluminescent product. The emitted photons are detected by the instrument and processed using the software Vision Launch Works / GenDoc (Euroclone), for converting the signal into an image. As housekeeping, or internal control, was assessed the expression of β-actin.

# 4. RESULTS

## 4.1 'EX VIVO' SMO INHIBITOR ORAL TREATMENT

Our hospital had opportunity to enroll selected patients in a phase I, dose-escalation, first-in-man clinical trials, that allow oral administration of a new selective inhibitor of Smo (SMOi).

### 4.1.1 'EX VIVO' SMO INHIBITOR SPECIFICALLY TARGETS THE HEDGEHOG PATHWAY IN LEUKEMIC STEM CELL-ENRICHED CD34$^+$ FRACTION

In order to evaluate '*ex vivo'* SMOi efficacy and to identify new potential clinical biomarkers of responsiveness, we separated, before and after 28 days start of treatment, highly purified (98%) bone marrow CD34$^+$ cells from 5 acute myeloid leukemia (AML), 1 myelofibrosis (MF), 2 blastic phases chronic myeloid leukemia (CML) patients treated with SMOi by immunomagnetic separation, and we analysed their gene expression profile using Affimetrix HG-U133 Plus 2.0 platform. We analysed the gene expression data by Partek Genomics Suite and pathway analysis software GeneGo.

| Patient | Disease | Age/Sex | Drug-dose | Cycle |
|---|---|---|---|---|
| Pt ID 1003 1001 | AML | 73 Y/F | 5 mg | V |
| Pt ID 1003 1002 | AML | 73 Y/F | 10 mg | V |
| Pt ID 1003 1003 | CML | 71 Y/F | 20 mg | V |
| Pt ID 1003 1004 | AML | 79 Y/F | 20 mg | IV |
| Pt ID 1003 1006 | CML | 72 Y/F | 80 mg | I |
| Pt ID 1003 1007 | AML | 67 Y/F | 80 mg | V |
| Pt ID 1003 1008 | AML | 41 Y/F | 80 mg | III |
| Pt ID 1003 1010 | MF | 68 Y/M | 120 mg | II |

Table 7: Characteristics of patients enrolled in Phase I Clinical Trial that allow oral administration of SMOi.
The column 'cycle' represent the number of therapy's cycles. Each cycle is 28 days long.

Comparing the gene expression data obtained on CML CD34$^+$ cells separated after 28 days start of therapy versus the gene expression data obtained on CML CD34$^+$ cells harvested at the beginning of treatment, we observed a differential expression of 1197 genes with a p-

value < 0.05. This differential expression are mainly related to the Hedgehog pathway with a p-value = 0.003 (Table 8).

| name | p-value |
|---|---|
| Development_Role of Activin A in cell differentiation and proliferation | 0.002052003 |
| Development_Ligand-independent activation of ESR1 and ESR2 | 0.002923441 |
| Development_Hedgehog signaling | 0.003441742 |
| Regulation of lipid metabolism_Insulin signaling:generic cascades | 0.003722921 |

Table 8: GeneGo MetaCore pathway analysis, report on CML patients.

Comparing the gene expression data obtained on AML $CD34^+$ cells separated after 28 days start of therapy versus the gene expression data obtained on AML $CD34^+$ cells harvested at the beginning of treatment, we observed a differential expression of 589 genes with a p-value < 0.05. This differential expression are mainly related to the Hedgehog pathway with a p-value = 0.0002 (Table 9).

| name | P-value |
|---|---|
| Development_Hedgehog signaling | 0.000223512 |
| Development_Regulation of epithelial-to-mesenchymal transition (EMT) | 0.000324707 |
| Neurophysiological process_GABA-A receptor life cycle | 0.000718989 |
| Proteolysis_Putative SUMO-1 pathway | 0.001011649 |

Table 9: GeneGo MetaCore pathway analysis, report on AML patients

This data providing evidences that SMOi tratment really target the Hedgehog pathway in leukemic stem cell-enriched $CD34^+$ fraction.

### 4.1.2 GAS1 AND KIF27 GENES ARE STRONGLY UPREGULATED BIOMARKERS OF HEDGEHOG PATHWAY INHIBITION IN 'EX VIVO' LEUKEMIC STEM CELL - ENRICHED CD34+ FRACTION

We observed that among the genes differentially expressed on CML $CD34^+$ after 28 days start of SMOi treatment, and related to Hh pathway, there are Gas1 (growth arrest specific 1 gene) and Kif27 (kinesin family member 27) genes. These genes were strongly

upregulated (fold change 1.0947 and 1.12757 respectively; p-value 0.01 and 0.02 respectively) (Figure 17). Gas1 and Kif 27 genes may be the new potential biomarkers of responsivness of SMOi treatment.

Gas-1 inhibits the Hh pathway by binding and blocking Sonic Hedgehog (Shh). Kif-27 acts as a negative regulator of the Hedgehog pathway inhibiting the nuclear translocation of Gli1.

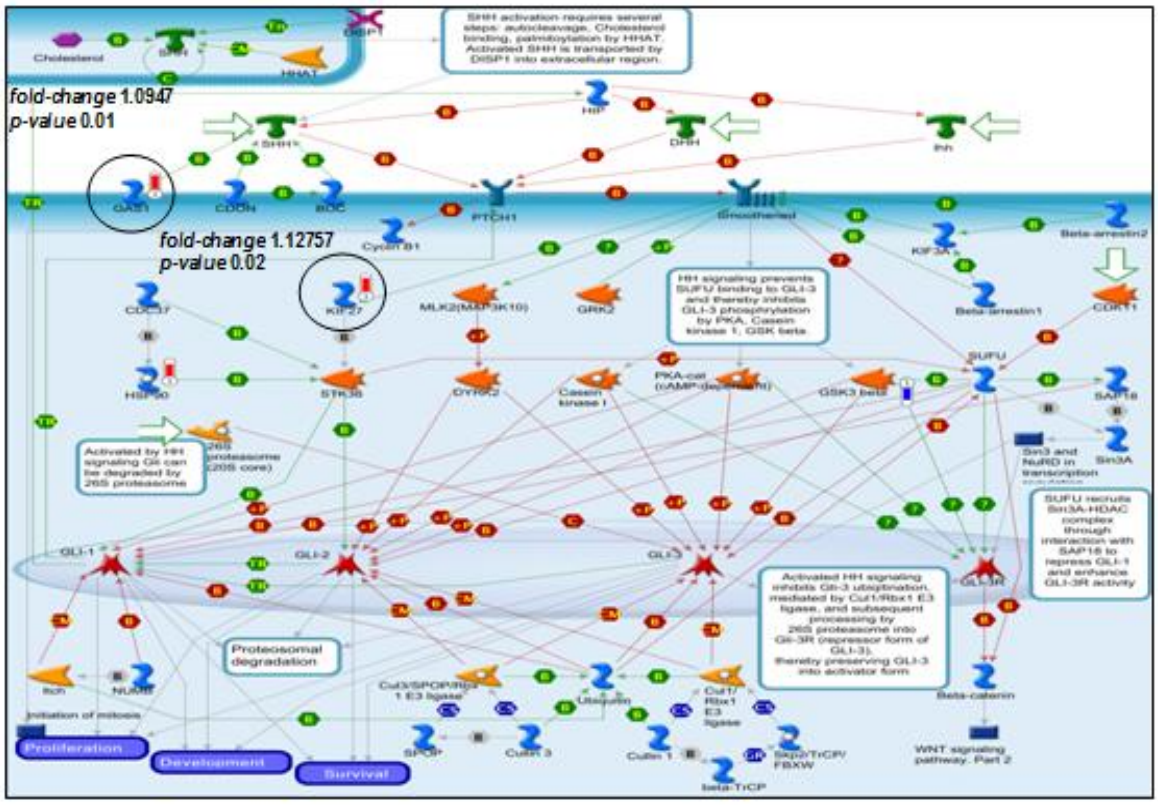



Figure 17: GeneGo MetaCore pathway analysis, report on CML patients. Gas 1 and Kif27 (circled). Blue hourglass= down-regulation; Red hourglass= up-regulation.

### 4.1.3 CASEIN KINASE I, GLI3 AND β-CATENIN GENES ARE STRONGLY DOWN-REGULATED IN LEUKEMIC STEM CELL-ENRICHED CD34+ FRACTION AFTER SMO INHIBITOR TREATMENT

We observed that among the genes differentially expressed on AML $CD34^+$ after 28 days start of SMOi treatment, and related to Hh pathway, there are Casein kinase I, Gli3 and β-catenin genes. These genes were down-regulated (fold change -1.22553, -1.122553 and -1.25495 respectively; p-value 0.012, 0.012 and 0,043 respectively) (Figure 18).

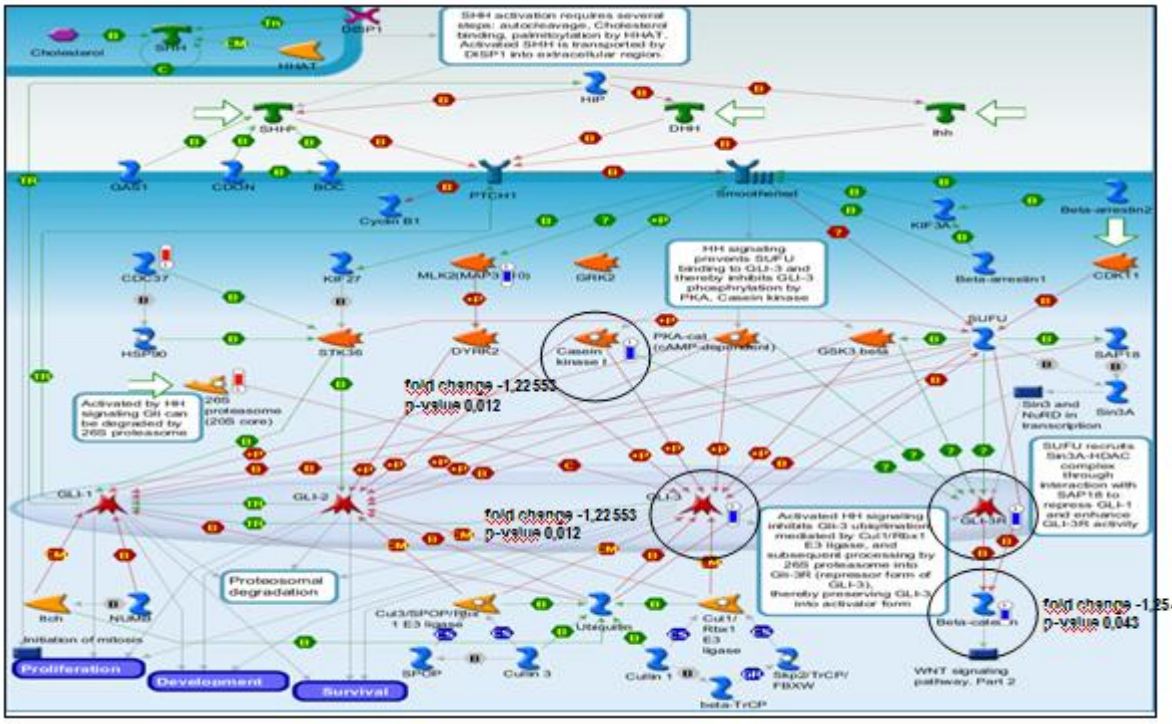



Figure 18: GeneGo MetaCore pathway analysis, report on CML patients. Casein kinase I, Gli3 and β-catenin genes (in circled). Blue hourglass= down-regulation; Red hourglass= up-regulation.

Therefore, SMOi treatment targets the Hedgehog pathway down-regulating Casein kinase I and Gli3 genes, both players of the Hedgehog pathway.

β-catenin protein is a subunit of the cadherin protein complex, which is important in the Wnt pathway\β-catenin, associated with cell survival and the self-renewal, suggesting a connection between the Hedgehog and Wnt pathway.

### 4.1.4 THE PHARMACOLOGICAL INHIBITION OF HEDGEHOG SIGNALING REDUCES BCL-2 EXPRESSION IN LEUKEMIC STEM CELL-ENRICHED CD34$^+$ FRACTION

It has been demonstrated that Hh signaling up-regulates Bcl-2 to promote cell survival. Bcl-2 is relevant in response to Hh signaling since the expression of Bcl-2 was proven to be up-regulated via direct binding of Hh-activated transcription factor Gli1 or Gli2 in solid cancer cells [124, 139-140].

Comparing the gene expression data obtained on CML and AML CD34$^+$ cells separated after 28 days start of therapy versus the gene expression data obtained on CML and AML CD34$^+$

cells harvested at the beginning of treatment, we observed a statistically significant (p value ≤0.05) down regulation of Bcl2 (fold change -1.03004), Abca2 (fold change -1.08966), Gli1 (fold change -1.0775), Smo (fold change -1.07702), and an up-regulation of Gli2 (fold change 1.08191) (Figure 19).

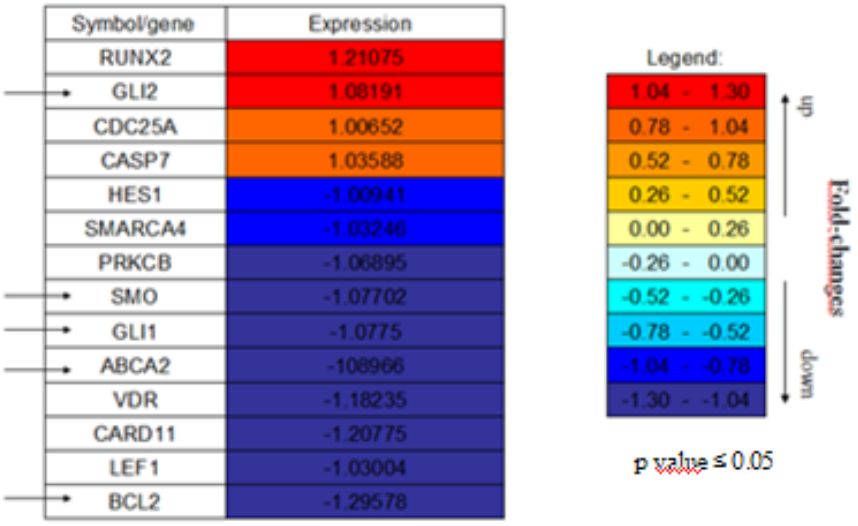



Figure 19: GeneGo MetaCore pathway analysis, report on CML and AML patients.

The pharmacological inhibition of Hh signaling reduces expression levels of its target genes, such as Gli1 and Smo, farther down-regulates Bcl-2 and Abca2 (ATP-binding cassette sub-family A member 2) genes**,** whose expression could be correlated with resistance of cancer therapy. In conclusion, Hh signaling could be involved in the survival and drug resistance of $CD34^+$ leukemic cells. Inhibition of Hh signaling can be a therapeutic option to directly induce apoptosis and reduce drug resistance of leukemia cells.

### 4.1.5 THE PHARMACOLOGICAL INHIBITION OF HEDGEHOG SIGNALING REDUCES EXPRESSION LEVELS OF TWO ABC TRASPORTER (ABCG2 AND ABCB1) IN LEUKEMIC STEM CELL-ENRICHED $CD34^+$ FRACTION

We confirmed the 'ex vivo' gene expression results for Gli1, Gli2 and Smo by real-time PCR. This evaluation included also Gli3, Abcb1 and Abcg2 genes. Abcb1 (ATP-binding cassette sub-family B member 1, MDR1), Abcg2 (ATP-binding cassette sub-family G member 2) belong to the superfamily of adenosine triphosphate-binding cassette (ABC) transporters. The pharmacological inhibition of Hh signaling showed a statistically

significant reduction of its target genes (Gli1, Gli2 and Gli3) and of Abcg2 and Abca2 genes. (Figure 20).

| Time points | n° pts |
|---|---|
| Screening | 8 |
| Post I cycle | 7 |
| Post II cycle | 6 |
| Post III cycle | 5 |
| Post IV ctcle | 4 |
| EOT (end of treatment) | 8 |

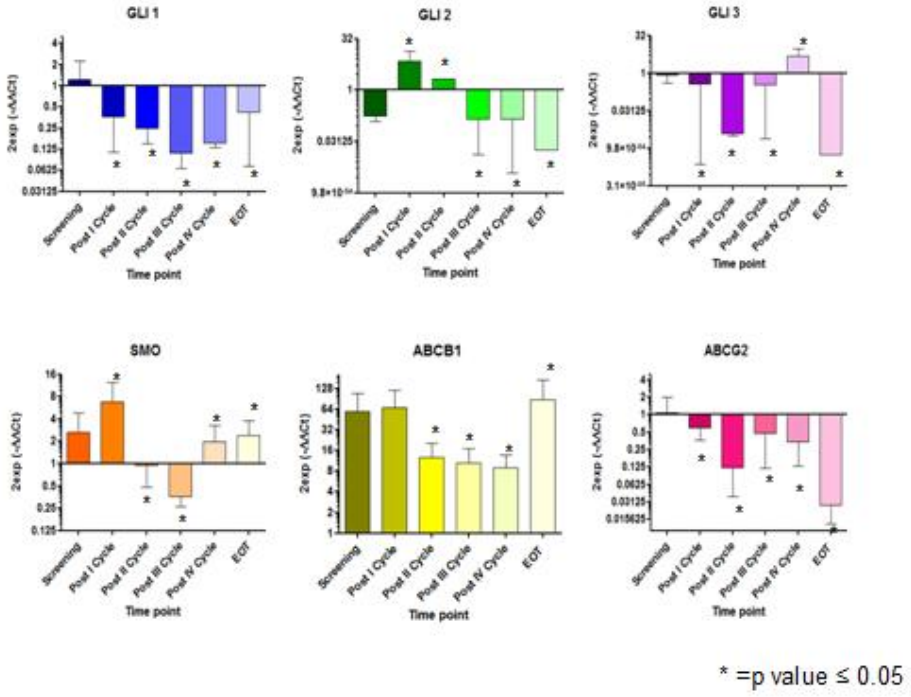



Figure 20: real time PCR analisys.

Activation of the Hh pathway is both sufficient and essential for resistance to classical chemotherapy in leukemia cells. Hh activation increases chemotherapeutic resistance by regulation intracellular drug uptake in an ABC transporter-dependent manner. The discovery that Hh signaling reduced expression levels of two ABC trasporters, Abcg2 and Abcb1, in leukemic stem cell-enriches $CD34^{+}$ fraction could provide potentially a novel therapeutic strategy because inhibiting Hh signaling may not only affect tumor proliferation but may also increase chemotherapeutic effect, and result in improved treatment response.

## 4.2 OVER-EXPRESSION OF HEDGEHOG PATHWAY GENES IN PRIMARY ACUTE MYELOID AND LYMPHOBLASTIC CELLS

In order to determine whether in primary acute myeloid and lymphoblastic cells, obtained from patients at diagnosis, there was a de-regulation in the components of Hedgehog pathway, has been evaluated, by real-time PCR, the Gli1, Gli2, Gli3, Smo mRNA expression, and only in primary acute myeloid cells also Abcg2, Abcb1 mRNA expression.

The expression results were stratified according to a cut-off generated by the median value of primary cells obtained from healthy donors in order to identify specific patterns related to leukemia.

The results, on primary acute lymphoblastic cells, show Gli1 mRNA over-expression (values higher than the cut-off of 0.3636) in 51% of cases, Gli2 mRNA over-expression (values higher than the cut-off of 0.0094) in 80, 4% of cases, Gli3 mRNA over-expression (values higher than the cut-off of 0.0365) in 59, 6% of cases and finally Smo mRNA over-expression (values higher than the cut-off of 0.0112 ) in 87, 2% of cases (Figure 26).

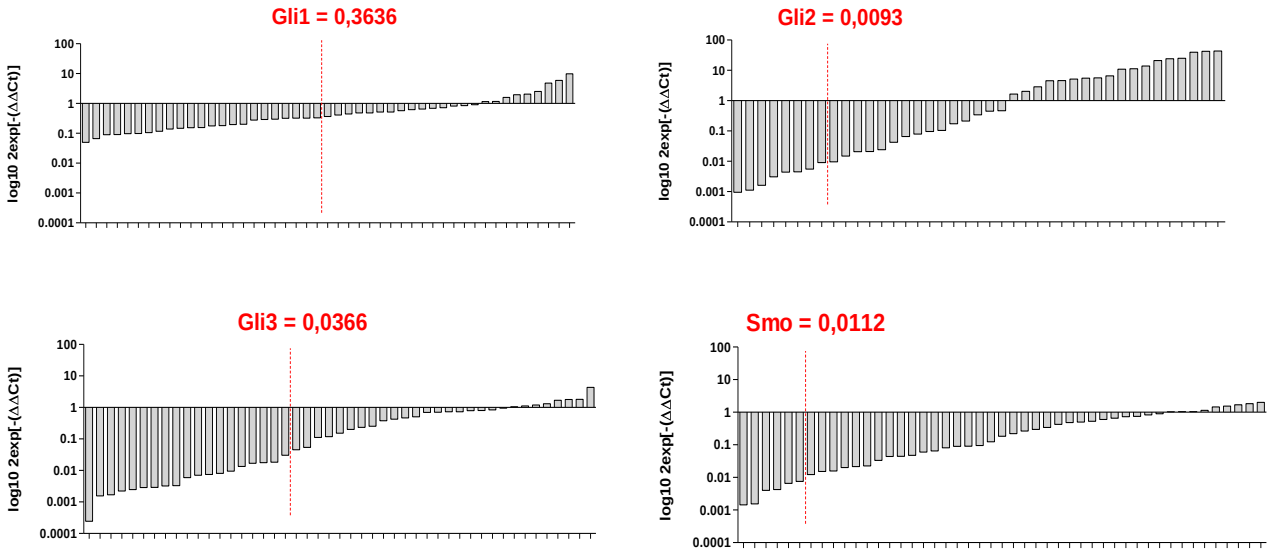



Fig. 26**:** real-time PCR results, on primary acute lymphoblastic cells, stratified according to the cut-off generated by the median value of primary cells obtained from 10 healthy donors. Gli1, Gli3 and Smo mRNA expression were analyzed in 47 patients at diagnosis, Gli2 mRNA expression was analyzed in 41 patients at diagnosis.

The results, on primary acute myeloid cells, show Gli1 mRNA over-expression (values higher than the cut-off of 0.093) in 58.3% of cases, Gli2 mRNA down-regulation (values lower than the cut-off of 0.364) in 100% of cases, Gli3 mRNA over-expression (values higher than the cut-off of 0.0019) in 100% of cases, Smo mRNA over-expression (values higher than the cut-off of 0.0089) in 100% of cases, Abcg2 mRNA over-expression (values higher than the cut-off of 0.151) in 90.0% of cases, and finally Abcb1 mRNA over-expression (values higher than the cut-off of 0.764) in 80% of cases (Figure 27).

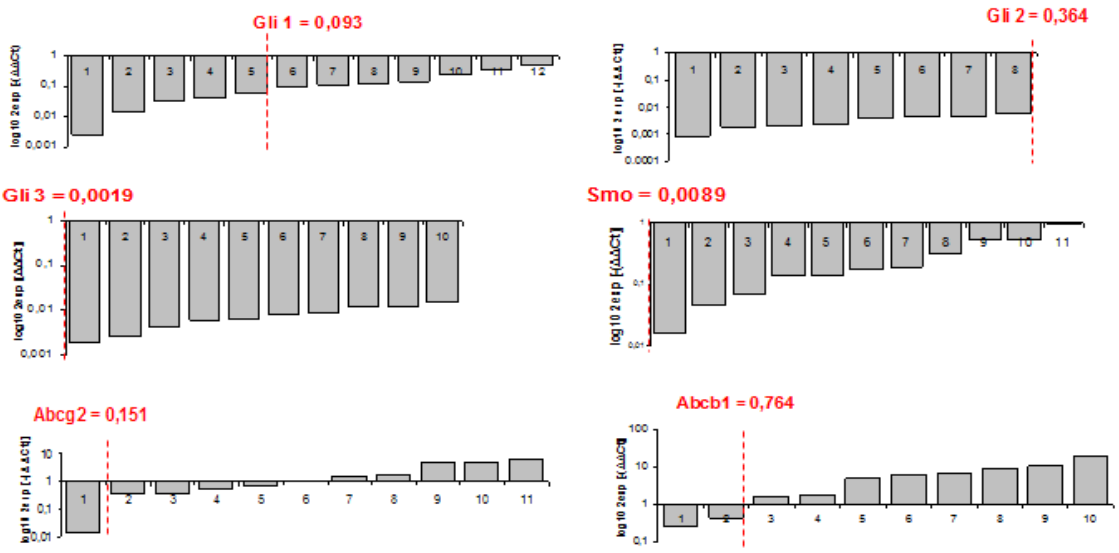



Fig. 27: real-time PCR results, on primary acute myeloid cells, stratified according to the cut-off generated by the median value of primary cells obtained from 10 healthy donors. Gli1 mRNA expression was analyzed in 12 patients at diagnosis, Gli2 mRNA expression was analyzed in 8 patients at diagnosis, Gli3 and Abcb1 mRNA expression were analyzed in 12 patients at diagnosis, Smo and Abcg2 mRNA expression were analyzed in 11 patients at diagnosis.

Therefore, this gene expression analysis showed over-expression statistically significant of Hedgehog target genes, both in the myeloid and in lymphoblastic leukemia compared to healthy samples.

## 4.3 'IN VITRO' SMO INHIBITOR TREATMENT

To investigate 'in vitro' SMOi activity, we treated BV-173, SUP-B15, K562 ($Ph^+$ cell lines) and HL60, KG1, MOLM13 (AML cell lines).

### 4.3.1 'IN VITRO' SMO INHIBITOR HAS NO EFFICACY ON VIABILITY AND DOES NOT INDUCE APOPTOSIS

To evaluate the effect of SMOi, as single agent, on cell viability, we performed Cell Proliferation Reagent WST-1 and Annexin V/Propidium Iodide staining analysis. BV-173, SUP-B15, K562 ($Ph^+$ cell lines) and HL60, KG1, MOLM13 (AML cell lines) were incubated

with increasing concentration of SMOi (0.1-10 µM) for 24, 48 and 72 hours. To test the efficacy on apoptosis we treated cell lines with 10 µM of SMOi for 48 hours. (Figure 28, 29). Primary cells obtained from 3 healthy donors were incubated with increasing concentration of SMOi (1-10 µM) for 24 hours (Figure 30), to evaluate the SMOi efficacy on cell viability of this cells.

In Table 10 summarized the percentages of Annexin V/Propidium Iodide staining analysis on Ph+ and AML cells lines.

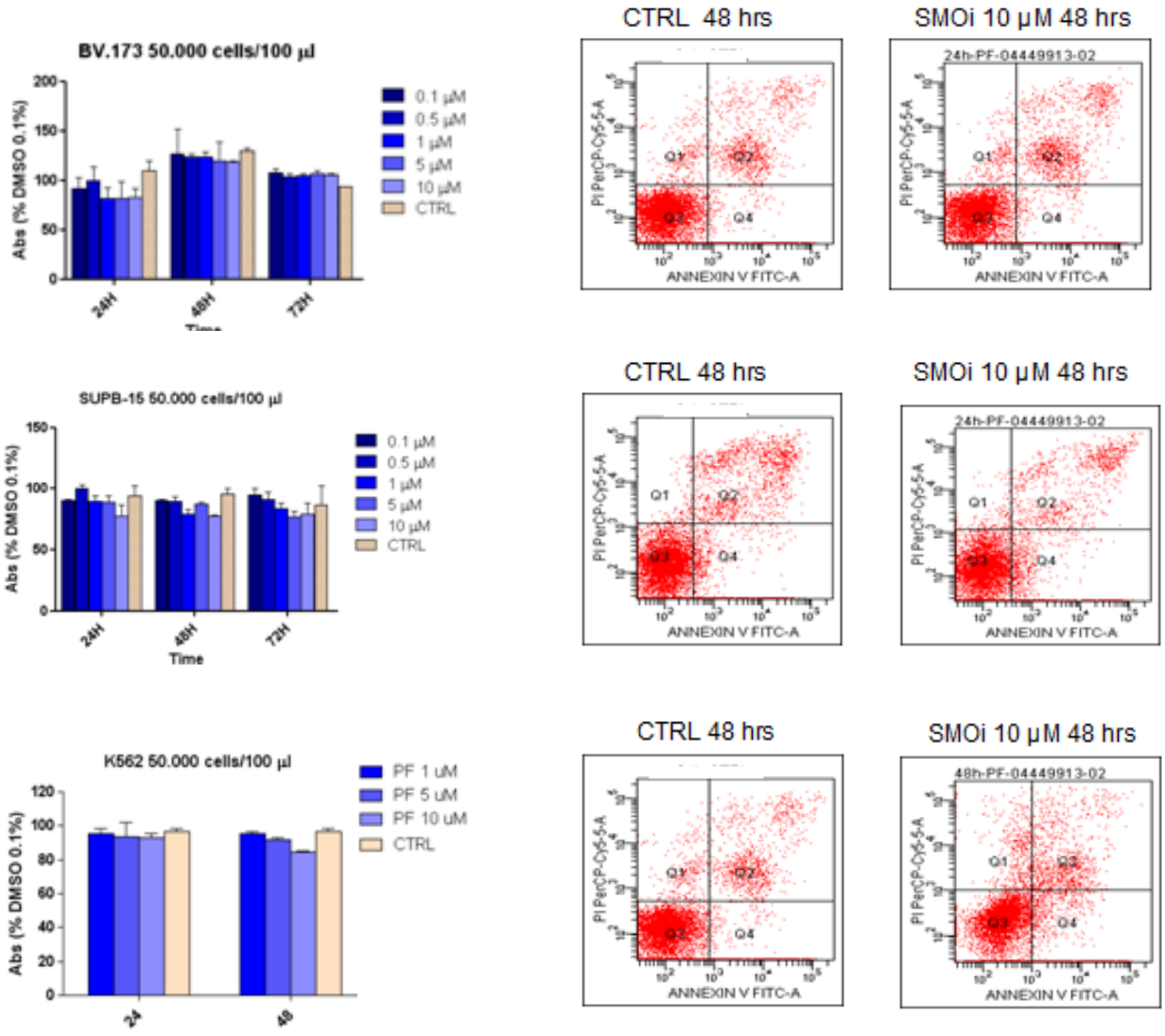



Fig. 28: Cell Proliferation Reagent WST-1 results (on the left). Annexin V/Propidium Iodide staining analysis results.(on the right). DMSO at 0.1% as a control.

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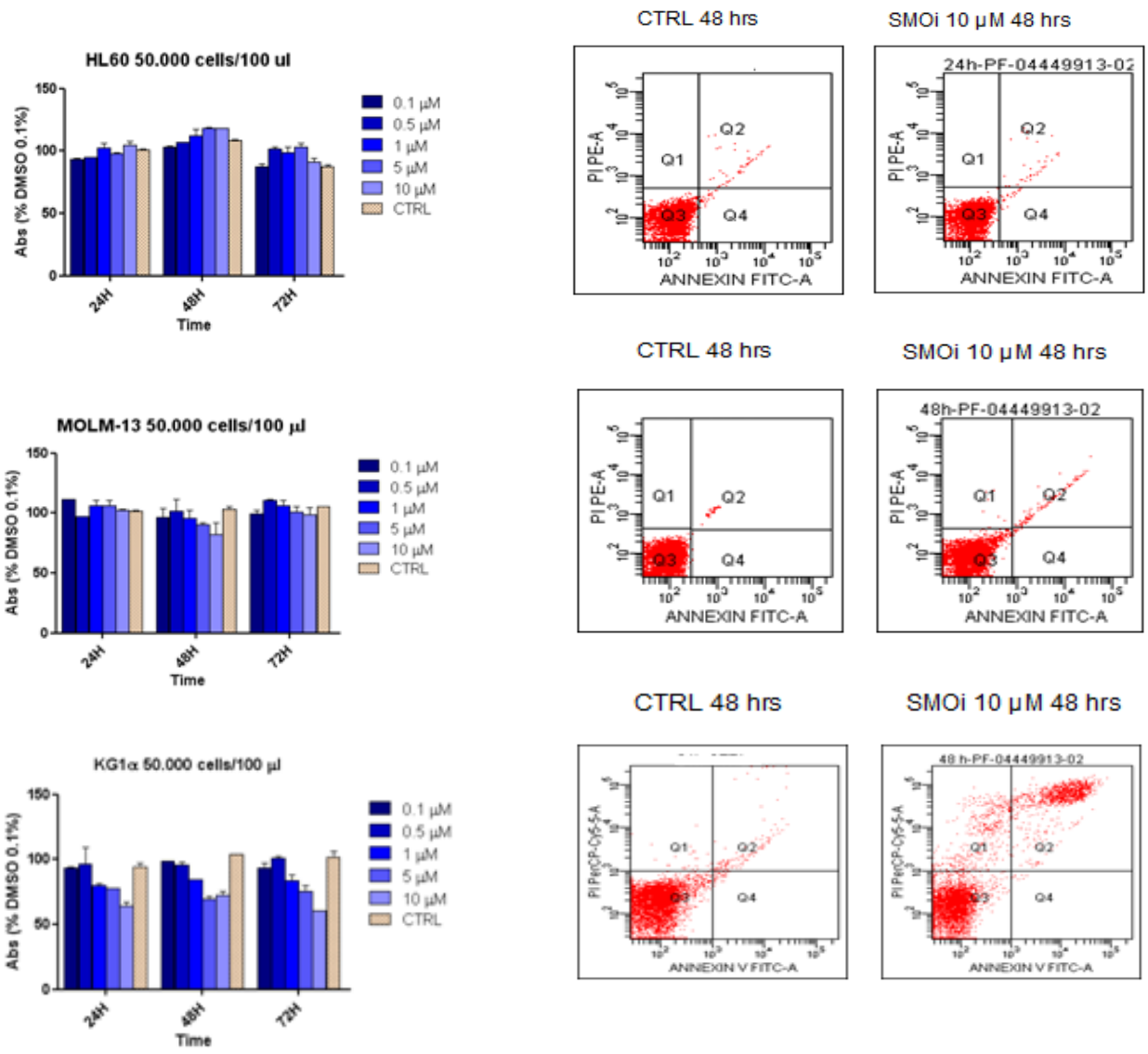


Fig. 29: Cell Proliferation Reagent WST-1 results (on the left). Annexin V/Propidium Iodide staining analysis results.(on the right). DMSO at 0.1% as a control.

SMOi 10 µM 48 hrs

| | LIVING CELL (Q3) | APOPTOTIC CELLS (Q2+Q4) | NECROTIC CELL (Q1) |
|---|---|---|---|
| KG1 | 61,2 | 32,8 | 5,8 |
| HL60 | 94,5 | 5,4 | 0 |
| MOLM13 | 94,5 | 4,1 | 1,4 |
| BV173 | 89,8 | 10 | 0,3 |
| SUPB15 | 82,5 | 13,7 | 3,7 |
| K562 | 65,1 | 22,5 | 12,4 |

Table 10: percentages of Annexin V/Propidium Iodide staining analysis.

SMOi had no effect on the dose and time-dependent cell viability. Consistent with the WST-1 results, Annexin V/Propidium Iodide staining analysis did not show a significant increase of apoptosis at 48 hours in Ph+, HL60 and MOLM13 cell lines.
Instead, WST-1 and Annexin V/Propidium Iodide staining analysis showed a significant increase of apoptosis at 48 hours in KG1 cell line.

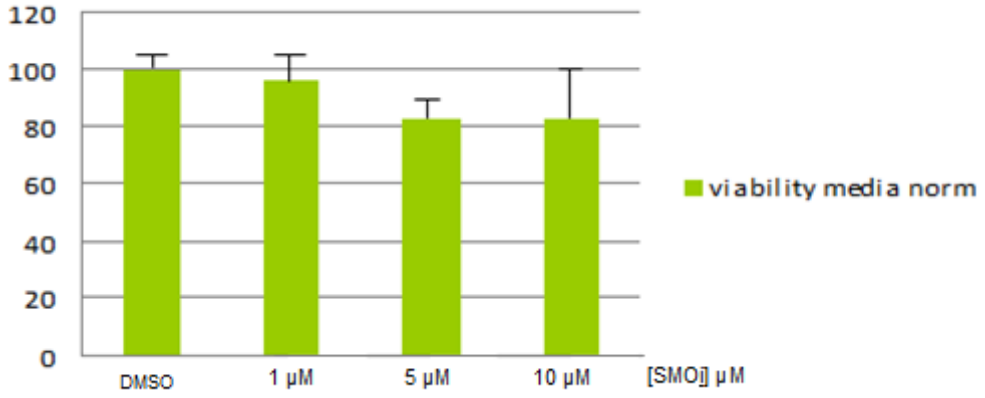


Fig. 30: Cell Proliferation Reagent WST-1 on 3 healthy donors bone marrow. DMSO at 0.1% as a control.

SMOi had no effect on the dose and time-dependent cell viability of primary cells from healthy donors bone marrow.

### 4.3.2 'IN VITRO' SMO INHIBITOR SPECIFICALLY TARGETS THE HEDGEHOG PATHWAY AND MODULATES THE EXPRESSION OF TWO ABC TRANSPORTERS

We examined 'in vitro' Gli1, Gli2, Gli3, Smo, Abcg2 and Abcb1 mRNA expression levels in two AML cell lines (HL60, MOLM13) by real-time PCR, after 48 hours SMOi treatment (we only show HL60 cell line, Figure 30).

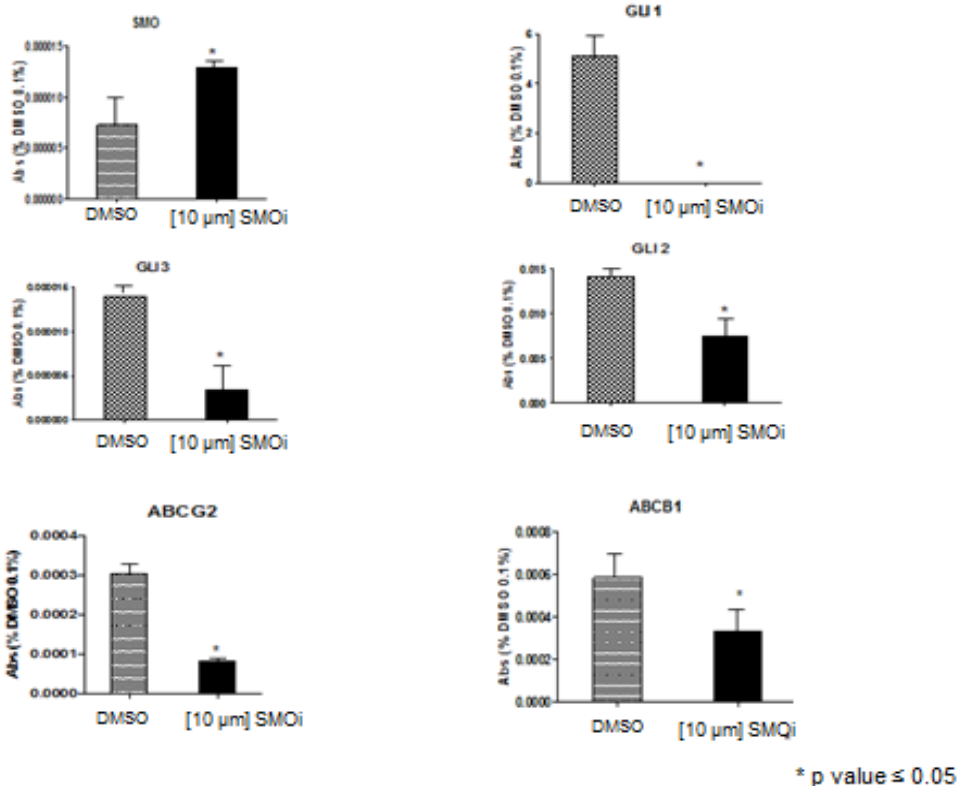


Fig. 30: real-time PCR for Smo, Gli1, Gli3, Gli2, Abcg2, Abcb1 mRNA expression in HL60 cell line, after SMOi treatment. DMSO at 0.1% as a control.

We observed a statistically significant reduction in Gli1, Gli2, Gli3 transcript levels, and a statistically significant increase in Smo transcript level. Furtheremore, we have seen a statistically significant reduction in Abcg2 and Abcb1 transcript levels. Therefore, 'in vitro' SMOi treatment specifically targets Hedgehog pathway and modulates the expression of two ABC transporters.

The gene expression data obtained 'ex vivo' showed up-regulation of two Hh pathway-related genes, Gas1 and Kif27. We analysed in 'vitro', Gas1 and Kif27 mRNA expression levels in AML cell lines (HL60, MOLM13, KG1) and Ph+ cell lines (BV-173, K562) by real-time PCR, after 48 hours SMOi treatment (Figure 31).

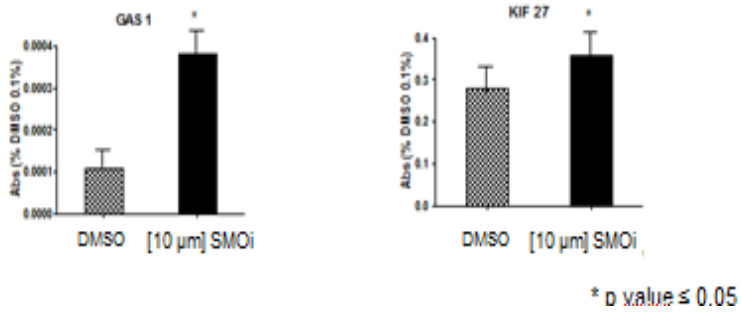


Fig. 31: real-time PCR for Gas1 and Kif27 mRNA expression in HL60 cell line , after SMOi treatment. DMSO at 0.1% as a control.

We observed a statistically significant increase in Gas1 and Kif27 transcript levels, thus confirming 'ex vivo' the data.

The expression data for Gli1, Gli2, Gli3, Smo, Abcg2 genes was also confirmed by western blotting analysis in $Ph^+$ cell lines (BV-172, K562) and in AML cell lines (HL60, MOLM13, KG1) (Figure 32).

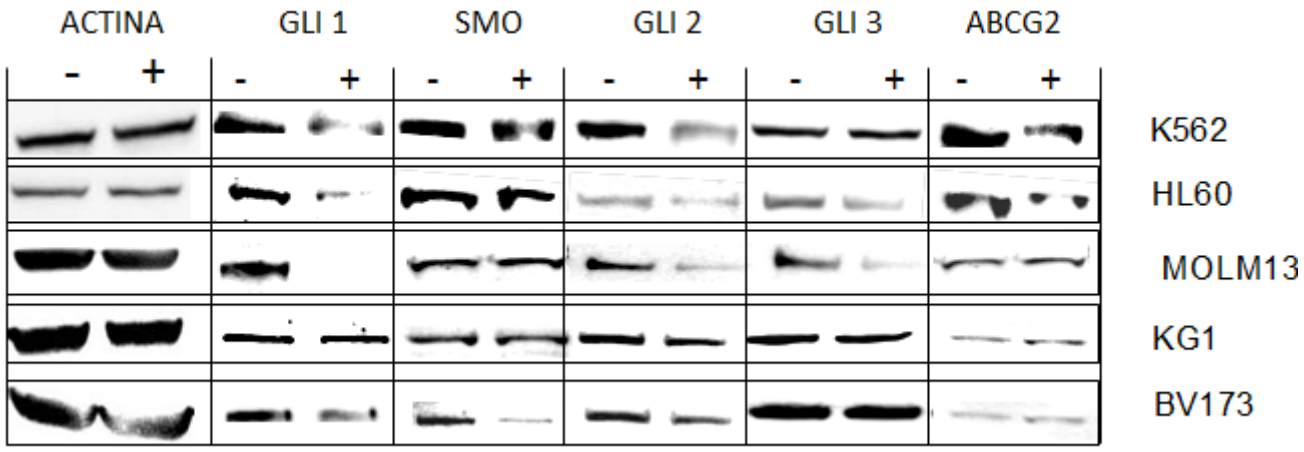


Fig. 32: western blot analysis for Gli1, Smo, Gli2, Gli3, Abcg2 protein expression in $Ph^+$ and AML cell lines, after SMOi treatment. β-actin as housekeeping, or internal control.

HL60, MOLM13, K562 and BV-173 cell lines are more sensitive to inhibition of the hedgehog pathway after 48 hours SMOi treatment, conversely, cell line KG1 that seems to be more resistant.

### 4.3.3 'IN VITRO' SMO INHIBITOR REVERTS K562 CHEMORESISTANCE TO TKIs

'Ex vivo' and '*in vitro*' data suggest that Hedgehog signaling modulate the expression of ABC-transporter family members, such as Abcb1, Abcg2 and Abcb1, whose expression has been correlated with clinical chemoresistance [136-137], this discovery could provide potentially a novel therapeutic strategy because inhibiting Hh signaling may not only affect self-renewal of leukemic stem cells but may also increase chemotherapeutic effect, and result in improved treatment responses [138].

Based on these results, we hypothesize that SMOi treatment in combination with conventional chemoterapy could be reverts the chemoresistance mechanism in K562 TKI-resistant cell line.

To this aim we treated, for 24 and 48 hours, K562 TKI-resistant cell line with SMOi, Imatinib, Dasatinib, Nilotinib used as single agent, and we tested also SMOi in combination with TKIs (Figure 33).

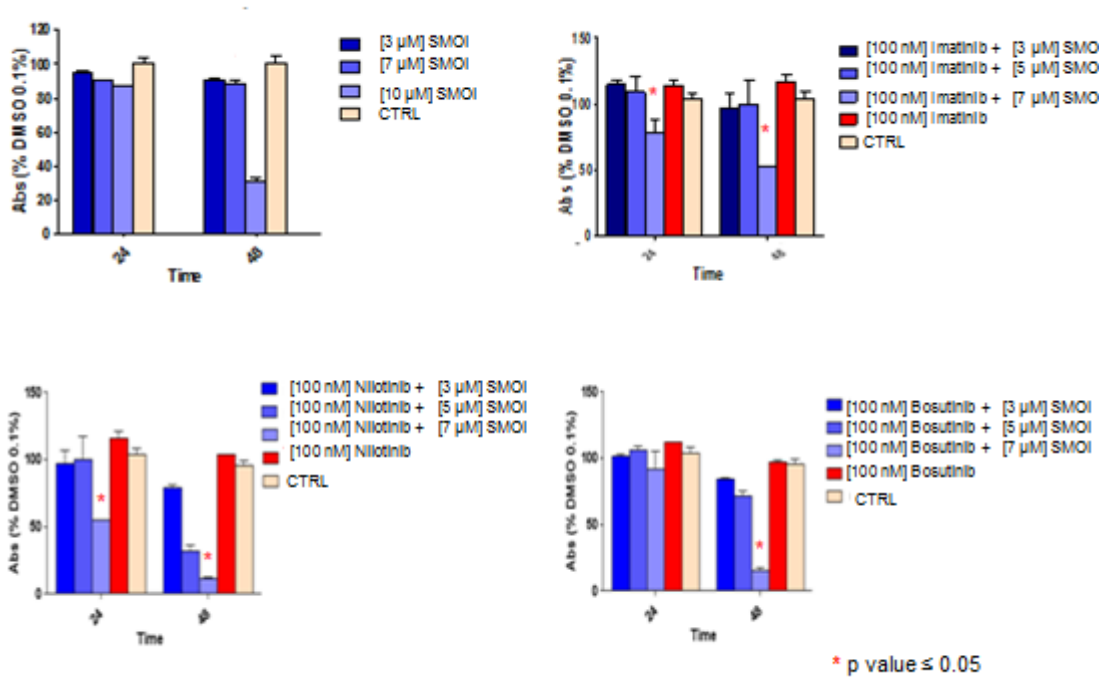


Fig. 33: Cell Proliferation Reagent WST-1 results on K562 TK-resistant cell line. Red histogram= TKIs used as single agent. DMSO at 0.1% as a control.

We observed that SMOi [7 µM] and TKIs [100 nM] used as single agent for 24 and 48 hours, had not efficacy on viability, instead SMOi [7 µM] treatment in combination with TKIs

[100 nM] showed a statistically significant decrease on viability, suggesting a synergic or additive effect between two drugs used in combination.

# 4. DISCUSSION

The Hedgehog pathway is a critical mediator of embryonic patterning and organ development, including hematopoiesis. In adult organisms, Hedgehog pathway activity is required for the tissue maintenance and regeneration. Over the past decade, the Hedgehog signaling pathway has attracted considerable interest because the pathway plays important roles in the tumorigenesis of several types of cancer as well as developmental processes. It has also been observed that Hedgehog signaling regulates the proliferation and self-renewal of cancer stem cells and may promote tumor repopulation after chemotherapy and contribute to chemotherapy resistance in cancers.

Smo, a key player of that signaling, is the most suitable target to inhibit this pathway. To this aim, several molecules antagonists of Smo have been synthesized and, some of them, have started the phase I in clinical trials. The first Smo inhibitors (SMOi), cyclopamine and jervine, were isolated from corn lilies as compounds causing teratogenic effects (including cyclopia) in lambs whose mothers had ingested this plant [143] and were subsequently shown to inhibit the Hh pathway [144] by binding to SMO [145], the GPCR-like structure of which makes it an ideal drug target [146]. The cyclopamine has poor oral bioavailability and suboptimal pharmacokinetics [50] for this reason, more potent and/or acid-soluble derivatives of it have been synthesized [147-149]. Among the inhibitors, several Smo antagonists have started the phase I in clinical trials. Our hospital participated to one of these studies which investigated the oral administration of a new selective inhibitor of Smo (SMOi).

In order to evaluate '*ex vivo'* SMOi efficacy and to identify new potential clinical biomarkers of responsiveness, we separated, before and after 28 days start of treatment, highly purified (98%) bone marrow $CD34^+$ cells from 5 acute myeloid leukemia (AML), 1 myelofibrosis (MF), 2 blastic phases chronic myeloid leukemia (CML) patients treated with SMOi by immunomagnetic separation and we analysed their gene expression profile using Affimetrix HG-U133 Plus 2.0 platform.

This analysis showed differential expression after 28 days start of therapy (p-value $\leq 0.05$) of 1,197 genes in CML patients and 589 genes in AML patients . This differential expression is related to Hedgehog pathway with a p-value = 0.003 in CML patients and with a p-value = 0.0002 in AML patients**,** suggesting that SMOi targets specifically this pathway.

Among the genes differentially expressed we observed strong up-regulation of Gas1 and Kif27 genes, which may work as biomarkers of responsiveness of SMOi treatment in CML $CD34^+$ cells.

Instead, among the genes differentially expressed and related to Hh pathway in AML patients, there are Casein kinase I, Gli3 and β-catenin genes, which were down-regulated after SMOi

treatment. Casein kinase I and Gli3 genes are both players of the Hedgehog pathway. β-catenin protein is a subunit of the cadherin protein complex, which is important in the Wnt pathway\β-catenin, associated with cell survival and the self-renewal, suggesting a connection between the Hedgehog and Wnt pathway. Therefore, 'ex vivo' SMOi therapy specifically targets the Hedgehog pathway in leukemic stem cell-enriched $CD34^+$ fraction obtained from CML and AML patients.

Activation of Hh pathway is both sufficient and essential for resistance to classical chemotherapy in leukemia cells. The mechanism of action of Hh inhibitors is probably dependent on its effect on P-gp expression levels, belong to the ABC transporters superfamily proteins[135].

Recent studies show that Hh signaling modulates the expression of two ABC transporters, multi-drug resistance protein 1 (MDR1; p-Glycoprotein, ABCB1) and breast cancer resistance protein (BCRP; ABCG2), whose expression has been correlated with clinical chemoresistance [136-137]. Hh activation increases chemotherapeutic resistance by regulating intracellular drug uptake in an ABC transporter-dependent manner.

Moreover it has been demonstrated that Hh signaling promotes up-regulation of Bcl-2 which is relevant in response to Hh signalling. The expression of Bcl-2 was proven to be up-regulated via direct binding of Hh-activated transcription factor GLI1 or GLI2 in solid cancer cells

Comparing the gene expression data obtained on $CD34^+$ cells separated from CML and AML patients after 28 days start of therapy versus the gene expression data obtained on $CD34^+$ cells harvested at the beginning of treatment, we observed a statistically significant down-regulation of Bcl2 and Abca2 genes. In conclusion, Hh signaling could be involved in the survival and drug resistance of $CD34^+$ leukemic cells. Inhibition of Hh signaling can be a therapeutic option to directly induce apoptosis and reduce drug resistance of leukemia cells.

We confirmed, by real-time PCR, that 'ex vivo' SMOi treatment led to statistically significant reduction of Hedgehog target genes (such as Gli1, Gli2 and Gli3) and to Abcg2, Abca2 mRNA down-regulation.

In order to highlight a gene expression alteration of the Hedgehog target genes in ALL and AML patients at diagnosis compared to healthy donors, we performed real-time PCR. The data obtained showed that, despite a large heterogeneity between the levels of expression of the genes analyzed, there is strongly down-regulation of hedgehog target genes, both in AML and ALL primary cells; and a significant up-regulation of Abcg2 and Abcb1 genes in AML primary cells. This results again confirms the importance to target this pathway to eradicate

leukemic stem cells, since the drugs currently in clinical use are able to effectively counteract the leukemic progenitors, but are not able to eradicate leukemic stem cells.

To investigate SMOi activity '*in vitro*', we treated $Ph^+$ (BV-173, SUP-B15, K562) and AML (HL60, KG1, MOLM13) cell lines and we showed that SMOi has no efficacy on viability and not induces apoptosis in all cell lines analyzed. We only showed a significant increase of apoptosis at 48 hours for KG1 cell line. We also observed that SMOi had no effect on the dose and time-dependent cell viability of primary cells from healthy donors bone marrow.
'Ex vivo' data, which showed that SMOi specifically targets Hedgehog pathway, down-regulates important ABC transporters (Abca2, Abcg1, Abcb1) and up-regulates strongly two potential biomarkers of response to therapy (GAS1 and Kif27), have been confirmed with '*in vitro*' experiments on AML cell lines (HL60 and MOLM13) treated with SMOi 10 µM for 48 hours.
The inhibition of Hedgehog pathway and the down-regulation of its target genes by SMOi treatment, was also demonstrated analyzing protein expression levels by Western blot assay on all cells lines taken as model in our studies. HL60, MOLM13, K562 and BV-173 cell lines are more sensitive to inhibition of the Hedgehog pathway after 48 hours SMOi treatment, conversely, KG1 cell line seems to be more resistant.
Finally, we hypothesize that SMOi treatment in combination with conventional chemoterapy could reverted the chemoresistance mechanism in K562 TKI-resistant cell lines. To this aim we treated, for 24 and 48 hours, K562 TKI-resistant cell lines with SMOi, Imatinib, Dasatinib, Nilotinib, used as single agent; and we tested also SMOi in combination with TKIs. The combination with TKIs showed a statistically significant decrease on viability, suggesting a synergic or additive effect.
Considering all the results discussed above, an innovative therapeutic approach should be suggested: the use of TKIs, highly effective to reducing the leukemic progenitors, in combination with SMO inhibitors molecule, able to counter the self-renewal stem cell, would be an innovative therapeutic approach in haematological malignancies, maintaining long-term remission, preventing possible relapses and, although ambitious, leading to a cure.

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