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PRE-CLINICAL AND CLINICAL DEVELOPMENT OF LEUKEMIA STEM CELL INHIBITORS IN ACUTE LEUKEMIAS

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1.INTRODUCTION

1.1 Hematopoietic stem cells (HSC)

In humans, the hematopoietic system is a highly structured hierarchy which originates from hematopoietic stem cells (HSC). HSCs are characterized by the peculiar and unique ability to self-renew and differentiate into mature and functional blood cells of many different lineages. Moreover, they are capable of reconstituting the hematopoietic system in recipients after myeloablative irradiation [1]. From a numerical point of view, HSC is a rare population, accounting for 1 in 10000 to 1000000 cells in BM. They are physiologically quiescent but upon stimulation, they can enter into cell cycle and proliferate. These processes are strongly and deeply regulated, resulting, whenever abnormal regulation of HSC cell-fate occurs, in leukemia and other myeloproliferative disorders.

The studies of molecular marker expression performed by flow cytometry analysis have led the identification of each blood cell subpopulations in terms of their biology and potential when combined with other functional assays. As a result, schematic demonstration of hematopoietic hierarchy has been proposed (Figure 1). The origin of all blood cell in hematopoietic system is thought to be derived from HSCs that contain self-renewal capacity and give rise to multipotent progenitors (MPPs) which lose self-renewal potential but remain fully differentiate into all multilineages. MPPs further give rise to oligopotent progenitors which are represented by common lymphoid and myeloid progenitors (CLPs and CMPs, resp.). All these oligopotent progenitors therefore differentiate into their restricted lineage commitment: (1) CMPs advance to megakaryocyte/erythrocyte progenitors (MEPs), granulocyte/macrophage progenitors (GMPs), and dendritic cell (DC) progenitors, (2) CLPs give rise to T cell progenitors, B cell progenitors, NK cell progenitors and DC progenitors.

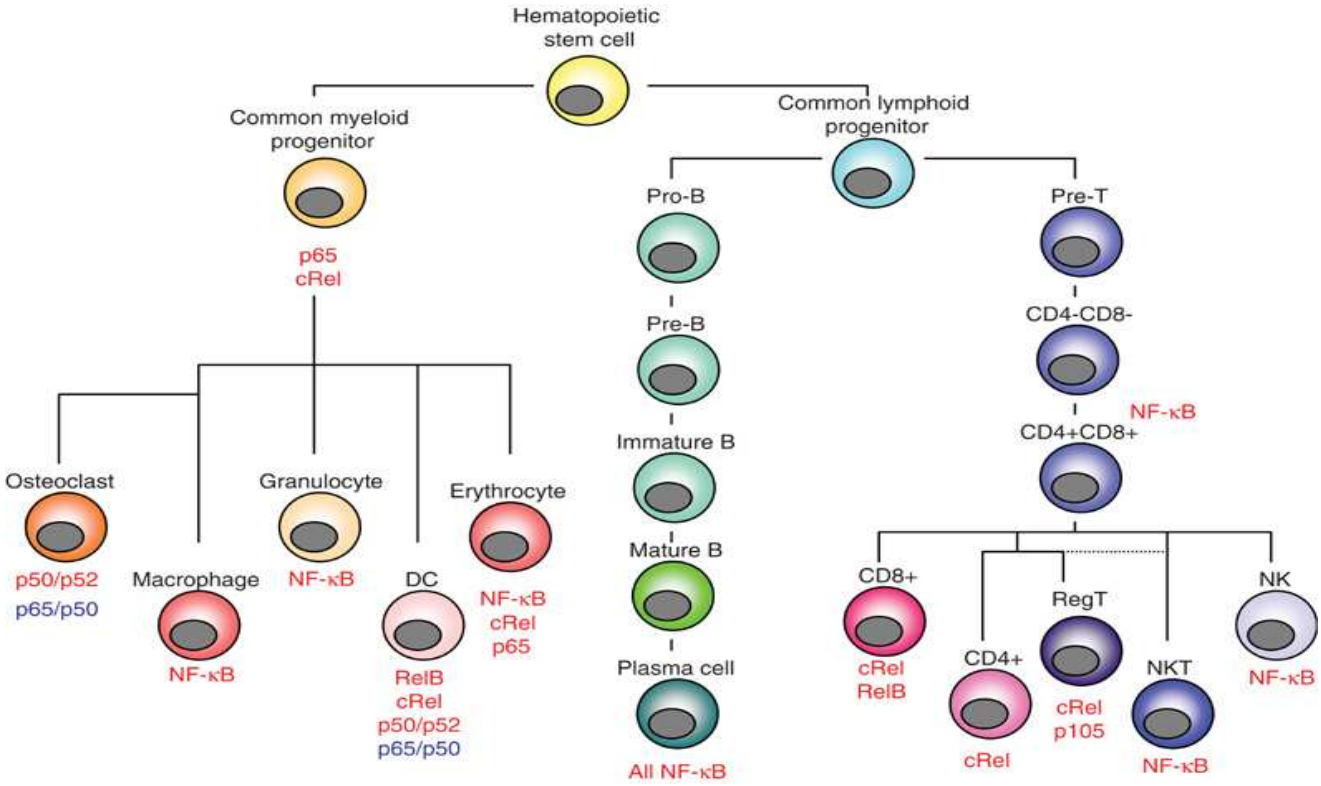

Figure 1: Hierarchy of hematopoiesis. The phenotypic cell surface marker of each population of human blood system is shown.

1.2 Cancer Stem Cells

Treatment of cancer still represents an ambitious challenge, since, both in solid and in haematological setting, the pathogenesis involves dysregulation of endogenous and essential cellular processes [2]. These alterations determine the difficulty, shared worldwide by scientists and physicians, to identify the potential targets to be hit in order to eradicate many neoplastic diseases.

According to the most recent literature, the development and maintenance of cancer can be explained by two different models. One model is the so called

"stochastic model", in which all cancer cells are able to form and maintain the tumor mass (Figure 2a). The second model, the so called "hierarchical model", identifies a small group of cells susceptible for transformation, which forms and maintains the tumor (Figure 2b). The hierarchical model is also called the "cancer stem cell" (CSC) model because the group of cells responsible for this maintenance of the tumor has stem cell like characteristics [3, 4, 5]

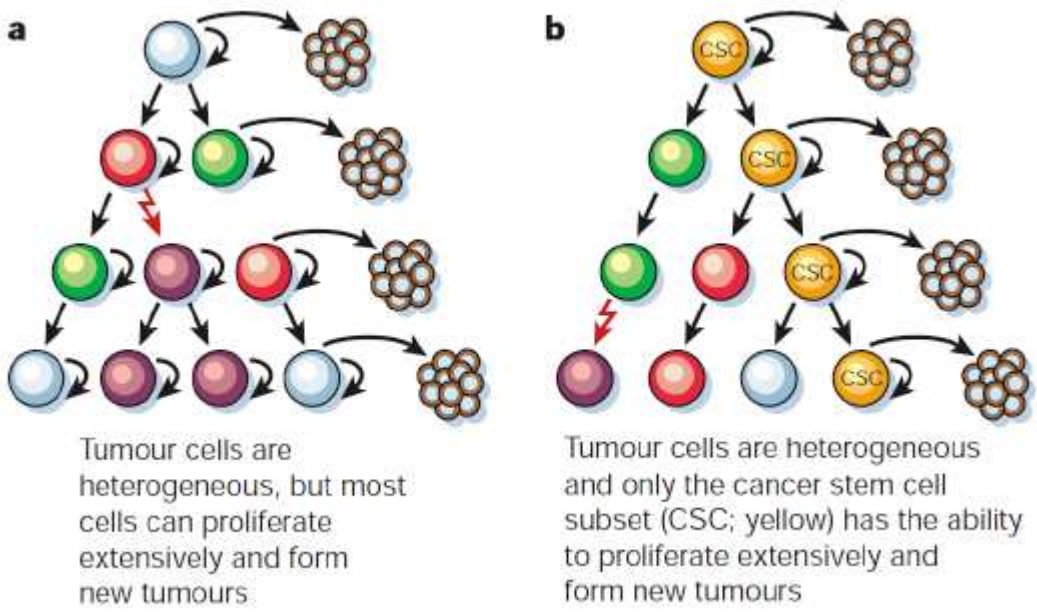

Figure 2 Two general model of heterogeneity in cancer cells.
a) "Stochastic model": cancer cells of many different phenotypes have the potential to proliferate extensively, but any one cell would have a low probability of exhibiting this potential in an assay of clonogenicity or tumorigenicity. b) "Hierarchical model": most cancer cells have only limited proliferative potential, but a subset of cancer cells consistently proliferate extensively in clonogenic assays and can form new tumors upon transplantation. This model predicts that a distinct subset of cells is enriched for the ability to form new tumors, whereas most cells are depleted of this ability. Existing therapeutic approaches, both in leukemia and in solid tumors, have been based largely on the first described model, but the failure of these treatments to eradicate most solid cancers suggests that the second mode may be more accurate[6]

More and more increasing evidences have been found, supporting the CSC hypothesis, and many features suggest an overlap between SCs and CSCs [6].

In details, normal SCs have the ability to proliferate life-long, are immortal and are mostly resistant to drugs by multiple mechanisms. SCs can divide asymmetrically and produce two cells: a daughter SC and a progenitor cell that can differentiate into different lineages but cannot self-renew. SCs have specific markers and are able to differentiate into certain tissues and cells due to the microenvironment and other factors.

CSCs are quite similar to these criteria. CSCs have the ability to proliferate and the peculiar property to self-renew, and are heterogeneous. The CSC develops along the differentiation path similar as normal SCs and finally the tumor includes tumor initiating cells (CSCs) and an abundant amount of non-tumor initiating cells. CSCs express specific markers, often also found on SCs and importantly CSCs are often more resistant to drugs then the bulk of the tumor [7, 8].

The main evidences for the existence of CSC emerge from *in vitro* and *in vivo* experiments. Bonnet *et al., i*n the mid-1990s, identified a stem cell-like population from a human acute myeloid leukemia (AML), proving that cells with the CD34+/CD38- phenotype are the cells that are able to proliferate and initiate AML. This population of cells represent 0.2% of the human leukemia population[5]. The isolated cells, CD34+CD38-, had a similar cell-surface phenotype to normal SCID-repopulating cells. They showed that these stem-like cells were capable of initiating human AML in NOD/SCID mice. In addition, the data they collected suggested that normal primitive cells, rather than committed progenitor cells, are targets for leukemic transformation. These cells homed to the bone marrow and proliferated extensively in response to *in vivo* cytokine treatment, resulting in a pattern of dissemination and leukemic cell morphology similar to that seen in the original patients [9].

Therefore, Zou postulated the definition of the CSC as follows:

"Cancer stem cells can be defined as the specific cell population inside cancer which has the capacity for self-renewal, the potential to develop into any cells in the overall tumor population, and the proliferative ability to drive continued expansion of the population of malignant cells."[10].

It is not known whether CSCs really arise from SCs however it is possible that deregulation of the normal SC gives the development of cancer[9]. Tumorigenesis process begins either with transformation of a multipotent SC which leads to uncontrolled self-renewal or transformation of a more downstream progenitor cell leading to acquired self-renewal of a cell that did not have self-renewal capacity [6,11]

In 1988, Pierce and Speers defined the CSC concept as follows:

"A concept of neoplasms, based upon developmental and oncological principles, states that carcinomas are caricatures of tissue renewal, in that they are composed of a mixture of malignant stem cells, which have a marked capacity for proliferation and a limited capacity for differentiation under normal homeostatic conditions, and of the differentiated, possibly benign, progeny of these malignant cells." [12]

Recently, and to summarize, Weissman and colleagues have proposed that a candidate CSC population should exhibit the following properties:

1) The unique ability to engraft;

2) The ability to recapitulate the tumor of origin both morphologically and immunophenotypically in xenografts;

3) The ability to be serially transplanted[13].

1.3 Leukemic Stem Cells (LSCs) in AML

AML represents a group of clinically, cytogenetically and molecularly heterogeneous diseases, affecting mostly adult people worldwide, with a mild prevalence in male sex and an estimated incidence of 3,7 cases out of 100,000 persons. The pathogenesis of this rare disease involves a block of differentiation at the myeloid lineage, which results into growth of abnormal white blood cells called blasts, which disrupt the growth of normal blood cells, white and red blood cells. This leads to a default in the immune system resulting in infections, bleeding or organ infiltration and a defect in the formation of red blood cells resulting in anaemia. AML patients outcome are very poor, mainly in elderly subgroups, unfit for aggressive chemotherapeutic approaches and allogeneic stem cell transplantation.

The AML phenotype is extremely various, since the disease develops by sudden genetic and/ or epigenetic events leading to the transformation of the HSC or progenitor. In the case of transformation of the progenitor, LSCs maintain the identity of the progenitor from which they arose, while acquiring SC-like features such as self-renewal [14]. The phenotype of the LSC is much more heterogeneous than expected and can vary even within a single AML sample.

At present, in AML treatment, despite a high initial complete remission rate obtained after induction chemotherapy, many patients relapse afterwards, resulting in a still dismal overall outcome. Several relapse related factors have been so far identified, such as certain cytogenetic and molecular aberrations at diagnosis, lack of early treatment response and high level of minimal residual disease (MRD, small number of leukemic cells that remain in the patient) after treatment. Moreover, high frequencies of LSCs at diagnosis and after treatment have been shown to predict relapse in AML[15]. Indeed, since conventional intensive chemotherapy primarily targets at the proliferative leukemic blasts, it has led to the concept of

chemo-resistant leukemia initiating cells, which can give rise to leukemic blasts and hence sustain the haematological malignancy.

Quiescence of LSCs, also called dormancy, is probably the most important reason for treatment failure of AML cells because these cells are not in cycle. As known, most conventional chemotherapy (e.g. anthracyclines, cytarabine) triggers cell death by inducing DNA damage which leads to interference with cell growth and keeping the cells in S-phase resulting in the induction of cell death. Therefore, rapid dividing cells, such as tumor cells, are most affected by chemotherapy. The LSC are non-cycling. Therefore, most chemotherapeutic agents do not trigger cell death in these cells as easy as in rapidly dividing cells. The LSC escapes the induction of cell death, resulting in disease relapse. After chemotherapy treatment in AML, quiescent LSC's often survive because of their dormant properties.

It's still a very debating object the development of agents which could bring normal SCs into cell cycle and awake the quiescent cells. To the best of our knowledge, some Cytokines like Interferon (INF)α and granulocyte colony stimulating factor (G-CSF) are able to activate HSCs. Moreover, the compound arsenic trioxide (As2O3), which is very active in APL, both at diagnosis and at relapse, targets the promyelocytic leukemia (PML) protein resulting in degradation of the protein and the increase of mRNA 15. This results, like INFα and G-CSF, in cells getting into cycle.

The initial stem cell idea of leukemia stem cell was first iterated in 1981, when Fialkow et al first demonstrated the clonal nature of AML by X-linked inactivation gene pattern [16].

Following published studies, showed that only a small percentage of AML blast cells can proliferate and form colonies in *in vitro* methylcellulose culture [17, 18].

Thereafter, Bonnet D and Dick JE, as already mentioned, firstly demonstrated the existence of putative LSC by showing that irrespective of the morphologic subtypes,

only a small fraction of LSCs, bearing the HSC-like phenotype (CD34+CD38-), can recapitulate the leukemia when injected into sublethally irradiated NOD/SCID mice [5]. Based on this discovery, Acute myeloid leukemia (AML) would be initiated and maintained by a small population of cells that have SC-like characteristics (Figure 3). These cells proliferate, divide asymmetrically and are able to self-renew and are called LSCs [19,20]. Due to their persistence despite the administration of high dosage chemotherapy schedules, it is essential to develop new therapeutic strategies to eradicate LSCs.

LSCs are located at the endosteal region of the bone marrow and are mainly non-cycling [21, 22, 23]. Due to this quiescent property, their escape from therapy could be explained. Thus, their release from this quiescence state could represent an opportunity to lead the LSCs into cell death.

As previously described, LSCs and normal HSCs share several features, since they both reside in the AML bone marrow and present similar cell surface markers. The success of anti-LSC therapy would rely on functional manipulation of genes that lead to specific killing of LSCs while saving normal HSCs (Figure 4).

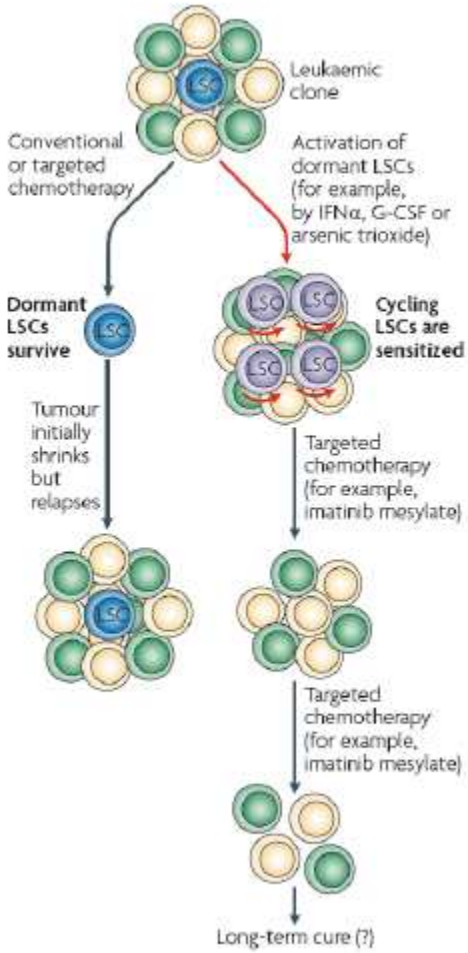

Figure 3 Leukemic stem cells may be targeted by a two-step strategy. Leukemic stem cells (LSCs), which may be a minority within the leukemic clone, show significant resistance to anti proliferative chemotherapy regimes that are thought to be the cause of frequently observed tumor relapse. In analogy to normal dormant hematopoietic stem cells (HSCs), resistance of LSCs may, at least in part, be mediated by a state of deep dormancy. Thus to specifically target dormant LSCs one may postulate a two-step therapy model. First, dormant LSCs would be activated by factors, such as Interferon α (IFNα), granulocyte colony-stimulating factor (G-CSF) or arsenic trioxide (As2O3) and exit from their niche. Once they are cycling, treatment would be continued through administration of targeted chemotherapy, such as Imatinib or cytarabine [19].

At present, the characteristics of LSC have not yet been fully and clearly defined. From the mid 90's, many studies have identified, in AML, potential phenotypic signatures which might concur in the definition of a LSC panel. Among these, Blair and co-authors showed that AML LSCs were highly enriched in the CD34+CD38–CD90–CD117– population [24, 25].

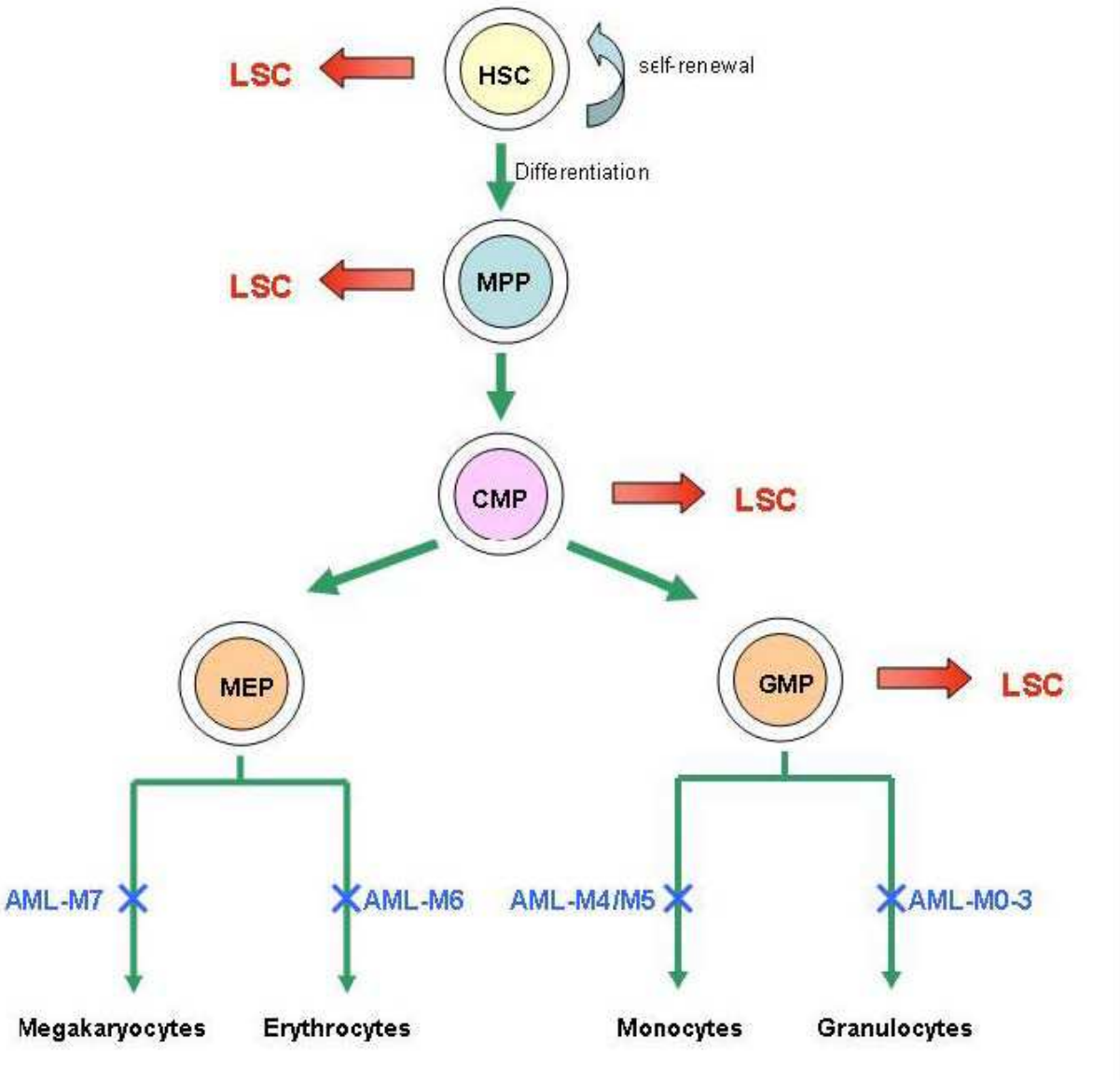

Figure 4 Development of LSC in AML. AML is organized in a hierarchy as normal hematopoiesis. Leukemic transformation into LSC can occur either at the primitive HSC level, multipotent progenitor (MMP), or even at the committed progenitor levels (CMP and GMP). Leukemic blasts of the different subtypes of AML (M0-M7) arise from a block in differentiation along the various hematopoietic lineages.

More recently, LSC have been characterized based on other phenotypic markers, including interleukin-3 (IL-3) receptor CD123 [26], early myeloid antigen CD33[27], the C-type lectin-like molecule-1 (CLL-1)[28], the pan-T cell marker CD96[29] and CD44[30] (Table

1). These data result in the lack of a unique surface phenotype in LSC population, suggesting that additional functional markers may be important in defining LSC.

$CD34^+CD38^-$	• Only $CD34^+CD38^-$ cells from AML samples gave rise to leukemic engraftment, but not $CD34^+CD38^+$ cells (Bonnet and Dick, 1997).
$CD34+CD90^-$	• AML cells that were capable of generating CFU-blast and engrafting NOD/SCID mice were $CD34+CD90^-$ (Blair et al., 1997)
$CD34^+CD71^-$/HLA-DR^-	• Only $CD34^+CD71^-$/HLA-DR^- AML cells were capable of long-term proliferation in vitro and in vivo (Blair et al., 1998).
$CD34^+CD117^-$	• $CD34^+CD117^-$ AML cells capable of long-term proliferation in vitro and NOD/SCID repopulation (Blair and Sutherland, 2000)
$CD34^+CD123^+$	• $CD34^+CD123^+$ leukemic cells were capable of generating leukemia upon transplantation into NOD/SCID (Jordan et al., 2000).
$CD33^+$	• In most AML cases, NOD/SCID repopulating cells were restricted to the $CD33^+$ cell fractions (Taussig et al., 2005)
$CD34^+CLL\text{-}1^+$	• The $CD34^+CLL\text{-}1^+$ population, engrafted in NOD/SCID mice with outgrowth to $CLL\text{-}1^+$ blasts (van Rhenen et al., 2007).
$CD96^+$	• Only $CD96^+$ cells, but not $CD96^-$ cells, engrafted significantly in the bone marrow of NOD/SCID (Hosen et al., 2007).
CD44	• In vivo administration of anti-CD44 antibody to NOD/SCID mice transplanted with human AML reduced leukemic repopulation (Jin et al., 2006).

Table 1 Surface phenotype of LSC

1.3.1 Regulations of LSC in AML

Many studies, performed on transgenic mouse models and exogenous gene manipulatons, have identified a number of genes involved in regulating LSC in Acute Leukemia setting. Among these, the overexpression of HOXA9 in donor mouse BM cells, and resulted in long latency leukemia in recipient after transplantation have been demonstrated[31]. Moreover, deletion of the polycomb group gene PTEN in murine HSC has been showed to led to the rapid development of myeloproliferative disease and eventually leukemia that are transplantable into secondary recipients [32]. Interestingly, human lin- UCB cells that were retrovirally transduced with the mixed-lineage leukemia (MLL)-AF9 fusion might be able to induce both acute lymphoid leukemia (ALL) and AML upon transplantation into immunodeficient mice [33].

Many other relevant studies have been conducted in primary AML samples, in order to identify the altered expression of genes and the involvement of the related signalling pathways in maintaining LSCs.

For instance, CD34+ AML cells have been showed to present at least three activated pathways, if compared with normal HSC:

-Activated NF kappa B activity [34];

-Activated phosphoinositide *3*-kinase (PI3K) [35]

-Constitutive activated Wnt/β-catenin pathway [36]

Recent studies have also shown that, analogous to HSC, LSCs from AML homed to and engrafted in the BM endosteal region upon transplantation into immunodeficient Mice [23]. More importantly, it has been demonstrated by the same authors that chemoresistant leukemic cells were clustered around the putative HSC niches in the BM endosteum and the sinusoidal endothelium[23]. This has highlighted

the important role of the BM microenvironment in regulating LSC. In addition, gene expression analysis have been performed, and data obtained have also revealed changes in the molecular signatures in leukemic stromal cells as compared to the normal counterpart [37], strongly suggesting the involvement of a leukemic 'niche' signal in leukemic transformation as well as LSC maintenance.

In this regard, genetic profiling comparing the BM and PB leukemic cells in AML has been performed [38]. Despite of a highly similar gene expression profile, the study showed that expression of *c-myb, HOXA9, LYN, cystatin C* and *LTC4s* were significantly different between BM and PB samples. Nevertheless, the use of the heterogenous non-purified mononuclear cell (MNC) fraction for comparison has largely limited the interpretation of such data. Hence, definitive evidence is still lacking and that the mechanisms whereby regulatory signals are translated to impact on LSC in the BM milieu are far to be elucidated.

1.4 Leukemia stem cells in Chronic Myeloid Leukemia (CML)

Philadelphia-positive (Ph+) CML is a myeloproliferative disease characterized by granulocytosis and splenomegaly. The disease course is divided into three phases, triphasic, starting with a chronic phase, progressing to an accelerated phase, and ultimately ending in a terminal phase called blast crisis, which presents clinical and features comparable with Acute Leukemia. The Ph chromosome is present in over 90% of CML cases, being the diagnostic molecular hallmark of the disease. After the introduction, 13 years ago, of selective drugs with tyrosine-kinase inhibitory activity on BCR-ABL fusion gene, the prognosis of this haematological malignancies dramatically improved both in young and adult patients. Nevertheless, a definitely cure for CML is still lacking, due to the apparent and demonstrated persistence, even after allogenic stem cell transplantation, of quiescent LSCs [39]. Although BMT is

considered a “curative” therapy for CML, the cure is apparently “relative” due to the existence of residual LSCs.

From a practical point of view, indeed, in the current clinical management, the BCR-ABL kinase inhibitor imatinib and many second generation TKIs have been developed to treat CML and now serves as the frontline therapy for the patients with chronic phase CML. Despite their ability to control CML, these compound do not appear to cure the disease, as LSCs evade treatment, as demonstrated in many reports [40]. Moreover, both *in vitro* and *in vivo* studies showed that CD34+ cells derived from the bone marrow of CML patients could not be effectively killed by imatinib treatment. CD34+ CML stem cells, especially the non-dividing CD34+ cell population, were not sensitive to imatinib-inhibition *in vitro*, and this was further confirmed by the detection of BCR-ABL mRNA transcripts in CD34+ bone marrow cells from CML patients after a long-term treatment with imatinib [41]. The minimal effect of BCR-ABL kinase inhibitor on LSCs was also observed in the CML mouse model [42].

The second generation TKI, dasatinib, is a dual BCR-ABL/SRC kinase inhibitor, whit demonstrated activity in controlling CML. In CML mouse model, mice treated with dasatinib lived significantly longer than those treated with imatinib [42]. These data correlated with significantly lower numbers of BCR-ABL-expressing leukemic cells in peripheral blood compared with placebo- and imatinib-treated mice. However, all dasatinib-treated CML mice eventually died of this disease, meaning that, like imatinib, this drug is not able to completely eradicate LSCs in CML mice. This conclusion on the failure of dasatinib to eradicate LSCs is supported by the observation that quiescent human CD34+CD38- CML cells are resistant to dasatinib treatment [43]. So, neither imatinib nor dasatinib are able to completely eradicate BCR-ABL-expressing HSCs, suggesting that neither drug alone will cure CML and targeting of multiple pathways in LSCs is required to cure the disease.

It is still unclear why imatinib and dasatinib do not completely eradicate LSCs, but several reasons should be excluded, based on the data available. First of all, the drug can access stem cells, as inhibition of intracellular BCR-ABL phosphorylation by dasatinib in the stem cells were detected [42], but its activity is not enough to eradicate the LSC compartment. Second, the inability of dasatinib to cure CML mice is not due to the appearance of mutations (e.g. BCR-ABL-T315I resistant clone) in the mice because CML mice treated with dasatinib for about 3 months contained >40% of GFP+Gr-1+ cells, among which there were large numbers of LSCs. Sequencing analysis of isolated genomic DNA from bone marrow cells of these mice did not show the T315I mutation in the BCR-ABL kinase domain[42]. In addition, the failure of imatinib to eradicate LSCs is not related to the c-*kit* function, because both imatinib and dasatinib inhibit c-*kit* [44]. These results confirm that inhibition of BCR-ABL kinase activity alone is insufficient to eradicate LSCs [45].

The current challenge, therefore, is to identify the molecular pathways which are involved and contribute to the maintenance, survival and self-renewal in CML LSCs, in order to discover molecular target potentially to be hit by specific drugs, in combination with TKIs. Therefore, as in AML, a real deep and complete cure may require complete eradication of these stem cells.

Thus, since CML patients receiving BMT can relapse LSCs are not eliminated.

CML is defined as a stem cell disease that results in the clonal expansion of BCR-ABL expressing cells, resulting from the t(9;22)(q34;q11.2) balanced translocation. BCR-ABL rearrangement occurs in a pluripotent hematopoietic stem cell, and LSCs in CML could be defined as part of properties of normal HSCs. Various subpopulations of CD34+ cells from CML patients have been isolated, and cells in each of the CD34+ subpopulations were examined for the presence of BCR-ABL mRNA [46], showing that BCR-ABL mRNA could be found in CD34+CD38- and CD34+CD38+ cells. Furthermore, other colleagues reported that enriched CD34+ cells from patients with CML could

be transplanted into NOD/SCID mice [47], although the mice did not develop lethal CML-like disease. The failure of BCR-ABL to induce typical CML in NOD/SCID mice does not necessarily indicate that this model is not suitable for examining LSCs, as human leukemia cells were indeed transplanted and survived in the recipient mice. It is obvious that the use of a NOD/SCID strain that allows more efficient engraftment of donor cells would likely improve the engraftment of human CML cells [48]. Therefore, a full understanding of the biology of LSCs requires the development of a good animal model that allows analysis of CML LSCs in the future.

1.4.1 Critical molecular pathways in CML LSCs

BCR-ABL plays a critical role in the maintenance of survival of LSCs, as all critical molecular pathways identified so far can be activated by BCR-ABL. It is reasonable to think that the stemness of BCR-ABL-expressing HSCs is maintained by a complex molecular network involving BCR-ABL and its interaction with other downstream signaling pathways. Thus, these pathways would be specifically involved in the survival regulation of LSCs but not normal stem cell counterparts. In other words, it could be possible to identify genes that play critical role in the regulation of LSC function. This idea is supported by the identification of the *Alox5* gene as a key regulatory gene for LSCs but not normal hematopoietic stem cells. So far, there have been no data that do not support a role of BCR-ABL in the maintenance of the stemness of LSCs. However, a critical question to ask is why BCR-ABL kinase inhibitors such as imatinib and dasatinib are incapable of eradicating LSCs, if BCR-ABL kinase activity is inhibited? It has been shown that the inhibition of BCR-ABL kinase activity by imatinib in LSCs does not completely compromise BCR-ABL function [42], indicating the kinase-independent function of BCR-ABL.

A complete removal of BCR-ABL protein would have a much stronger inhibitory effect on LSCs. Besides targeting BCR-ABL, the identification and inhibition of key BCR-ABL downstream signaling molecules/pathways will offer effective therapeutic strategies aiming to eradicate LSCs. Below are some examples of the key pathways activated by BCR-ABL in LSCs (Figure 5, 6).

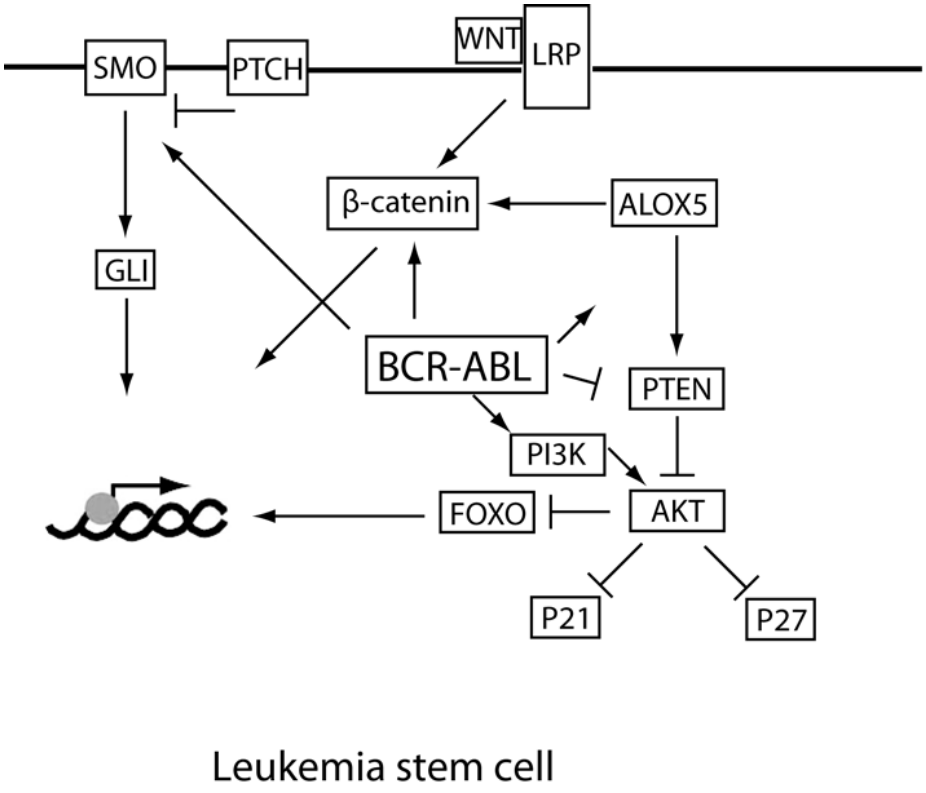

Figure 5 Critical molecular pathways in LSCs

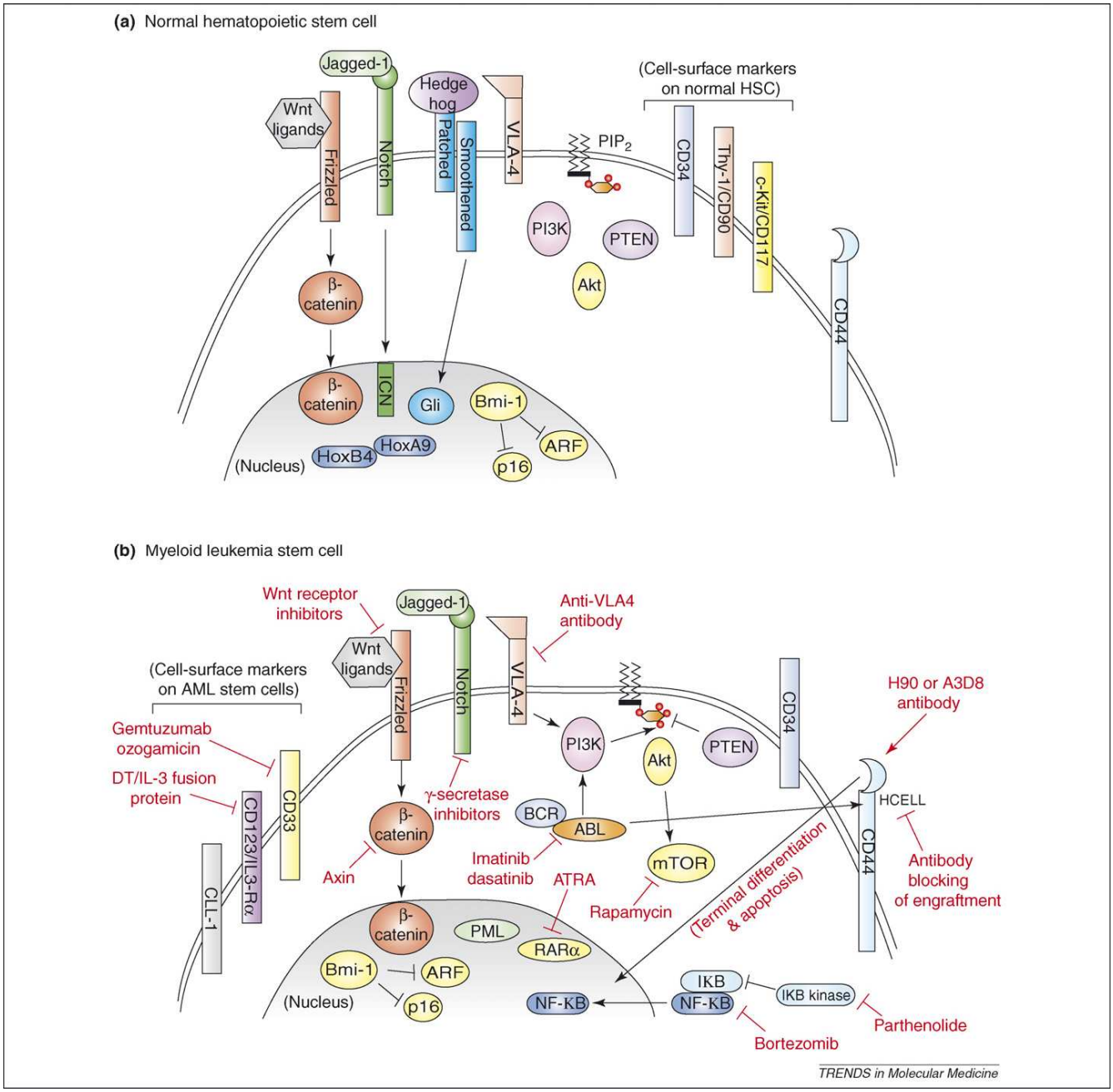

Figure 6 Cell-surface markers and therapeutic targets of myeloid leukemia stem cells.
Potential agents and strategies for the eradication of LSCs are depicted in red and include antibodies and fusion proteins as well as small-molecule inhibitors. (Adapted from Daniela S. Krause and Richard A. Van Etten Right on target: eradicating leukemic stem cells, TRENDS in Molecular Medicine Vol.13 No.11)

1.5 Hedgehog pathway

Many emerging laboratory and clinical investigations, in the recent years, have shown that Hedgehog signaling (Hh) represents a novel therapeutic target in various human cancers [49,50]. It's a signaling pathways which physiologically regulates self-renewal and terminal differentiation during the embryonic development, becoming typically silenced after birth, in adult tissues. Its reactivation, infact, usually may appear only during tissue repair [51].

Interestingly, aberrant Hh pathway signaling has been implicated in the pathogenesis, self-renewal, and chemotherapy resistance of a growing number of both solid and hematologic malignancies, defining a potential target not only for preclinical studies but also for identification of new tailored therapies.

Major components of the Hh pathway, which will be further described in details, include the Hh ligands (Sonic, Desert, and Indian), the transmembrane receptor Patched, the signal transducer Smoothened (Smo), and transcription factors Gli1–3 which regulate the transcription of Hh target genes. Mutations in Hh pathway genes, as expected, increased Hh signaling in tumor stroma, and Hh overexpression in self-renewing cells (cancer stem cells) have been described. These different modes of Hh signaling, therefore, have implications for the design of Hh pathway inhibitors and their integration into conventional treatment regimens.

The presence of a naturally-occurring Smo inhibitor, cyclopamine, has encouraged the development of several derivative compounds, which are currently objectives of clinical trials. Relevant and interesting encouraging laboratory and in vivo data has resulted in Phase I and II clinical trials of Smo inhibitors, but the optimal combination and sequence of these targeted therapies into current treatment algorithm still remain a challenge.

1.5.1 The pathway description

The Hh signaling pathway is activated by the binding of Hh ligand to the transmembrane receptor, Patched (Ptc). In the absence of ligand, Ptc suppresses the Hh signaling cascade by inhibiting the activity of a second transmembrane protein, SMO. Binding of Hh ligand to Ptc alleviates repression of SMO, allowing SMO to transduce the signal to the cytoplasm resulting in activation of zinc finger transcription factor, Gli. Gli translocates into the nucleus and activates transcription of target genes including additional Gli family members and Ptc (Figure 7).

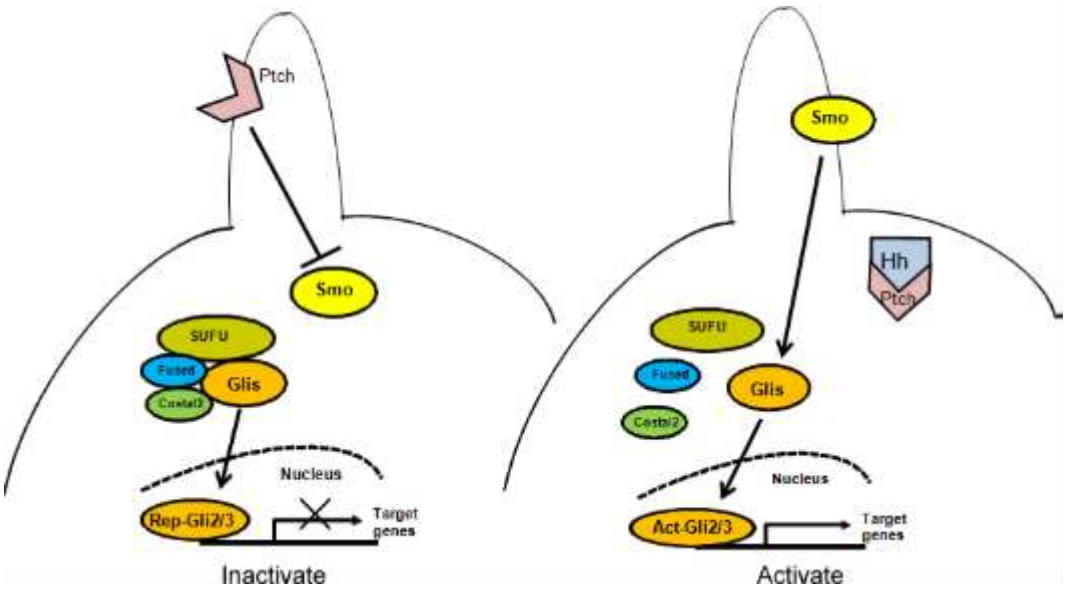

Figure 7 Proposed mechanism for Hedgehog (Hh) pathway activation. In the absence of Hh ligands, Gli2 and Gli3 (Glis) form a protein complex with other proteins, and the suppressor of Fused (SUFU). Furthermore, Patched (Ptch) suppresses the signaling activity of Smo. Costal2 might promote the degradation or proteolysis of Glis, which generates the repressor form of Glis (Rep-Gli2 □3). The translocation of Rep-Gli2 □3 to the nucleus inhibits the transcription of target genes, including Gli1 and Ptch, and cancer proliferation- and invasion-related genes (left panel). In the presence of Hh, it binds to Ptch, activating Smo, and both degradation of Gli2 and proteolytic processing of Gli3 into its repressive form are inhibited, thereby permitting Gli2 to function as a strong activator of Hh signaling (Act-Gli2) and allowing full-length Gli3 to serve as an activator (Act-Gli3) (right panel). (Modified from Onishi H, Katano M: Hedgehog signaling pathway as a therapeutic target in various types of cancerCancer Sci. 2011 Oct;102(10):1756-60)

The Hh signaling pathway is essential for multiple functions in embryonic development, and is therefore considered a key developmental pathway. Disruption of this crucial pathway during human development results in severe birth defects represented by holoprosencephaly, polydactyly, cranial defects, and skeletal malformations[52]. Aberrant activation of the Hh pathway has also been implicated in a lot of human malignancies, and can occur by two mechanisms, both of which result in cancer.

First, mutations in Ptc or SMO genes result in constitutive activation of the pathway and upregulation of Gli. Mutations in Hh signaling members have been reported in basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma [53, 54]. Moreover, a direct link between mutation driven aberrant Hh signaling and human tumorgenesis is found in Gorlin Syndrome, characterized by the development of multiple basal cell carcinomas (BCC) and a predisposition to medulloblastoma and rhabdomyosarcoma. These patients present a germline inactivating mutation in the repressor Ptc which results in constitutively active SMO and upregulation of Hh target genes. The link between mutations in Ptc and BCC was also reported in a majority of sporadic BCC tumors [53, 54]. A significant number of sporadic medulloblastomas, a pediatric cancer of the cerebellar granule neuron progenitor cells, can also be attributed to inactivating mutations in Ptc gene [54].

The second method of Hh activation is through autocrine or paracrine mechanisms of ligand driven Hh pathway activation in adult tissues with a normally dormant Hh pathway. In some cancers Hh pathway members are expressed in the tumor cells and directly affect the growth of the tumor, resulting in an autocrine positive feedback loop. In other cases, Hh ligand produced by tumor cells may activate the pathway in adjacent stroma, leading to the release of growth factors that support tumor growth or angiogenesis as part of the tumor microenvironment [55] (Figure 8).

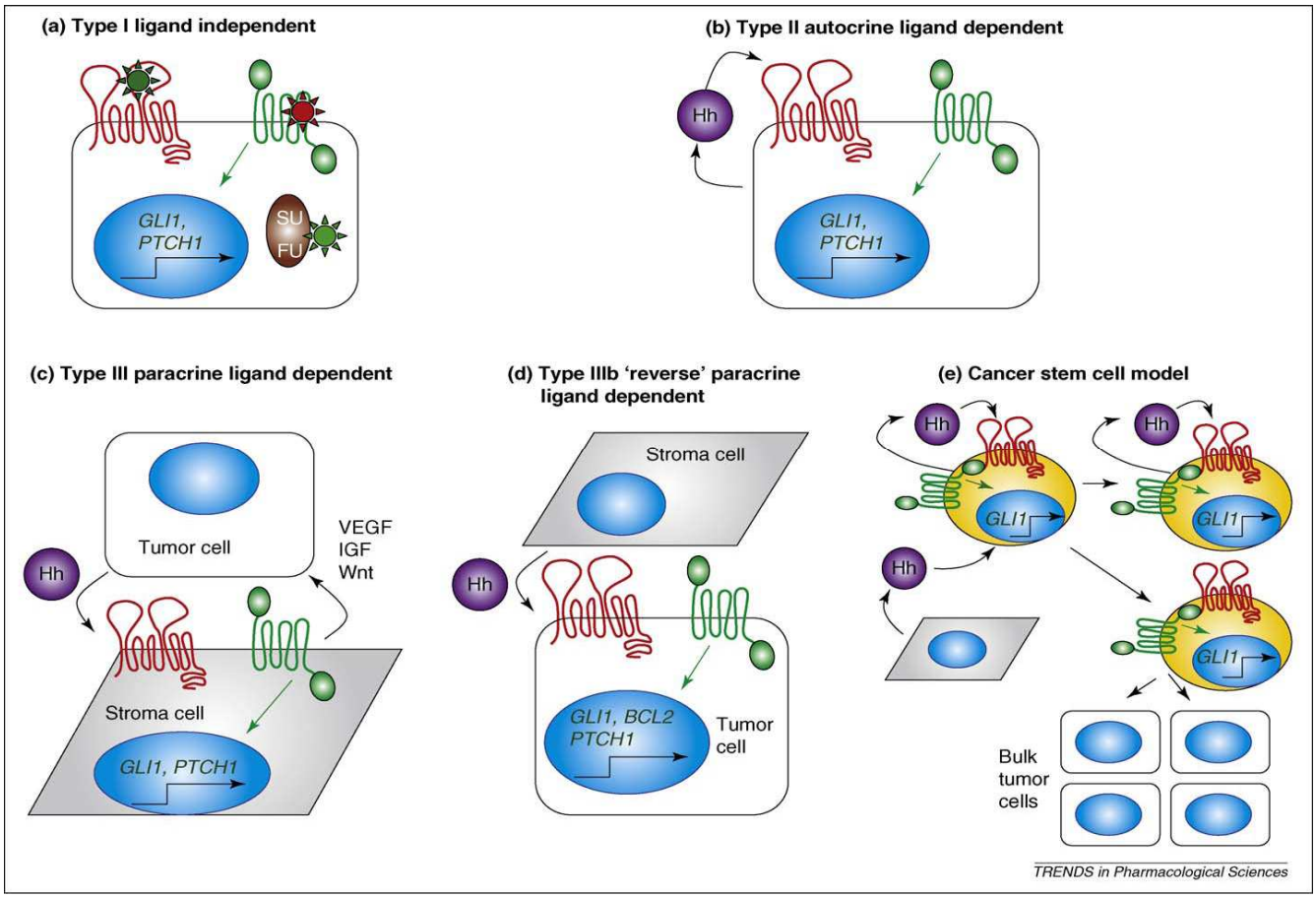

Figure 8 HH pathway regulation in cancer (Adapted from Scales S. et al, Trends Pharmacol.Sci., 2009)

This has been reported in pancreatic, breast, prostate, small cell lung cancer and more recently in hematopoietic malignancies [56, 57, 58, 59,60, 61] (Figure 9).

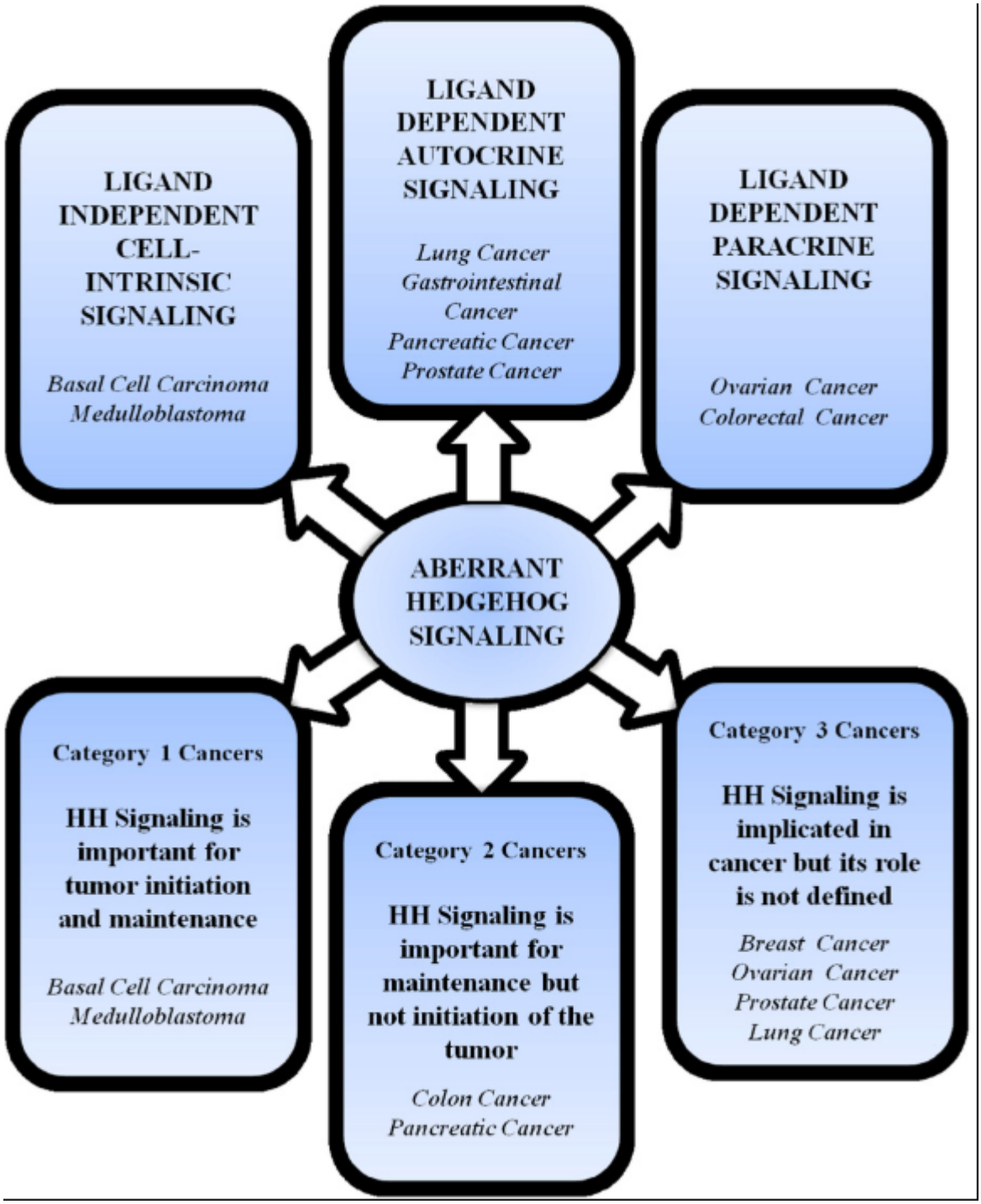

Figure 9 A schematic presentation of the different modes of aberrant HH signaling and the subsequent different types of cancers which can be categorized on the basis of at which stage the HH signaling influences the neoplastic progression (Adapted from Swayamsiddha Kar, Moonmoon Deb et al., Intricacies of hedgehog signaling pathways: A perspective in tumorigenesis, Experimental Cell Research 318 (2012) 1959–1972).

1.5.2 Role of Hedgehog pathway in Hematologic Malignancies

As previously assessed, the identification of cancer stem cells that self-renew, reinitiate tumor development and give rise to the neoplastic bulk, represented a milestone in the understanding of leukemia pathogenesis. Ultimately, this biological capacity can contribute to resistance and metastatic spread, affecting, at the end, overall survival. Standard chemotherapy, radiotherapies and some targeted therapies, as widely demonstrated, can greatly reduce tumor bulk but may be less effective on quiescent cancer stem cells. The key challenge has been, therefore, in identifying the molecular mechanisms that maintain and support cancer stem cell self-renewal and survival, in order to hit the initiating cell, responsible for tumor appearance.

In Ph+ CML subset, it has been widely demonstrated the ability of leukemic BCR-ABL clone to escape from direct tyrosine kinase inhibition, induced by first and second generation TKIs. For this reason, stem cell transplantation (with its high morbidity and mortality rates) remains the only potential cure for the relatively few patients with this option. Thus, the identification of less toxic and potentially curative therapies which target this disease remains of great interest. Several studies [60, 61] have identified components of the Hh pathway as potential drug targets in BCR-ABL-positive CML as it may play a key role in leukemic stem cells (LSC's). Dierks *et al* described a 4-fold induction of Gli1 and Ptc1 in CD34+ chronic or blast crisis CML cells. BCR-ABL expression induced SMO, Gli1 and Ptc levels within the stem cell compartment in a mouse model of CML. The Ptc1 expression level is 20-fold higher in patient derived CD34+ blast-crisis CML cells compared to CD34+ chronic-phase CML cells [62]. Gli2 expression increases progressively in chronic phase and blast crisis CML samples, and SMO is essential for expansion of the leukemic stem cell pool as compared to normal hematopoietic stem cells (HSC's). Zhao et al demonstrated that Hh signaling is activated in LSCs through up regulation of SMO. While SMO does not

impact long-term reconstitution of regular hematopoiesis, the development of retransplantable BCR-ABL-positive leukemia was abolished in the absence of SMO expression. Furthermore, loss of SMO impairs HSC renewal and decreases induction of CML by the BCR–ABL oncoprotein and by depletion of the CML stem cell, whereas constitutively active SMO augments CML stem cell number to accelerate disease. The cell fate determinant Numb**,** which depletes CML stem cells, is increased in the absence of SMO activity. Pharmacological inhibition of Hh signaling impairs not only the propagation of CML driven by wild-type BCR–ABL, but also the growth of Imatinib-resistant mouse and human CML by reducing the number of LSCs in vivo [61]. Finally, SMO was significantly upregulated in CML patients, suggesting activation of the Hh pathway may be associated with CML progression[63]. Collectively, these data indicate that Hh pathway activity is required for maintenance of normal and leukemic stem cell populations in CML, and raise the possibility that drug resistance and disease progression associated with TKI failure might be avoided by targeting this essential stem cell maintenance pathway.

In addition to CML, aberrant Hh signaling has been described in a variety of human leukemia and leukemia stem cells. Expression levels of Ptc, SMO and Gli1 were examined in several leukemic cell lines [64]. Ptc and SMO were expressed in Jurkat T-ALL cells, and Shh and Gli1 were expressed in human promyelocytic leukemia (HL-60) and KG-1 cells. Hh signaling is up-regulated in several subtypes of human AML cells, including primary CD34+ leukemic cells and cytokine-responsive CD34+ cell lines such as Kasumi-1, Kasumi- 3 and TF-1. These CD34+ cells express Gli1 and Gli2, indicative of active Hh signaling. Inhibition of Hh signalling induced apoptosis after 48 h of exposure, although these CD34+ cell lines exhibited resistance to cytarabine (Ara-C). This data was confirmed by reverse transcription– polymerase chain reaction (RT-PCR) for Hh pathway components and a Gli-responsive reporter assay [65]. Finally, upregulation of Hh pathway components has been observed in

chemoresistant AML cell lines in vitro, and pharmacological inhibition of the Hh pathway resulted in decreased multi-drug resistance (MDR-1) or P-glycoprotein (Pgp) expression in these cells [66]. Given the central role that Hh signalling plays in cell differentiation, Hh inhibition represents a mechanistically novel approach to eliminate the LSC population and thus abrogate tumor proliferation in at least a subset of CD34+ myeloid driven hematapoietic malignancies.

Additional evidence for the importance of Hh in leukemia's is derived from patients enrolled in a recently closed single agent hematology trial with a Smo-inhibitor molecule, which will be the object of this research activity. The results will be showed in the related chapter.

1.6 New Smo-inhibitor drugs in clinical development

After the identification of a natural Hh pathway antagonist, represented by Cyclopamine, many synthetic and semi-synthetic derivatives, with increased potency and bioavailability have been introduced in clinical research, both in hematological malignancies and in solid tumors.

Among these, in Table 2 are reported the most promising compounds, currently objectives of company sponsored experimental clinical trials.

All these molecules act at the level of Smo, after oral administration, according to different schedules. As expected, similar to challenges underlined in other neoplastic malignancies, an open question about the potential benefit and the most appropriate combination chemotherapy approach, currently hasn't led to any answer.

Drug (Sponsor)	Indications	Phases
GDC-0449 (Genentech)	Advanced solid tumors, BCC, breast, chondrosarcoma, colorectal, gastric,	Phase II
LDE-225 (Novartis)	Advanced solid tumors, BCC, chronic myeloid leukemia, pancreatic	Phase I, II
BMS-833923 (Bristol-Myers Squibb)	Advanced solid tumors, BCC, chronic myeloid leukemia, esophageal,	Phase I, II
XL139 (Exelixis)	gastric, multiple myeloma, small cell lung	
IPI-926 (Infinity)	Advanced solid tumors, chondrosarcoma, head and neck, myelofbrosis, pancreatic	Phase I, II
SMO-INHIBITOR (Pfizer)	Advanced solid tumors, hematologic malignancies	Phase I
LEQ-506 (Novartis)	Advanced solid tumors	Phase I
TAK-441 (Millenium)	Advanced solid tumors	Phase I
Itraconazole	BCC, metastatic prostate cancer, non-small cell lung cancer	Phase II

Table 2 Smoothened inhibitors currently in clinical trials for cancer (Modified from Lin TL, Matsui W. Hedgehog pathway as a drug target: Smoothened inhibitors in development. Onco Targets Ther. 2012;5:47-58.

1.7 A new Smo-inhibitor: molecular and PK features

The hereby described Smo inhibitor compound is a potent and selective inhibitor of Hedgehog (Hh) signaling *in vitro*, and has showed significant antitumor efficacy *in vivo*. The first experience in a mouse model of Hh pathway-driven tumor has been performed in medulloblastoma setting, demonstrating that the drug inhibits pathway activation (Gli1 expression) and produces rapid and complete tumor regression. Moreover, the compound was studied in haematological malignancies, demonstrating its capability of reducing leukemic burden in a blast crisis chronic myeloid leukemia mouse model, and of inhibiting tumor formation in secondary recipients.

Preclinical pharmacokinetic/ pharmacodynamic (PK/PD) modelling suggests a target human dose of 15 mg/day, projected to yield at least 50% of tumor Gli1 mRNA inhibition from baseline levels. A 15 mg dose is projected to result in a Cmin (minimum plasma concentration) of 62 ng/mL total (5.6 ng/mL free) and a Cav (Average drug concentration) of 79 ng/mL total (7.2 ng/mL free). The anticipated maximum dose in the clinic is 200 mg/day which is projected to result in a Cmax (maximum plasma concentration) of 2150 ng/mL total (196 ng/mL free) and a Cave of 967 ng/mL total (88 ng/mL free).

Absolute oral bioavailability of the molecule following single dose oral administration was 33% in rats and 68% in dogs. Plasma protein binding of the drug in mouse, rat, dog, and human plasma ranged from 85% to 93%. A volume of distribution at steady state (Vss) of 4.78 and 4.21 L/kg was observed in rats and dogs, respectively. Hepatic metabolism is predicted to be the major clearance pathway for the drug in humans. Its *in vitro* metabolism was consistent across preclinical species and humans.

1.7.1 Drug Metabolism

This Smo-inhibitor appeared to be metabolized to several oxidative metabolites. Preliminary assessment using individual recombinant P450 enzymes suggests that CYP3A4 plays a major role in mediating the metabolism of the drug. All metabolites observed in human in vitro incubations were present in one or more of the evaluated preclinical species. In vitro, the molecule did not inhibit CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, or CYP3A4 at concentrations up to 30 µM. Based on *in vitro* and *in vivo* evaluations of the drug, systemic plasma clearance, volume of distribution at steady state, elimination half-life and oral bioavailability in humans are projected to be 1.03 mL/min/kg, 2.7 L/kg, 30 hours and 55%, respectively.

The compound was evaluated in rat and dog repeat-dose toxicity studies up to 1 month in duration. The Smo-inhibitor compound was well tolerated up to 50 mg/kg/day in the rat and 5 mg/kg/day in the dog. In both the rat and the dog, a greater than proportional increase in exposure occurred with increasing dose. In the 1-month rat study, the increase in mean AUC(0-24) values were approximately 175-fold and 232-fold greater than the dose (50-fold) range evaluated for Day 1 and Day 29, respectively. In the 1-month dog toxicity study, the mean AUC(0-24) values were approximately 165-fold (male) and 360-fold (female) greater than the dose (30-fold) range evaluated on Day 1. Deaths and/or moribund euthanasia occurred in the 7-day and 10-day rat studies, and the 1-month dog study at 250, 500 or 30/15 mg/kg/day, respectively. Cause of death/morbidity in both species was attributed to kidney toxicity. The target organ in the rat 1-month toxicity study included kidney (tubular degeneration/necrosis, cytomegaly, inflammation, regeneration) and bone (decreased/disorganized chondrocytes in epiphysis); the no observed adverse effect level (NOAEL) was 10 mg/kg/day (less than the projected human Cave at steady state at a 200 mg dose). The kidney changes showed some signs of reversibility but did not entirely reverse, while the bone changes persisted.

The target organ in the 1-month toxicity study in the dog was limited to the kidney (tubular necrosis, granular/mineralized casts, dilated tubules); the NOAEL was 1 mg/kg/day which is less than the projected human Cave at steady state at a 200 mg dose. Mild changes in the kidney were observed at 5 mg/kg/day which are also below the projected Cave at a 200 mg dose. The kidney changes in the dog were completely reversed in males and partially reversed in females after a 6-week reversal period.

In the acute central nervous system (CNS) and respiratory studies in the rat, no effects were observed at the high dose of 50 mg/kg. The maximum plasma concentration at 50 mg/kg (group mean free Cmax of 617 ng/mL) from the 1-month rat study is 3-fold above the projected human free Cmax concentration of 196 ng/mL at a 200 mg dose. Increases in QT and QTc were noted. The Smo inhibitor compound was negative in the definitive in vitro bacterial mutagenicity assay, human lymphocyte assay and the in vivo rat micronucleus. The molar extinction coefficient for the Smo inhibitor compound at 290 nm is 9622 L/mol/cm; therefore, the Smo-inhibitor compound has the potential to be phototoxic.

2. AIMS OF THE STUDY

The role of Hedgehog pathway in LSCs maintenance and self renewal both in AML and CML have been demonstrated by *in vitro* and *in vivo* studies.
In clinical development, data concerning hematological malignancies are still lacking. The aims of this study have included both preclinical and clinical activities, in order to:

1. Conduce a first in man Phase I clinical trial in patients with hematological malignancies, based on the administration, as a single agent, of a new Smo-inhibitor drug, aimed to assess:
    a. the safety and tolerability profile of the compound
    b. the maximum tolerated dose (MTD1) of the compound
    c. the pharmacodynamics and the pharmacokinetics of the compound
    d. the clinical and the hematological response observed in treated patients
2. Identify useful biomarkers related to Hh pathway in order to detect stem cell persistence in myeloid hematological malignancies
3. Evaluate GEP of CD34+ cells before and after treatment with Smo-inhibitor drugs
4. Evaluate the rationale for combination schedules of Smo-inhibitor with TKIs in Ph+ Leukemias, according to innovative phase I/II clinical trials.
5. HH pathway contribution to functional human LSC propagation

3. MATERIALS AND METHODS

3.1 First in man, Phase I Multi-Center Clinical Trial

The above described innovative Smo-inhibitor drug has been tested in an open-label, multi-center, Phase 1 study. The compound was administered orally as single agent to adults with select advanced hematologic malignancies (Phase 1a), after the obtainment of a signed informed consent. A second part (Phase 1b), consisting in the combination treatment with dasatinib or bosutinib for CML patients in AP/BC was planned, but has not been performed yet. The protocol, therefore, was designed for previously strongly treated patients with relapsed or refractory advanced hematological malignancies, such as AML, CML, MDS or PMF.

Phase 1a was aimed to assess the above mentioned Smo-inhibitor administered as single agent once daily in a continuous regimen. A dose escalation design was applied in 3-6 patient cohorts up to identification of the maximum tolerated dose (MTD1). The starting dose was 5 mg once daily. A lead-in period on Day -6 for each dose escalation cohort was planned, in which the single-dose pharmacokinetics and pharmacodynamics of the compound was characterized prior to initiation of continuous dosing in the first cycle of treatment. The lead-in period duration, PK time-points, doses and/or regimens used in subsequent cohorts could be modified based on the exposure (AUC) observed during the lead-in period (although the number of PK samples will not be increased).

Once the MTD1 is established, the cohort will be expanded with at least 8 additional patients to further characterize safety and tolerability at the MTD1 and to collect blood and urine for metabolite profiling/urine PK.

Phase 1b will assess the drug in combination with dasatinib or bosutinib as two separate arms, both combinations administered orally once daily with food in a continuous regimen to patients with AP/BC CML.

Treatment with the compound single agent or in combination with dasatinib or bosutinib (Phase 1a and 1b, respectively) could continue for up to 1 year (52 weeks) or until disease progression, patient withdrawal or unacceptable toxicity occurs. Patients who complete treatment for 1 year (52 weeks) will be considered to have completed the trial. Patients who are still on trial at 1 year and who continue to benefit from treatment may have the option to continue treatment upon agreement between the investigator and sponsor, and pending study drug availability.

Pre- and post- Smo inhibitor dose blood, bone marrow and normal skin was obtained for biomarker assessments and evaluating potential genetic changes that could correlate to clinical outcome. These assessments included pharmacodynamic analyses of Hedgehog target genes and other signaling pathways which may interact with the Hedgehog pathway.

Schedule

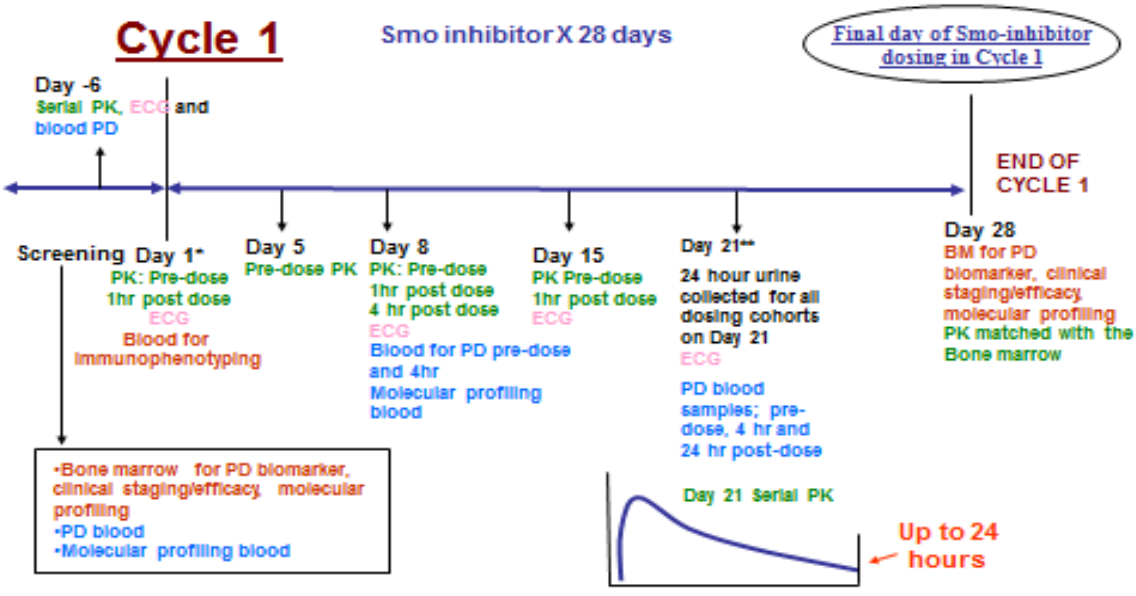

Figure 10 Treatment schedule of cycle 1

3.1.1 Statistical Methods: Sample Size Determination

The number of patients to be enrolled in the study depended on the observed safety profile, and the number of dose escalations in the two phases of the study.
The expected number of patients for Phase 1a was estimated to be 42.

3.1.2 Rationale for Selection of the Starting Dose

The starting dose for the Smo inhibitor compound in this first-in-patient trial in cancer patients has been determined to be 5 mg daily, based on information derived from the 1-month repeat dose toxicology studies in rats and dogs (Data coming from Investigators' Brochure).
The doses tested in the 1-month toxicology study in the rat were 1, 10, and 50 mg/kg/day orally, and in the 1-month dog study were 1, 5, and 30/15 mg/kg/day orally.
According to DeGeorge et al (1998) , the currently accepted algorithm for calculating a starting dose in clinical trials for cytotoxic agents is to use one-tenth of the dose that causes severe toxicity (or death) in 10% of the rodents (STD10) on a mg/m basis, provided this starting dose does not cause serious, irreversible toxicity in a non-rodent species. If irreversible toxicities are produced at the proposed starting dose in non-rodents or if the non-rodent is known to be the more sensitive animal model, then the starting dose would generally be one-sixth of the highest dose tested in the non-rodent that does not cause severe, irreversible toxicity. The human equivalent starting dose was calculated to be ~ 55 mg based on the rat STD10, and ~6 mg based on the NOAEL of 1 mg/kg in the dog.
Because the dog was determined to be the more sensitive species, the starting dose of the compound was 5 mg and used as the starting dose for the FIP study.

3.1.3 Inclusion Criteria

Patient eligibility was reviewed and documented by an appropriately qualified member of the investigator's study team before patients are included in the study.

Patients enrolled had to meet all of the following inclusion criteria to be eligible for enrollment into the study:

1. Phase 1a: Patients with select advanced hematologic malignancies who are refractory, resistant or intolerant to prior therapies. They may be newly diagnosed (patients with AML must be in compliance with national treatment guidelines, see below) and previously untreated (for all diseases with the exception of non-T315I CML (see below)), but not eligible for standard treatment options, or for whom standard therapies are not anticipated to result in a durable response. Eligible patients are limited to 1. Myelodysplastic Syndrome (any MDS International Prognostic Scoring System or IPSS score), 2. Myleofibrosis, 3. Chronic Myelomonocytic Leukemia (CMML), 4. CML T315I mutants (may be previously untreated), 5. non-T315I CML (any phase; must have received at least one prior treatment), 6. Acute Myeloid Leukemia (AML; not eligible to receive standard therapy based on national treatment guidelines [Morra et al, 2008; NCCN guidelines, AML 2010).

Patients with CML:

- ✓ Must have a confirmed diagnosis as evidenced by the presence of the BCR-ABL translocation [t(9;22)] by fluorescence in situ hybridization (FISH), cytogenetics, or quantitative polymerase chain reaction (QPCR) for chronic myeloid leukemia in either chronic, accelerated or blast phase.
- ✓ Non-T315I CML must have received at least one prior therapy.
- ✓ May be resistant or intolerant as defined by:

a. In CML-CP, primary resistance is defined as failure to achieve a complete hematologic response (CHR) following 3 months on therapy; failure to achieveany

cytogenetic response (CyR) following 6 months on therapy, failure to achieve a major cytogenetic response following 12 months on therapy, or failure to achieve a complete cytogenetic response following 18 months on therapy.

b. Secondary resistance is defined as a loss of CHR (defined by leukocytosis confirmed with at least one WBC>15K not felt to be due to a secondary cause); loss of a MCyR (defined by ≥30% increase in the number of metaphases); or disease progression to AP or BP.

c. In CML-AP or CML-BC, resistance is defined as the failure to achieve a hematologic response, an increasing WBC, or an overt disease progression.

d. Intolerance for all phases is defined as discontinuation of prior therapy due to adverse events at the lowest approved dose or if a patient can only tolerate prior therapy at less than the lowest approved dose.

In addition, for all phases (except patients with T315I mutations), patients are eligible in the case of unsatisfactory clinical response to the initial course of TKI, but who do not meet the definition for refractory, resistant or intolerant (eg, a CML CP patient who rapidly progresses on primary therapy, but does not meet the criteria for primary resistance because they have not been on TKI for 3 months; or patients with co-morbid diseases who cannot tolerate TKI therapy).

3.1.4 Exclusion Criteria

Patients presenting with any of the following were not be included in the study:

1. Patient has undergone a donor lymphocyte infusion (DLI) in the prior 30-days;

2. Patient is known to be refractory to platelet or packed red cell transfusions per Institutional Guidelines;

3. Patient with active malignancy with the exception of basal cell carcinoma, non-melanoma skin cancer, carcinoma-in-situ cervical or skin cancer. Other concurrent malignancies will be considered on a case-by case basis;

4. Any one of the following currently or in the previous 6 months: myocardial infarction, congenital long QT syndrome, torsades de points, arrhythmias (including sustained ventricular tachyarrhythmia and ventricular fibrillation), right bundle branch block and left anterior hemiblock (bifascicular block), unstable angina, coronary/peripheral artery bypass graft, symptomatic congestive heart failure (CHF NY Heart Association class III or IV), cerebrovascular accident, transient ischemic attack or symptomatic pulmonary embolism;

For Phase 1a: QTc interval of >470 msec and for Phase 1b: QTc interval of >450 msec;

7. Bradycardia defined as HR <50 bpm;

8.For Phase 1b: Uncorrected serum calcium, potassium, magnesium or phosphate below institutional LLN;

9. Patient with an active, life threatening or clinically significant uncontrolled systemic infection;

10. Patients with active central nervous system (CNS) involvement by leukemia. Patients with prior history of CNS disease will qualify if active disease is ruled out by imaging studies or spinal tap;

11. Active graft versus host disease other than Grade 1 skin involvement;

12. Patients taking immunosuppressants for GVHD (including but not limited to: steroids, cyclosporine, tacrolimus, methotrexate or mycophenolate mofetil) from 14-days prior to the first dose of TKI until study treatment discontinuation;

13. Known human immunodeficiency virus (HIV) or acquired immunodeficiency syndrome (AIDS)-related illness or with active Hepatitis B or C infection;

14. Known malabsorption syndrome or other condition that may impair absorption of study medication (eg, gastrectomy or lap band);

15. Prior or concurrent anti-cancer treatment with a Hedgehog inhibitor or concurrent treatment with other investigational (excluding bosutinib) or approved

oncology agents. Prior treatment with SMO-INHIBITOR is acceptable for inclusion in Phase 1b if the patient is withdrawn from the Phase 1a portion of the study for disease progression, meets Phase 1b inclusion criteria, has tolerated SMO-INHIBITOR as a single agent and following sponsor approval;

16. Concurrent administration of herbal preparations;

17. Current use or anticipated need for food or drugs that are known strong/moderate CYP3A4 inhibitors, including their administration within 7-days prior to the first compound/TKI dose (ie, grapefruit juice, ketoconazole, itraconazole, voriconazole, posaconazole, clarithromycin, telithromycin, indinavir, saquinavir, ritonavir, nelfinavir, nefazodone, lopinavir, troleandomycin, mibefradil, conivaptan, erythromycin, azithromycin, verapamil, atazanavir, fluconazole, darunavir, diltiazem, delavirdine, aprepitant, imatinib, tofisopam, ciprofloxacin and cimetadine);

18. Current use or anticipated need for drugs that are known strong CYP3A4 inducers, including their administration within 7-days prior to the first drug/TKI dose (ie, phenobarbital, rifampin, phenytoin, carbamazepine, rifabutin, rifapentin, St. John's Wort);

19. Current use or anticipated need of drugs that are P-gp inhibitors (cyclosporine, tacrolimus, ritonavir, verapamil, erythromycin, ketoconazole, itraconazole, quinidine, elacridar and valspodar) or P-gp inducers (rifampin and St. John's Wort), including their administration within 7-days prior to the first SMO-INHIBITOR/TKI dose;

20. Current use or anticipated need for drugs that are CYP3A4 substrates and have a narrow therapeutic index, including their administration within 7-days prior to the first drug/TKI dose (eg, cyclosporine, sirolimus, astemizole, terfenadine, cisapride, pimozide, quinidine or ergot alkaloids);

21. Chronic systemic corticosteroid treatment, although topical applications, inhaled sprays, eye drops, local injections of corticosteroids and systemic steroids required for acute medical interventions are allowed;

22. Current non-prescription drug or alcohol dependence;

23. For Phase 1b only: Concurrent use of aspirin, clopidrogrel, dipyridamole, ticlopidine or other platelet inhibitors;

24. Pregnancy or breastfeeding. Female patients must be surgically sterile or be postmenopausal, or must agree to the use of effective contraception during the period of therapy. All female patients with reproductive potential must have a negative

pregnancy test (serum or urine) prior to enrollment. Male patients must be surgically sterile or must agree to use effective contraception during the period of therapy;

25. Other severe acute or chronic medical or psychiatric condition or laboratory abnormality that may increase the risk associated with study participation or study drug administration, or may interfere with the interpretation of study results, or in the judgment of the investigator would make the patient inappropriate for entry into the study.

3.1.5 Study drug formulation

The drug is formulated in tablets containing 5 mg, 10 mg, 25 mg and 100 mg of study medication. The tablets are packaged in High-density polyethylene (HDPE) bottles, with protection from moisture.

3.1.6 Laboratory Safety Assessments

According to the clinical trial, laboratory values were checked at defined time points. These parameters involved, blood chemistry, coagulation tests, hematology.

In details: White blood cell count plus differential (including neutrophils, lymphocytes, eosinophils, basophils, monocytes), platelet count, hemoglobin, sodium, potassium, chloride, blood urea nitrogen, creatinine, glucose, uric acid, calcium, phosphorus, magnesium, total protein, albumin, total bilirubin, direct and indirect bilirubin, aspartate transaminase, alanine transaminase, alkaline phosphatase, lactate dehydrogenase, international normalized ratio, prothrombin time, and partial thromboplastin time. Microscopic urinalysis: pH, specific gravity, protein, glucose, ketones, red and white blood cells, leukocyte esterase, casts, crystals and nitrite. If the urinary protein is ≥2+, then a 24-hour urine is required for quantitative measurements of protein, creatinine and glucose.

Pregnancy test: Serum or urine pregnancy test for women of childbearing potential.

3.1.7 Other Safety Assessments: ECG

Triplicate 12-lead (with a 10-second rhythm strip) tracing in the supine position were performed for all ECGs, to determine the mean QTc interval. If any patient had a mean pre-or post dose QTc value >480 msec (using both Fridericia and Bazett correction methods), immediate correction for reversible causes (including electrolyte abnormalities, hypoxia and concomitant medications for drugs with the potential to prolong the QTc interval) should be performed.

3.2 Blood for PK Analysis

Blood samples (2 mL whole blood sufficient to provide a minimum of 1 mL of plasma) were collected for PK analysis of the Smo-inhibitor for all cohorts as outlined in the Schedule of

Activities: during the lead-in period on Day (-6), in Cycle 1 on Day 1, Day 5, Day 8, Day 15 and Day 21. For cycles 2 and above a PK samples were also collected on Day 1 and Day 15 matched with the ECGs as well as at End of Treatment.

3.2.1 Urine for Analysis of Smo inhibitor and Metabolite Profiling

Urine samples were collected in all cohorts in Phase 1a, on Cycle 1/Day 21 over 0-24 hours post Smo-inhibitor dosing.

At the end of the urine collection period, the total volume of urine was measured and total volume recorded in the CRF. The urine were then mixed thoroughly and a 20-mL aliquot were withdrawn for PK analysis and a further 20-ml aliquot withdrawn for metabolite profiling (if there is insufficient urine collected, the urine volume apportioned for PK analysis will be prioritized as first). The samples were then frozen at -20°C.

3.2.2 Pharmacodynamic Biomarker Assessments

Pharmacodynamic biomarker assessments were performed in patients enrolled in the Phase 1a. These assessments included evaluation of the effects of single agent Smo-inhibitor alone on Hh pathway related genes.

Blood samples (~10 mL) were collected for pharmacodynamic biomarker assessments during screening, the lead-in period on Day (-6), Cycle 1/Day 8, Cycle 1/Day 21 and at End of Treatment. Bone marrow was collected at screening, on Day 1 of every even cycle, End of Treatment and at investigators discretion for the AML and CML AP/BC patients. For all other patients the bone marrow was collected at screening, Day 1 of Cycle 2, 6, 10, End of Treatment and at investigators discretion.

3.3 Efficacy Assessments

The study has enrolled patients with select hematologic diagnoses, each having specific clinical response criteria. The response criteria for CML are derived from Faderl et al [67](1999) and Cohen et al (2005) [68]. CMML/MDS, MF, Ph+ ALL and AML are derived and defined by the disease specific International Working Groups and World Health Organizations (WHO) Guidelines.

3.4 Real Time PCR or quantitative PCR with Taqman probes

The gene expression profiling has been quantified through a Real-Time PCR method and by the use of Taqman, selective for the following genes: Smo, Gli1, Gli2, Gli3, Abcb1, Abcg2 (Applied Biosystems). Moreover, it was necessary to have a reference gene, with a stable expression. The most suitable gene for this aim is the one codifying for GAPDH. The amplification has been performed loading every sample, in double, on a plate consisting in 96 wells (MicroAmp Optical 96-well reaction plate, Applied Biosystems), with a whole reaction volume of 25 mcl. The Real-Time PCR has been performed on a tool ABI prism 7300 SDS (Applied Biosystems) using these amplification procedures: 50°C 2 minutes, 95°C 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The quantitative analysis has been performed through $\Delta\Delta$Ct.

4. RESULTS

4.1 Clinical Trial

This first-in-patient Phase 1a dose-escalation study [69] was aimed designed to assess the dose-limiting toxicities (DLTs) and the recommended Phase 2 dose (RP2D) of the above mentioned Smo inhibitor compound, in patients with select hematologic malignancies (primary endpoint). Secondary endpoints included safety, pharmacokinetics (PK), pharmacodynamics, and preliminary signs of efficacy as defined by disease-specific guidelines. Patients had refractory, resistant, or intolerant select hematologic malignancies and could be previously untreated but not candidates for standard therapies: CML including T315I mutations (any phase), acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), myelofibrosis (MF), or chronic myelomonocytic leukemia (CMML). Cohorts of 3 patients received the drug alone, administered continuously in 28-day cycles, starting at a dose of 5 mg orally once daily.

Thirty-five patients have been enrolled at doses up to 400 mg: 21 males/14 females; AML, 20; MF, 6; CML, 5; MDS, 3; CMML 1, with a median age of 69 (35–79) years. ECOG PS was 0/1/2: n=11/16/5 (Table 3).

The majority of the patients, according to inclusion and exclusion criteria, presented with strongly pretreated high risk hematological malignancies, refractory to standard chemotherapy or resistant to standard therapeutic approaches. Clinical general conditions of the patients were in line with the advanced status disease.

About one half of the entire enrolled population was affected by AML, and the risk stratification of these patients is reported in Table 4, according to cytogenetics.

Characteristic	(N = 35)
Age, median (range)	**69 (35-79)**
Gender, n (%)	
Female	**14 (40)**
Male	**21 (60)**
ECOG, n (%)	
0	**12 (34)**
1	**18 (51)**
2	**5 (14)**
Malignancy, n (%)	
AML	**20 (57)**
CML	**5 (14)**
CMML	**1 (3)**
MDS	**3 (9)**
MF	**6 (17)**
Prior therapy for primary diagnosis	
No	**10 (29)**
Yes	**25 (71)**

Table 3 Characteristics of enrolled patients

Preliminary indications of efficacy were observed across all hematologic diseases studied (Figure 11 and Table 6).

One patient with AML (evolved from CMML and with a concurrent diagnosis of systemic mastocytosis) achieved a complete remission with incomplete blood count recovery; bone marrow blast count decreased from 92% to 1%. Five AML patients

had a ≥50% reduction in bone marrow blast counts (20% to 10%, 70% to 20%, 44% to 8%, 14% to 7%, 40% to 10%). One patient with low-risk MDS, currently remaining on study after 335 days, achieved significant reduction in spleen size and a hematologic improvement in platelets (from 98.5 to 369 $x10^9$/L) and neutrophils (ANC from 410 to 5490), and is no longer granulocyte colony-stimulating factor (G-CSF) dependent. Five patients with MF attained stable disease; an additional MF patient, currently remaining on study after 385 days, achieved clinical improvement with a >50% reduction in extramedullary disease (spleen size decreased from 10 cm to 3.5 cm sustained over 8 weeks). One patient with T3151 lymphoid blast crisis CML on study for 115 days achieved a major cytogenetic response with loss of their T3151 mutation.

Characteristic	(n = 20)
Poor risk cytogenetics, n (%)	**10 (50)**
Type of AML, n (%)	
De novo	8 (40)
Developed from AHD	7 (35)
Unknown	5 (25)

Table 4 AML patients characteristics

Treatment duration ranged from 1 to 387 days (AML: 1–266; CML: 1–281; MDS: 2–335; MF: 44–387 days). One patient discontinued the study due to a treatment-related adverse event (AE) after 137 days of therapy at the 10 mg dose level (grade [G] 3 hemorrhagic gastritis in the setting of chronic proton pump inhibitor administration prior to and during the study). One AML patient evolved from CMML on 80 mg had a DLT comprising G3 hypoxia and G3 pleural effusion. The majority of

AEs were of G1/2 severity; the most frequent treatment-related AEs included dysguesia (16%), alopecia (6%), arthralgia (6%), decreased appetite (6%), nausea (6%), and vomiting (6%) (Table 5).

Event, n (%)	Grade 1	Grade 2	Grade 3	Grade 4
Dysgeusia	4 (11%)	2 (6%)	0	0
Alopecia	3 (9%)	0	0	0
Muscle spasms	1 (3%)	1 (3%)	0	0
Nausea	2 (6%)	0	0	0
Vomiting	2 (6%)	0	0	0

Table 5 Adverse Events

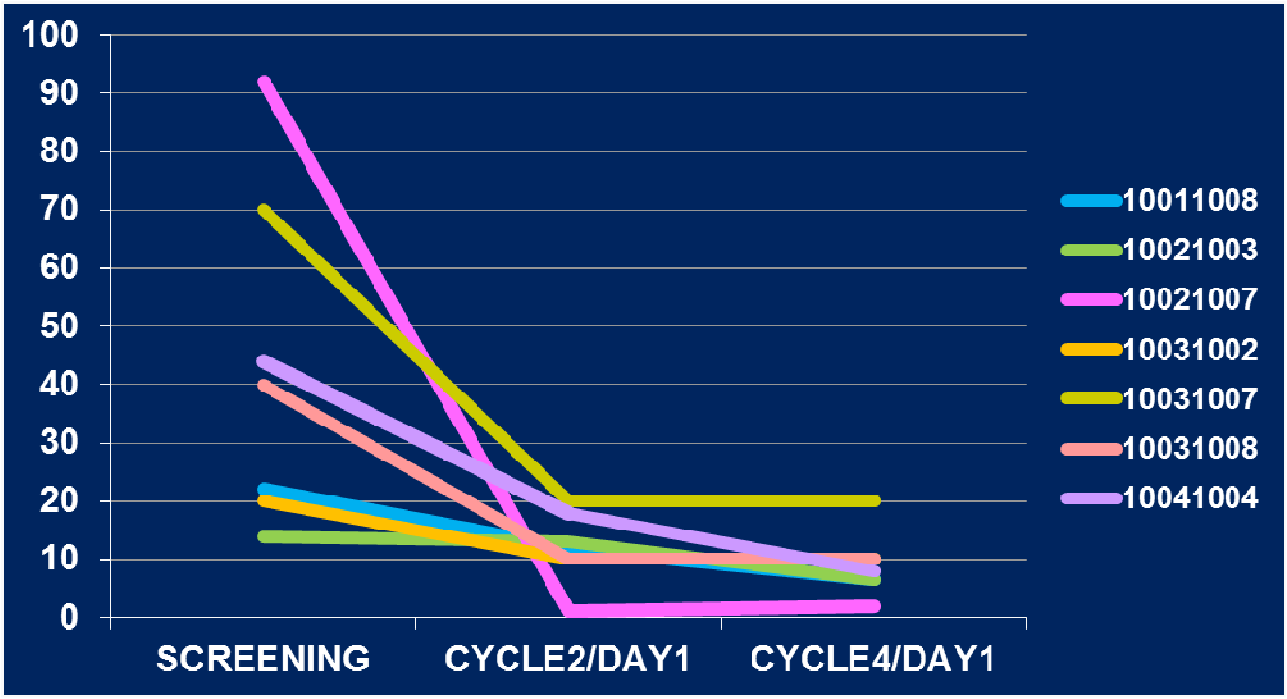

Figure 11 **Bone marrow blast cells percentage in AML enrolled patients**

Disease	Response	Dose(s)
MDS (Low Risk)	Hematologic improvement in platelets and neutrophils, G-CSF independent (1 patient)	**40 mg**
MF	Clinical improvement with ≥50% reduction in spleen size (1 patient)	**20 mg**
	Stable disease (5 patients)	**5, 5, 10, 80, 120 mg**
CML	Major cytogenetic response (1 patient with T315I BC CML)	**20 mg**

Table 6 Patients who obtained a clinical and hematological response

4.1.1 PK

Preliminary PK parameters for enrolled subjects come from single and multiple dosing of Smo inhibitor administration in all cohorts tested (5, 10, 20, 40, 80, 120, 180, 270, 400, and 600 mg QD) [70].

The drug was rapidly absorbed following oral dosing with a median Tmax of 1 to 2 hours after single and multiple dose administration. Following attainment of Cmax, plasma concentrations of the compound showed a bi-exponential decline. It was eliminated with a mean terminal half-life ranging from 17.4 to 34.2 hours. Following repeated daily dosing, the drug steady state was achieved by Day 8 and showed a median drug accumulation of 1.4 to 2.9 fold, which is consistent with the estimated half-life. In general, low to moderate inter-individual variability were observed in Cmax and AUC following single and multiple dose administration, though higher variability was observed at 180 and 270 mg.

4.2 Identification of biomarkers related to Hh pathway

In order to identify new potential clinical biomarkers for the above mentioned Smo-inhibitor compound, the leukemia stem cell population (CD34+ cells) collected before and after 28 days treatment in the already described first in men phase I dose escalation protocol was studied. Highly purified (98%) bone marrow hematopoietic progenitor cells (CD34+ populations) were collected and separated from 5 AML, 1 MF and 2 CML patients by immunomagnetic separation, and the analysis for gene expression profile (GEP) using Affimetrix HG-U133 Plus 2.0 platform was performed [71]. 1197 genes resulted differentially expressed between CD34+ cells separated from CML samples, collected before and after 28 days of the experimental therapy (Table 7, Table 8, Figure 12). Clustering of their expression profiles showed that mostly genes differentially expressed are mainly related to Hh signaling, thus providing relevant evidences that the compound really

therapeutically targets the Hh pathway. As far as the genes involved in Hh pathway are concerned, it was observed that *Gas1* and *Kif27* were strongly upregulated (fold change 1.0947 and 1.12757 respectively; p-value 0.01 and 0.02 respectively) in CD34+ leukemia stem cells after 28 days exposure to treatment as compared to baseline, suggesting the potential role of these two genes as new biomarkers of activity. *GAS-1* (growth arrest specific 1 gene) is a Sonic Hedgehog (Shh)-binding protein; it acts to sequester Shh and inhibit the Shh signalling pathway. *Kif27* (kinesin family member 27) mainly acts as a negative regulator in the Hh signaling pathway, and inhibits the transcriptional activator activity of *Gli1* by inhibiting its nuclear translocation. Other genes were differentially expressed after *'ex- vivo'* treatment with the molecule as compared to baseline: we observed a down regulation of *Bcl2* (fold change -1.03004), *ABCA2* (fold change -1.08966), *LEF1* (fold change -1.28457), *Gli1* (fold change -1.0775), *Smo* (fold change -1.07702), and an upregulation of *Gli2* (fold change 1.08191).

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 7 **CML- experiment contains 1197 genes with the specified threshold (p-value < 0.05)**

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 8 **AML- experiment contains 589 genes with the specified threshold (p-value < 0.05)**

Moreover, genes involved in multidrug resistance pathway were found to be differently expressed depending on treatment with the described Smo inhibitor compound. In details, ABCB1 (fold change 1,46592) and ABCG2 (fold change -1,16103) are respectively up and down regulated, with a not statistically significant p-value (0,35375 and 0,288194 respectively).

Bcl2 (B-cell lymphoma 2), Bcl2l2 (Bcl2-like protein 2) and Bcl2l13 (Bcl2-like 13) are the founding members of the Bcl-2 family of apoptosis regulator proteins. Recent studies showed that Hh signals upregulate Bcl2 to promote cellular survival.

Casp 4,7,10 (Caspases, or cysteine-aspartic proteases) are a family of cysteine proteases that play essential roles in apoptosis, necrosis, and inflammation.

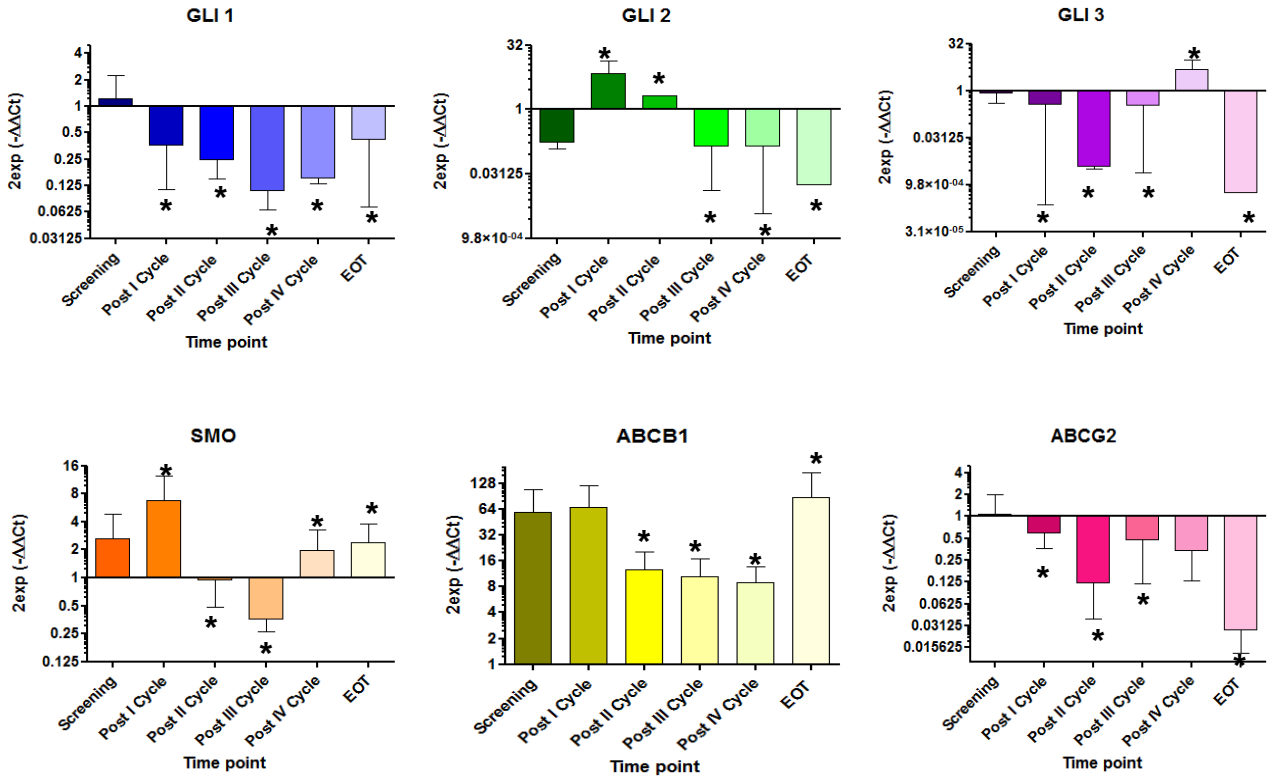

Figure 12 Different expression if genes involved in Hh pathway in samples of patients treated with Smo-inhibitor compound. Comparison at different timepoints.

ABCA2 (ATP-binding cassette sub-family A member 2), ABCF1 (ATP-binding cassette sub-family F member 1), 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. ABC proteins transport various molecules across extra- and intracellular membranes. One mechanism of MDR is the increased expression of ABC drug transporters that mediate energy-dependent transport of drugs out of the cells against a concentration gradient, resulting in low intracellular drug concentrations. This is a common finding in LSC, and represents an important clinical problem for disease eradication.

Furthermore, we evaluated Gli1, Gli2 and Smo expression by GEP, comparing data before and after 28 days of treatment with SMO-INHIBITOR and, as expected, we observed a down regulation of Gli1 (fold change -1.0775), Smo (fold change -1.07702), and an up regulation of Gli2 (fold change 1.08191).

Our results suggest that the compound is able to revert MRD mechanisms of LSC by a strong down regulation of genes (Bcl-2, Bcl-2l13, Bcl-2l2, ABCA2, and ABCF1), which are critical for chemoresistance in acute and chronic leukemia patients[72]. Therefore, the combination of SMO-INHIBITOR with Tyrosine Kinase inhibitors or conventional chemotherapy could represent a valid new therapeutic approach in these haematological malignancies.

4.3 Rationale for combination approach based on Smo inhibitors and TKIs in Ph+ Leukemias

Pre-clinical studies showed that the compound has significant activity in imatinib resistant CML blast crisis disease (C. Jamieson et al, personal communication). Patient derived CD34+ imatinib resistant blast crisis CML cells xenotransplanted into immunocompromised mice treated with the drug alone or in combination with

dasatinib significantly reduced primary leukemic tumor burden. This combination therapy also was able to reduce the leukemic stem cell population. In addition, treatment with the molecule alone as compared to vehicle reduced leukemic tumor formation in secondary recipients, suggesting that the drug is able to inhibit the LSC population necessary for tumor propagation. Finally, treatment with single agent in a CML model of T315I ABL mutant disease (known to have escaped TKI inhibition and for which no therapeutic options other than stem cell transplant exist) significantly reduced leukemic tumor burden, suggesting that the drug may be a viable option for these patients. Similar data have been demonstrated for other TKI's including nilotinib in combination with hedgehog inhibitors.

Other compounds, also in our experience, have been administered, in clinical trials, in association with TKIs in Philadelphia positive Leukemias, resistant or refractory to TKIs alone. The rational was based on preclinical data, showing a synergistic activity, due to a double inhibition both on BCR-ABL domain and on self-renewal pathways. Data regarding our little experience on this topic are not available yet.

4.4 Pre-clinical studies

As previously reported, cumulative evidence coming from literature suggests that dormant self-renewing LSC contribute to relapse and blast crisis transformation by evading therapies that target cycling cells. Sonic hedgehog signaling was shown to modulate cell cycle regulation and self-renewal in normal mouse hematopoietic stem cells. However, its role in human LSC regeneration and quiescence had not been elucidated. This aim has been investigated (data submitted) in order to evaluate the role of Shh signaling in maintenance of dormancy [73]. Compared to chronic phase CML and normal progenitors, human blast crisis LSC harbor enhanced expression of the Shh transcriptional activator, GLI2, and decreased expression of a transcriptional repressor, GLI3. Treatment of human blast crisis LSC engrafted $RAG2^{-}$

$^{/-}$gc$^{-/-}$ mice with the selective Shh inhibitor, reduced leukemic burden in a niche-dependent manner commensurate with GLI downregulation. Full transcriptome RNA sequencing performed on FACS-purified human progenitors from Smo-inhibitor treated blast crisis LSC engrafted mice demonstrated greater Shh gene splice isoform concordance with normal progenitors than vehicle treated controls. In addition, RNA sequencing revealed significantly decreased cell cycle regulatory genes expression and splice isoform analysis demonstrated reversion towards a normal splice isoform signature for many cell cycle regulatory genes. Moreover, cell cycle FACS analysis showed that selective Shh inhibition permitted dormant blast crisis LSC to enter the cell cycle while normal progenitor cell cycle status was unaffected. Finally, the drug synergized with BCR-ABL inhibition to reduce blast crisis LSC survival and self-renewal in concert with increased expression of Shh pathway regulators. Therefore selective Shh antagonism induces cycling of dormant human blast crisis LSC, rendering them susceptible to BCR-ABL inhibition, while sparing normal progenitors. Implementation of novel LSC splice isoform detection platforms to assess efficacy of Shh inhibitor-mediated sensitization to molecularly targeted therapy may inform dormant cancer stem cell elimination strategies that ultimately avert relapse.

5.Discussion

Current available treatments in many haematological malignancies are still unsatisfactory, in terms of overall response rates and long term survival. In particular, as widely demonstrated, the complex molecular heterogeneity of AML requires the identification of molecular targets in order to perform tailored treatments approaches. At the same time, the mechanisms of resistance to TKIs in Philadelphia positive leukemias underline the need for the assessment of other targets, to be hit in order to obtain not only a deeper control of the disease but also to eradicate it definitely.

Therefore, the so called “cell of origin” key concept is assuming a more and more important role in the understanting of pathogenetic events which lead to the onset of leukemias. We know, infact, that in many cases of AML and CML, the first triggering neoplastic event occur to the most immature cell of the HSC, giving rise to a kind of leukemia intrinsically chemoresistant. Many molecular pathways seem to be involved in self renewal of the LSC, and among these the Hedgehog signalling was demonstrated to play a key role.

Many compounds are currently in clinical development, both in solid and in haematological neoplasia, showing preliminary promising results as single agents. No data were available, so far, on leukemias.

We’ve therefore participated in a first in man Phase I clinical trial, aimed, by definition, to define the safety profile in terms of adverse events of a new Smo antagonist compound, administered mainly at AML and CML relapsed or refractory patients. The drug was safe and well tolerated, with early signs of efficacy observed in all hematologic diseases studied. Several patients with aggressive malignancies remained on trial for prolonged durations with improved quality of life; some exhibited cellular differentiation as determined by flow cytometry. On-target AEs (e.g. dysgeusia and alopecia) were observed at multiple dose levels.

Pharmacokinetics were linear, predictable, and compatible with once-daily dosing. The limited number of patients enrolled, of course, requires further investigations in order to confirm these data.

The biological correlative studies have shown relevant results as well. Through the GEP analysis, performed on CD34+ separated CML or AML cells, before and after one month of therapy, a statistically different expression signature was found. The involvement of genes belonging to Hh pathway allowed us to confirm that the drug is active against the pathway it has been synthetized for. Moreover, our data demonstrate that the drug is reverts MDR by down-regulation of ABCA2 and BCL2 on leukemia stem cells in AML and CML treated patients. It's worldwide shared the challenge on MDR, mainly in elderly AML patients, with intrinsically resistant leukemias. Based on this, at least in this poor prognostic group, a combinatory approach consisting in Smo-inhibitor and chemotherapy could offer a relevant benefit to our patients with high risk AML or relapsed CML. Starting from this rationale, we're planning to participate in a second clinical trial, which is based on the concomitant administration of Smo-inhibitor compound and Cyatarabine in high risk AML patients.

In conclusion, in the landscape of the molecular pathways involved in leukemogenesis, the Hh pathway seems to have a key role in self renewal, as demonstrated by our biological experiments in terms of up and down regulation of specific pathway genes. A combination approach with some "milestones" drugs of leukemia might lead, through the applicability of a strong rationale, to the improvement of the clinical outcome of our patients.

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