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Role of ERK5/BMK1 in the survival, proliferation and stemness of chronic myeloid leukemia cells in hypoxia

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Dottorando
Dott. Ignazia Tusa

_____
(firma)

Tutore
Prof. Persio Dello Sbarba

_____
(firma)

Coordinatore
Prof. Persio Dello Sbarba

_____
(firma)

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Abbreviations

Abl	Abelson
AGM	Aorta Gonad Mesonephros
ALL	Acute Lymphoblastic Leukaemia
AML	Acute myeloid leukaemia
aV	Annexin V
BCR	Breakpoint Cluster Region
BM	Bone Marrow
BMK1	Big Mitogen-Activated Kinase-1
CDS	Coding Sequence
CFC	Colony Forming Cell
CFU	Colony Forming Unit
ChIP	Chromatin immuno-precipitation
CML	Chronic Myeloid Leukemia
CRA	Colture-Repopulating Ability
CRC	Colture-Repopulating Cell
CSC	Cancer Stem Cell
CXCL	CXC Chemokine Ligand
CXCR	Chemokine CXC-motif Receptor
ECM	Extra Cellular Matrix
EGF	Epidermal Growth Factor
EHT	Endothelial-Haematopoietic Transition
ERK	Extracellular signal-Regulated Kinases
FACS	Fluorescence-activated cell sorting
FAK	Focal Adhesion Kinase
FasL	Fas ligand
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
FL	Fetal Liver

Flt3	FMS-like tyrosine kinase 3
5FU	5-Fluorouracile
G-CSF	Granulocyte-Colony Stimulating Factor
HCC	Hepatocellular carcinoma
HIF	Hypoxia Inducible Factor
HSC	Haematopoietic Stem Cell
HPC	Haematopoietic Progenitor Cells
HSC	Haematopoietic Stem Cell
IL	Interleukin
IM	Imatinib-Mesylate
kDa	Kilo Dalton
KL	Kit Ligand
LC	Liquid Culture
LPC	Leukemic Progenitor Cell
LSC	Leukemic Stem Cell
LTC-IC	Long-Term Culture Initiating Cells
LTR	Long-Term Repopulating
MAPK	Mitogen-Activated Protein Kinases
MBP	Myelin Basic Protein
ME	Mercapto-ethanol
MEF2	Myocyte Ehnancer-binding Factor2
MEK	Mitogen/Extracellular signal-regulated Kinase
MEKK	MEK Kinase
MFI	Mean Fluorescence Intensity
MMP	Matrix Metallo-Proteinase
MoI	Multiplicity of Infection
MRA	Marrow-Repopulating Ability
MRD	Minimal Residual Disease
NES	Nuclear Exporting Signal
NFkB	Nuclear Factor k B
NGF	Nerve Growth Factor
NLS	Nuclear Localization Signal
NOD-SCID	Non Obese Diabetic-Severe Combined Immuno-Deficient
PAGE	Poly-Acrylamide Gel Electrophoresis
PBS	Phosphate-Buffered Saline
PDGF	Platelet Derived Growth Factor

PFU	Plaque-Forming Units
PI	Propidium Iodide
PI3K	Phosphoinositol-3-Kinase
PML	Promyelocytic Leukaemia Protein
PMSF	Phenyl-Methane-Sulfonyl-Fluoride
RSK	p90 Ribosomal S6 Kinases
SCF	Stem Cell Factor
SDF	Stromal cell Derived Factor
shRNA	short hairpin RNA
STR	Short-Term Repopulating
TEMED	Tetra-Metil-Etilen-Diamine
TGF	Transforming Growth Factor
VEGF	Vascular Endothelial Growth Factor
vHL	von Hippel-Lindau protein
VLA	Very Late Antigens

Abstract

We previously demonstrated that resistance to hypoxia is a common feature of Haematopoietic Stem Cells (HSC) and Leukemic Stem Cells (LSC), but also that a hypoxia-selectable LSC subset is likely to exists within any type of leukemia, including stabilized clonal cells lines. In Chronic Myeloid Leukemia (CML), either cell lines or CD34+ cells explanted from CML patients, hypoxia completely suppresses the expression of BCR/$Abl_{protein}$ but not that of BCR/Abl transcript, so that hypoxia-resistant cells, while remaining genetically leukemic, are independent of BCR/Abl signaling for their maintenance *in vitro* and resistant to treatment with Imatinib-Mesylate (IM). Thus, hypoxia-selectable LSC are refractory to IM due to the lack of its molecular target. This is very well in keeping with the notions that: (i) IM, despite its impressive efficacy as first-line therapy for patients with chronic-phase CML, induces a state of Minimal Residual Disease (MRD), rather than cure; (ii) LSC most likely sustain MRD and are thereby responsible for the late relapses of CML; (iii) tissue hypoxia provides optimal conditions for the homing of normal and neoplastic stem cells.

The Extracellular signal-Regulated Kinase 5 (ERK5) is a Mitogen-Activated Protein Kinase involved in the control of cell survival and proliferation, as well as in the pathogenesis of a number of cancers, including CML. ERK5 is activated by cytokines regulating stem cell compartments and participates to cell response to hypoxia.

The main targets of the experimental work of this thesis were: 1) to explore the role of ERK5 in the maintenance of leukemia stem and progenitor cells; 2) to address the effects of different pharmacological MEK5/ERK5 inhibitors on the survival and proliferation of CML cells, either bulk or LSC populations; 3) to gather information useful to identify innovative (combination) treatment capable to eliminate the IM-insensitive LSC and thereby MDR.

The selection of leukemic progenitor cells (LPC)/LSC, as well as the effects of pharmacological ERK5 pathway inhibition on hypoxia-selected LPC/LSC, were estimated by the Culture-Repopulating Ability (CRA) assay, a non-clonal assay

which measures the culture-repopulating power of normal or leukemic hematopoietic cells. On the basis of entity and kinetics of repopulation of secondary cultures (LC2) where cell growth is unrestrained, it is possible to estimate the LSC or LPC content of cell populations subjected to a selective treatment in primary cultures (LC1). In the experiments reported here, the CRA assay was used to estimate the content of hypoxia-resistant CML cell populations with LSC/LPC and to test the effects on these cell subsets of genetic or pharmacologic inhibition of the ERK5 pathway. The inhibitors used were the ERK5-specific inhibitor XMD8-92 and the MEK5-specific inhibitorsBIX02188 and BIX02189. Genetic inhibition of ERK5 was obtained using lentiviral vectors expressing ERK5-specific short hairpin RNAs. The effects of the inhibiting treatments were tested on the KCL22 and K562 human stabilized CML cell lines, where ERK5 is constitutively activated.

The results obtained indicated that all three inhibitors were inactive on the bulk of CML cell population with respect to cell number in culture. On the other hand, XMD8-92 determined an appreciable increase of the percentage of KCL22, but not K562, cells in the G0/G1 phase of cell cycle and a decrease of the percentage of cells in S phase. BIX02188 and BIX02189 did not exhibit any appreciable effect on cell cycle phase distribution in either K562 or KCL22 cell line. XMD8-92, but not BIX02188 or BIX02189, also determined an increase of p27kip expression in both cell lines and reduced the basal apoptosis occurring in untreated K562 cell cultures. In hypoxia, where constitutive activation of ERK5 was suppressed, BIX02188 or BIX02189 did not affect the cell number significantly in either cell line. XMD8-92 treatment, on the contrary, resulted in a significant, although not marked, increase of viable cell number. A block of progression of CML cells to the S phase and the increase of p27Kip operated by XMD8-92 were observed in both cell lines. Consistently, neither BIX02188 nor BIX02189, which did not induce a detectable G0/G1 accumulation, increased the expression of p27Kip. Taken together with the data obtained in normoxia, these results point to a cytostatic, rather than cytotoxic, effect of ERK5-specific inhibition. Moreover, the treatment with XMD8-92, but not BIX02188 orBIX02189, resulted in a significant reduction of hypoxia-induced apoptosis. These results, together with those obtained in normoxia, suggest that the protective effect of XMD8-92 against basal apoptosis occurring in control cultures in normoxia is enhanced in hypoxia.

The treatment of KCL22 and K562 cells with BIX02188 or BIX02189 in normoxic LC1 reduced the ability to repopulate LC2, while that with XMD8-92 almost completely suppressed LC2 repopulation. On the other hand, when hypoxic LC1 were treated with BIX02188 or BIX02189, LC2 repopulation was almost immediate. On the contrary, the treatment with XMD8-92 completely suppressed LC2 repopulation. These results indicate that MEK5 inhibitors have a modest effect on CRA of BCR/$Abl_{protein}$-expressing CML, actually accelerating BCR/$Abl_{protein}$ re-expression in cells rescued from hypoxia. On the contrary, both BCR/$Abl_{protein}$-expressing and BCR/$Abl_{protein}$-negative subsets were markedly sensitive to the treatment with XMD8-92. Thus, XMD8-92 was inactive on the bulk of CML cell population, but capable to suppress completely the BCR/$Abl_{protein}$-positive LPC and the hypoxia-selected, BCR/$Abl_{protein}$-negative LPC/LSC.

To confirm the above results while overcoming problems of interpretation of data due to possible off-target effects of ERK5-inhibiting drugs, K562 cells were infected with lentiviral vectors expressing shRNA against ERK5 (shERK5). In hypoxia, genetic knockdown of ERK5, unlike its pharmacological inhibition, impaired CML cell survival. These differences are likely due to the well-known property of ERK5 to regulate a number of genes by direct interaction, independently of its kinase activity. On the other hand, ERK5 knockdown suppressed LC2 repopulation driven by hypoxia-selected cells as much as the inhibition of ERK5 enzymatic activity by XMD8-92.

Since we found that XMD8-92 does not inhibit the overall survival of CML cells, but suppresses hypoxia-selected LSC-like cell subsets, we tested the effects of the XMD8-92/IM combination. IM markedly reduced the number of viable cells in normoxia as well as in hypoxia. The combination with XMD8-92 determined a marginal, if any, enhancement of the inhibitory effect of IM. LC2 repopulation was reduced, but not abolished, by IM treatment of normoxic or hypoxic LC1. Importantly, XMD8-92, alone or in combination with IM, suppressed completely LC2 repopulation. The effectiveness of XMD8-92 demonstrated on hypoxia-selected cell subsets of stabilized CML lines was also tested on primary cells explanted from CML patients. In keeping with what observed for cell lines, XMD8-92, alone or in combination with IM, impaired culture repopulation driven by hypoxia-selected LSC from CML patients.

These results indicate that the combined treatment of XMD8-92 with IM may be an useful approach to try to eradicate CML together with the induction of remission, being XMD8-92 active on LPC/LSC but not cell bulk, and, *viceversa*, IM very active on the bulk but unable to suppress CML progenitors.

Introduction

Chronic Myeloid Leukemia

Chronic Myeloid Leukemia (CML) is a lethal hematological malignancy characterized by the increased and unregulated growth of predominantly myeloid cells in the bone marrow (BM) and the accumulation of these cells in the blood. The natural history of CML follows stages based on clinical characteristics and laboratory findings.

The clinical presentation often includes granulocytosis, splenomegaly and marrow hypercellularity; however about 40% of patients are asymptomatic and their diagnosis is based on elevated white blood cell count [1]. The natural course of disease usually involves this sequence: chronic phase, accelerated phase and blast crisis. Ninety percent of patients are diagnosed in chronic phase that normally lasts for 3 to 8 years. In this phase, blood cells retain their ability to differentiate until the illness progresses to the accelerated phase, which is characterized by the egress of immature cells into the bloodstream. Finally, the disease progresses to the blast crisis, defined by the presence of thirty percent or more leukemic cells in peripheral blood or marrow or extra-medullary infiltrates of blast. During this phase the survival of patients is reduced to months and even weeks [2].

CML was the first malignancy to be clearly linked to a genetic abnormality, the chromosomal translocation leading to the generation of an abnormal chromosome known as the Philadelphia chromosome. This chromosomal abnormality is so named because it was first discovered and described in 1960 by two scientists from Philadelphia (Pennsylvania, USA): Peter Nowell and David Hungerford. The Philadelphia chromosome is a shortened chromosome 22 originated from the reciprocal translocation between the long arms of chromosomes 9 and 22 [t (9; 22)] and involves addition of 3' segments of the *abl* gene (9q34) to 5' segments of the *BCR* gene (22q11). This gives rise to the *BCR-abl* fusion gene that transcribes a chimeric mRNA of 8.5 kb that, in turn, gives rise to the BCR/Abl fusion protein [2] (**Figure I**). t(9;22) is evident in

more than 95% of CML patients; between 5% and 10% of CML patients also present complex rearrangements that may involve one or more chromosomes in addition to 9 and 22 [3].

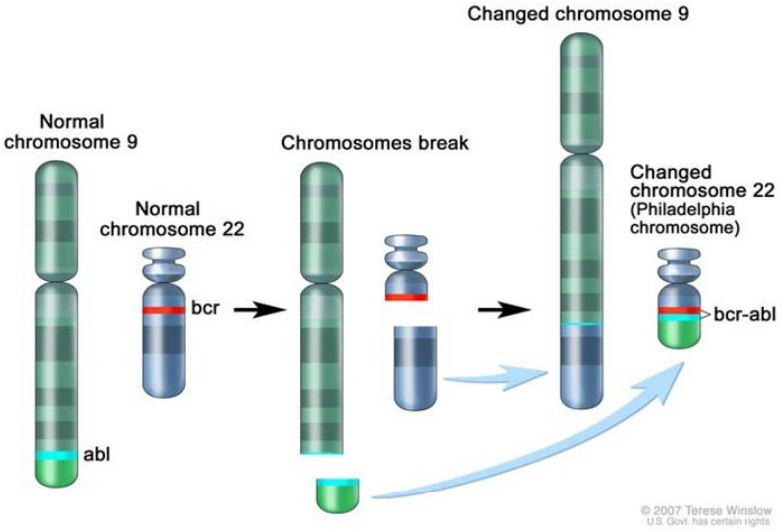


Figure I. The reciprocal translocation t(9; 22) (q34; q11). The chromosomes exchange the terminal portions of the respective long arms: a portion of c-abl gene translocates from chromosome 9 to chromosome 22, where it joins head-to-tail to bcr gene. The chromosome that carries this "fusion" gene is the altered chromosome 22 commonly called the Philadelphia chromosome. © 2007 Terese Winslow, National Cancer Institute.

The normal human Abl gene encodes for a 145 kDa non-receptor tyrosine kinase that is ubiquitously expressed, especially in hematopoietic cells [4] and neurons [5] and localized mainly at the cytoplasmic level [6]. Abl is involved in the regulation of cell cycle, response to genotoxic stress, and integrin-mediated intracellular signaling [7]. The normal human BCR gene encodes for a serine/threonine-kinase predominantly expressed in hematopoietic cells and neurons. It is localized mainly at the cytoplasmic level, where it plays an important role in the regulation of different signaling pathways, especially those that are regulated by G proteins [8-10]. There are three isoforms of the $BCR/Abl_{protein}$, all of which encode the same portion of the Abl tyrosine kinase, although they differ in the length of the BCR sequence at the N-terminus. p190 BCR/Abl is expressed in Acute Lymphoblastic Leukaemia (ALL), p210 BCR/Abl is characteristic of CML, and p230 BCR/Abl has been associated with a subgroup of CML patients with a more indolent disease (**Figure II**).

$BCR/Abl_{protein}$ is directly responsible for leukemogenesis and interferes with the regulation of different signaling pathways that promote the growth and survival of hematopoietic cells. Such an interference induces cell transformation.

Under physiological conditions, cells depend, for survival and proliferation, on the expression and ligand stimulation of plasma membrane receptors for growth factors. $BCR/Abl_{protein}$ suppresses this requirement, interacting with

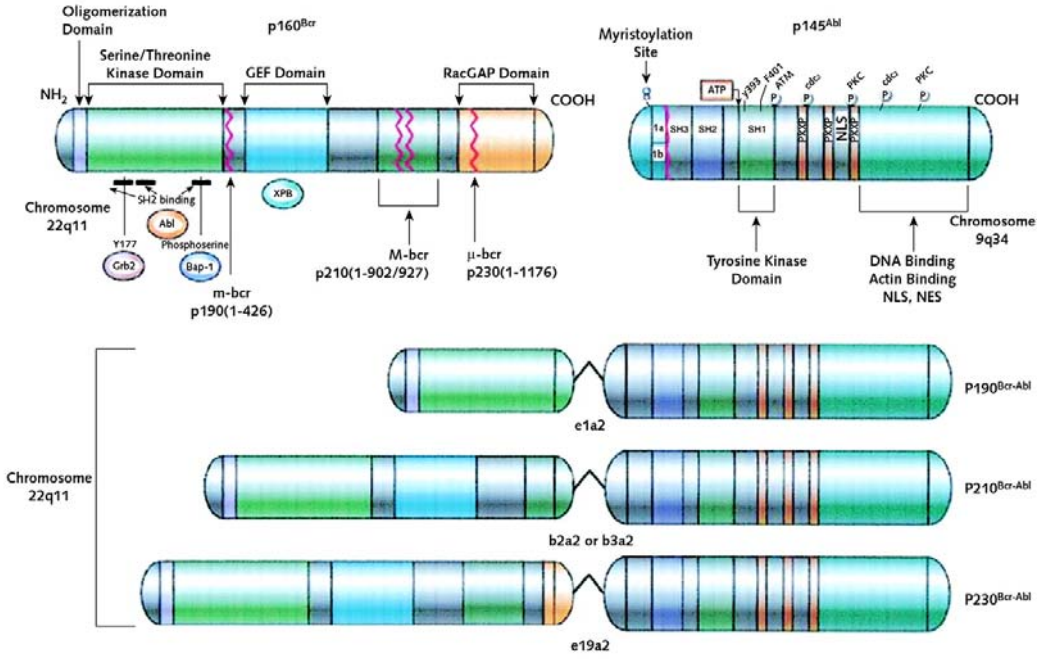


Figure II. Variants of the BCR/Abl fusion protein. Kurzrock R. et al., Ann Intern Med 003, 138:819-830.

receptors for growth factors such as the Interleukin-3 Receptor (IL-3R) or activating, in the absence of ligand, signaling pathways downstream of the receptor [11, 12]. $BCR/Abl_{protein}$, for example, is able to activate the Ras/Raf/MEK pathway that stimulates growth factor-independent cell growth [13, 14].

The leukemic transformation is accompanied by an increased damage to the DNA double helix. $BCR/Abl_{protein}$ alters the normal functions of various proteins involved in the mechanisms of DNA repair, by increasing the genetic instability of the cell, which results in the progression of CML to "blast crisis" [15]. Under physiological conditions, the damaged cell should undergo apoptosis or, in the case of slight damage, the block of mitotic cycle resulting in repair of the damage. Leukaemia cells lose this regulation as a result of BCR/Abl protein expression. $BCR/Abl_{protein}$, indeed, activates the AKT pathway constitutively, with two important effects: increase of glucose import from outside and block of the pro-apoptotic mechanisms controlled by the Bad and Bcl-2 proteins families (Bcl-x, Bax, Bcl-2). In the presence of cellular damage, the proteins belonging to the Bad family, in an unphosphorylated state, are normally associated with Bcl-2 and

Bcl-xL, inducing apoptosis. In leukemia cells, the phosphorylation of Bad due to the activation of AKT prevents this association, inducing instead Bcl-2 and Bcl-xL to interact with Bax, with the consequent interruption of the pro-apoptotic machinery [16] (**Figure III**).

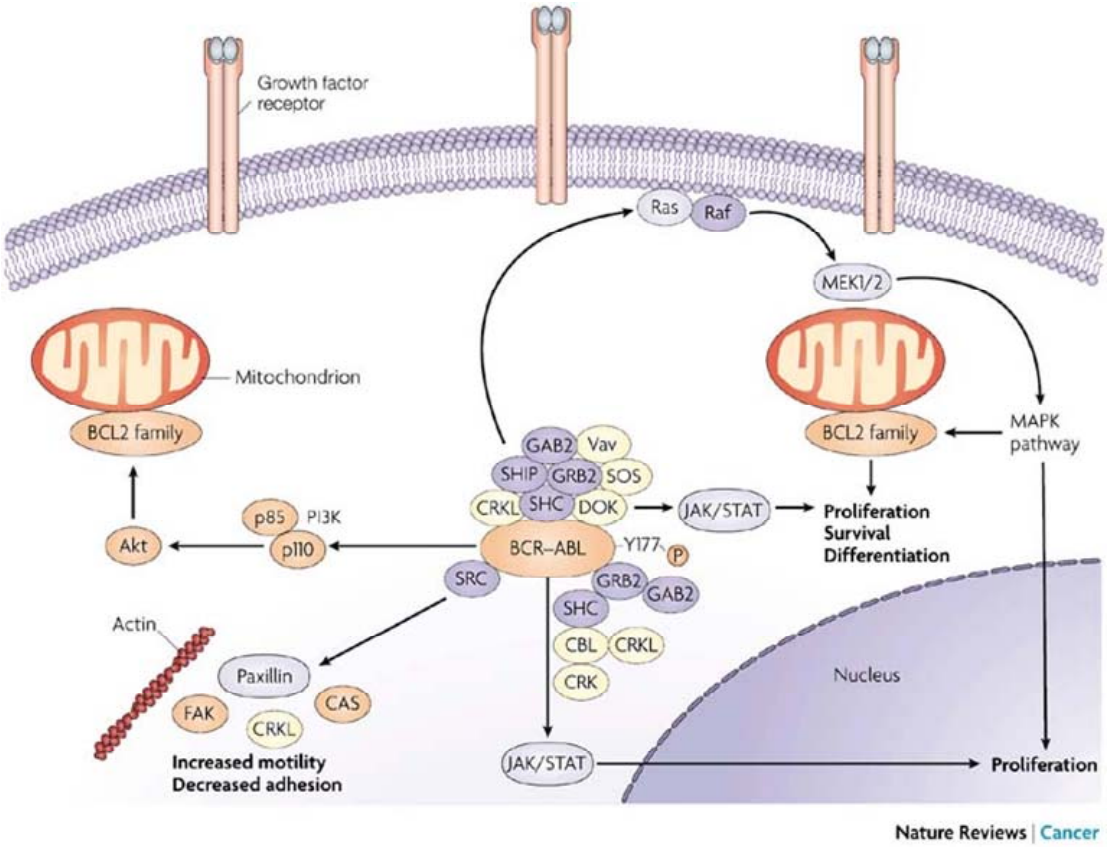


Figure III. BCR/Abl signaling in Chronic Myeloid Leukaemia. Weisberg E. et al., Nat Rev Cancer 2007, 7:345-356.

CML is characterized by the release into blood of immature progenitors normally confined within BM, a phenomenon that is attributed to defects in the mechanisms of hematopoietic cells adhesion, which were recently defined essential for the maturation of progenitors. $BCR/Abl_{protein}$ can cause indeed cytoskeletal alterations that modify the normal behavior of adhesion molecules, especially the β-integrins [17]. $BCR/Abl_{protein}$ is associated with components of the focal adhesions such as CRKL, actin, paxillin, Focal Adhesion Kinase (FAK) or the FAK-related PYK2 molecule; the activation of CRKL–FAK–PYK2 leads to a decrease in cell adhesion [18, 19].

Different biological actions of $BCR/Abl_{protein}$, as well as the normal tyrosine-kinase Abl, can be attributed to their different subcellular distribution. The

subcellular distribution of BCR/Abl is regulated by the presence in the protein of sequences which enable its translocation to and from the nucleus, such as Nuclear Localization Signal (NLS) and Nuclear Export Signal (NES) sequences [20, 21]. If activated within the nucleus, where it is imported in its dephosphorylated/inactivated state, Abl tyrosine-kinase activity stimulates apoptosis in response to double-helix DNA damage. On the other hand, when $BCR/Abl_{protein}$, which is predominantly cytosolic, is unable to determine such a pro-apoptotic effect and is responsible for the continuous generation of survival signals, via the constitutive activation of the Ras or Jak/STAT (signal transducer and activator of transcription) pathways [22]. However, when induced to translocate into the nucleus and remains there in its active form, even $BCR/Abl_{protein}$ is capable to induce pro-apoptotic stimuli [20]. This fact recently emerged as very relevant in view of the results obtained on cells of CML patients, for which the combined treatment with the BCR/Abl tyrosine-kinase inhibitor Imatinib-Mesilate (IM) and the nuclear export inhibitor leptomycin-B appears to have a synergistic effect in inducing apoptosis, drawing attention to a new strategy for the treatment of CML.

The treatment of CML has significantly changed over the past decade, perhaps more than any other cancer therapy. It is in this field, indeed, that the first molecularly-targeted drug (IM; STI571; Gleevec®) able to block specifically the cause of cell transformation has been developed [23]. IM binds BCR/Abl when it is in the “closed” conformation, preventing the transition to the “open” conformation necessary to bind ATP, and thereby blocks the kinase activity. IM ensures clinical, hematologic and cytogenetic remission of the large majority of chronic-phase CML cases. Although IM has proven to be an excellent treatment option for patients with CML, it was found that the emergence of resistance or intolerance to treatment might affect up to one third of patients [24]. Furthermore, some patients do not respond at the beginning of treatment or may never reach a complete hematologic, cytogenetic or molecular response. This is known as primary resistance to IM. Other patients, who initially respond to treatment, may lose response after a certain period of time and this is called secondary resistance [24]. The mechanisms of resistance are related to the occurrence of point-mutations in the catalytic domain of BCR/Abl, preventing the binding of drug [21], to the overexpression of the protein [25], or even to its suppression [26]. Subsequent researches led to the development of second-generation drugs, such as Dasatinib®, Nilotinib®, Bosutinib®, with the same

mechanism of action of IM, but endowed with molecular features which make them able to bind mutated IM-resistant forms of BCR/$Abl_{protein}$, and with a pharmacological potency 30- to 300-fold increased. The unique mutation-covering profiles of BCR/$Abl_{protein}$ inhibitors resulted in the development of protocols based on their combination. Further progress being made towards the development of a 'global' pan-BCR/Abl inhibitor that inhibits the full spectrum of identified Imatinib-resistant BCR-Abl point mutants. However, the potential for the emergence of new drug-resistant point mutations of BCR/$Abl_{protein}$ still exists. This justifies the continued development of more potent BCR/$Abl_{protein}$ inhibitors. However, even when the treatment with inhibitors of BCR/$Abl_{protein}$ is very effective in inducing remission, many patients undergo relapse of disease.

Leukemia and Hematopoietic Stem Cells

Hematopoiesis is a term used to describe the process of blood cell formation during both the embryonic and the adult stages of an organism.

All mature circulating blood cells derive from a relatively small number of Haematopoietic Stem Cells (HSC) via a series of intermediate progenitors displaying progressively increasing proliferation rate, lineage commitment and differentiation (**Figure IV**). Such an organization allows to produce a high number of mature cells starting from an extremely low number of stem cells and via a limited number of replications of stem cells. This protects the stem cell pool as much as possible from the risk of mutations, that typically occurs during cell replication.

In fetal and adult mammals, HSC reside predominantly in Fetal Liver (FL) and BM, respectively. In particular, human hematopoiesis is restricted to the proximal regions of long bones, cranium, sternum, ribs, vertebrae and ilium. The HSC sub-population in BM accounts to less than 0.01% of the total number of hematopoietic cells.

The original pool of HSC is formed during embryogenesis through a complex developmental process that involves several anatomical sites such the Yolk Sac, the Aorta-Gonad-Mesonephros region, the placenta. HSC then home transiently in FL, before being definitively established in BM and thymus. Recent work showed that HSC in the zebrafish embryo arise directly from haemogenic endothelium, lining the ventral wall of dorsal aorta. In particular, HSC seems to

emerge directly from the aortic floor, through a process that does not involve cell division but a strong bending. Consequently, a single endothelial cell finds its way out from the aortic ventral wall into the sub-aortic space, with its concomitant transformation into a hematopoietic cell. The process is polarized not only in the dorso-ventral, but also in the rostro-caudal versus medio-lateral direction and depends on Runx1 expression. During this cell transition, called the Endothelial-Haematopoietic Transition (EHT), cells express CD41, the earliest marker distinguishing blood progenitors from endothelial cells/progenitors. In mouse, these cells (expressing Sca1, c-Kit and CD41) emerge in the lumen of the aorta, budding directly from ventral CD31+/CD41- endothelial cells [27-29].

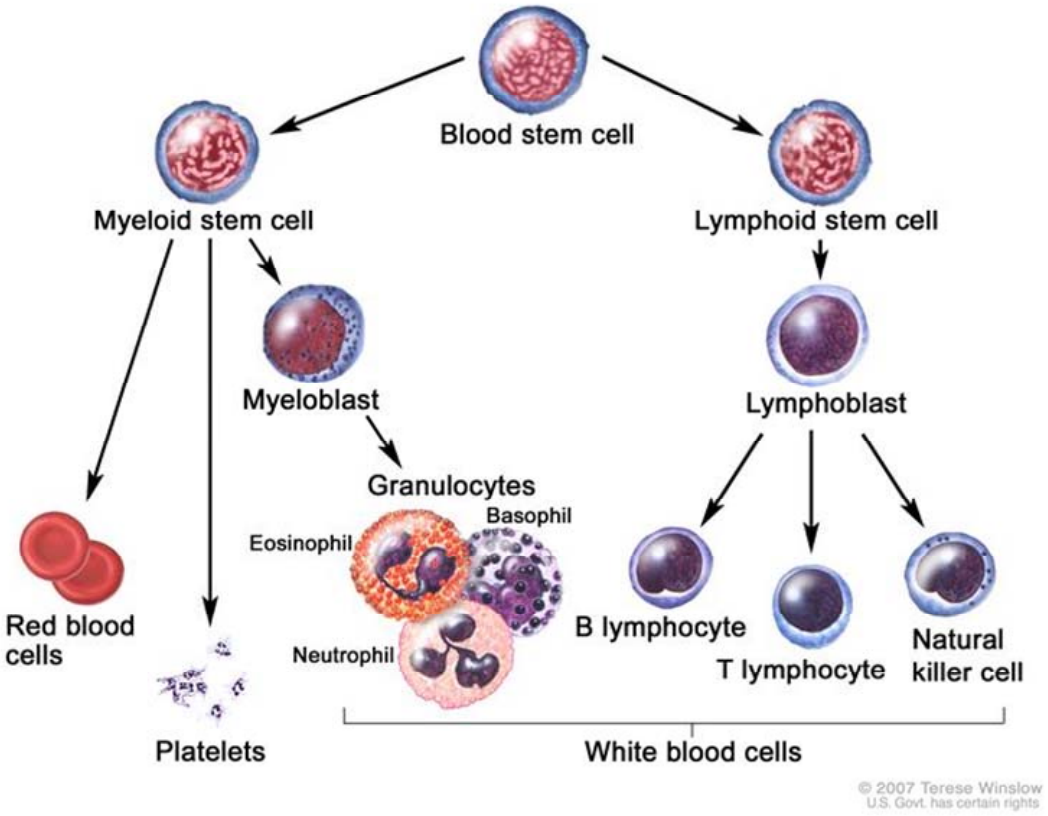


Figure IV. Blood cell development. Haematopopietic stem cell (blood stem cell in the pisture) goes through several steps to become a red blood cell, a platelet or a white blood cell. © 2007 Terese Winslow, National Cancer Institute.

The hierarchical model of hematopoiesis has provided a paradigm for the development and regeneration of other tissues as well as leukemogenesis, and, more in general, tumorigenesis. HSC self-renewal and concomitant clonal expansion are ensured by symmetric or asymmetric cell division: in the first case, molecular determinants of cell fate are redistributed unequally to the two daughter cells, while in the second case one daughter cell leaves the “niche” that

sustains HSC self-renewal and is then exposed to an environment that promotes clonal expansion and lineage commitment [30].

The stem cells of highest rank are the Long-Term-Repopulating (LTR)-HSC, able to completely reconstitute the hematopoiesis in the bone of a sub-lethally irradiated recipient. The LTR-HSC may undergo five different fates:

— *stem cell amplification*, through a symmetrical division which generates daughter cells both with LTR-HSC properties; this is the maximal expression of self-renewal;

— *clonal expansion*, through a symmetrical division which generates daughter cells both committed to tissue regeneration; these are called Short-Term Repopulating (STR)-HSC, are multipotent but with self-renewal ability limited to 6-8 weeks, which means that they are temporarily capable of maintenance but not amplification (see next paragraph); STR-HSC generate Haematopoietic Progenitor Cells (HPC) committed to the myeloid or lymphoid lineages; the differentiative potential of HPC is further and progressively restricted during clonal expansion, eventually to a single differentiative hematopoietic line [31];

— *stem cell maintenance/clonal expansion*, through an asymmetrical division in which one daughter cell remains LTR-HSC while the other becomes STR-HSC [32, 33]; in this case, self-renewal is limited to LTR-HSC maintenance, instead of driving LTR-HSC amplification;

— *induction of reversible quiescence*, when LTR-HSC exits the replicative cycle to remain in the G0 phase for an indefinite time;

— *induction of apoptosis*.

Normal HSC were the first stem cells described by Till and McCulloch in 1961. Likewise, Leukemic Stem Cells (LSC) were the first Cancer Stem Cells (CSC) described, in 1994, by Dick and co-workers, who dissociated LSC from the bulk of Acute Myeloid Leukaemia (AML) cells.

Leukemias are clonal disorders of hematopoiesis where a normal hematopoietic cell acquires mutations that confer the capacity for unlimited self-renewal, enhanced proliferation and impaired differentiation. LSC were discovered within the CD34+/CD38- sub-population as cells able to transmit AML to Non Obese Diabetic-Severe Combined Immuno-deficient (NOD-SCID) mice. By contrast, CD34+/CD38+ AML cells were found not competent in engrafting NOD-SCID mice, indicating that leukemic, like normal, hematopoiesis

is hierarchically organized and that LSC, like HSC, exhibit peculiar and detectable phenotypes [34-36] (**Figure V**).

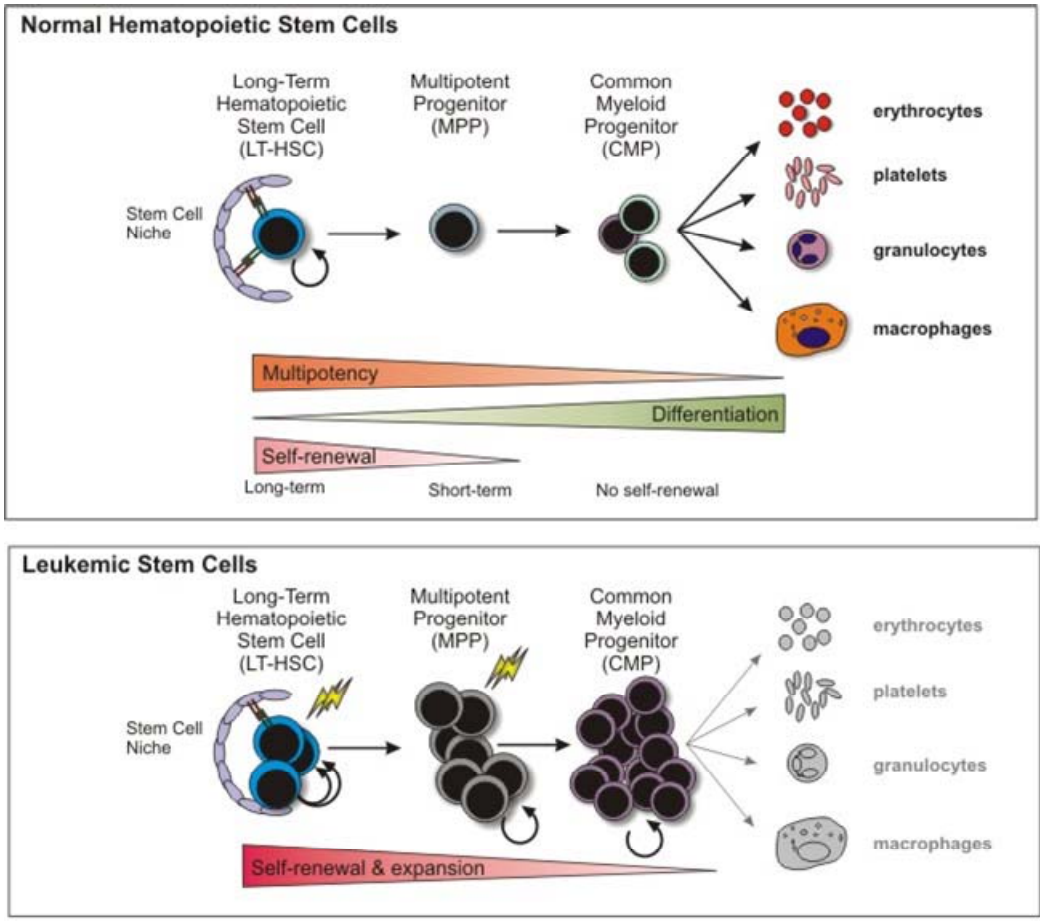


Figure V. Self-renewal and differentiation of normal and leukemic stem cells. Reya T. *et al.*, Nature 2001, 414, 105-111.

In the last years, a growing body of evidence suggested that a primitive population of cancer cells escaped the normal control of self-renewal, resulting in unrestrained clonal expansion. These studies led to the formulation of the CSC hypothesis, which postulates that tumors are maintained by a small minority of stem-like cancer cells, which possess the capacity for indefinite self-renewal. Among the cancer cell subsets, only CSC would have sufficient self-renewal capacity to maintain tumor growth while generating all the phenotypes typical of the cancer cell population. CSC could originate from the neoplastic transformation of a normal stem cell that physiologically possesses the capacity of self-renewal, or from a non-stem cell which acquires this capacity aberrantly as a consequence of oncogenesis. In any case, CSC are likely responsible for the primary resistance to therapy as well as for the maintenance of the so-called

Minimal Residual Disease (MRD) after successful response to treatment, and thereby late relapse of disease.

An alternative hypothesis, the stochastic model, could also explain the heterogeneous potential of tumor cells to self-renew. This model predicts that all cancer cells have the potential to self-renew and recapitulate all the tumor phenotypes, but that the probability that any particular tumor cell finds its way to reproduce the whole cancer varies strongly. Indeed, the functional assessment of a CSC requires not only the ability to form a new stem cell, but also to recapitulate precisely the phenotype of the initial disease, *i.e.* to balance self-renewal, proliferation and differentiation.

Human CSC are studied by three strategies. First, in animal models, evaluating transplanted cells on the basis of the capacity to engraft *in vivo* and pattern of engraftment for instance, the Marrow-Repopulating Ability (MRA) assay. Second, *in vitro*, by establishing developmental/functional assays, such as the Long-Term Culture Initiating Cell (LTC-IC) assays, the Culture-Repopulating Ability (CRA) assays [37] and the tumor sphere assays. Third, *in vitro,* by tracking detectable acquired mutations, oncogenes or normal lineage-specific genetic markers (*e.g.* the B cell receptor) back in the cellular hierarchy at the single cell level. This strategy is based on the idea that a single cell acquires a rearrangement or mutation, which is transmitted to the progeny and becomes detectable within the end-stage populations following clonal expansion. The assumption is that any cell surviving an acquired genetic event, at any step of the disease, may be responsible for a specific step of progression. However, this cell would be a CSC only if self-renewal is maintained, resulting in a sustained clonal expansion and making the mutations detectable as a part of the genetic profile of end-stage tumor cells. *In vitro* assays can be adapted to mimic individual conditions of the *in vivo* microenvironment.

The LSC have been described as HSC-like cells. However, in other BM disorders like multiple myeloma, it is likely that the cell of origin is a more differentiated cell, such as the post-germinal memory B cell or plasmablast. Likewise (see above), LSC can derive from either HSC or HPC, so that the phenotype of LSC usually matches that of the stage of differentiation at which oncogenic mutation occurs and self-renewal is acquired.

The hematopoietic stem cells niche

Tissue regeneration takes place in stages and self-renewal, commitment, clonal expansion, differentiation and maturation are under close regulation of a number of environmental stimuli, such as soluble cytokines, molecules expressed on the surface of stromal cells and molecules of the extracellular matrix [38]. Cells respond to these stimuli via the progressive expression or repression of transcriptional regulators, receptors for cytokines and autocrine cytokines. The production of mature cells in types and numbers adequate to meet the peripheral requests depends on the proper integration of all these factors [39, 40]. HSC self-renewal, for instance, requires a specific microenvironment, known as "stem cell niche", characterized by the presence of appropriate cell elements and signaling molecules.

The concept of the stem cell niche was first proposed in 1978 [41] for the human hematopoietic system by Schofield, who predicted that HSC would be protected from microenvironmental stimuli inducing commitment to differentiation within privileged tissue sites where the maintenance of their stem cell potential would be ensured.

To leave the stem cell niche was hypothesized to be necessary for the HSC progeny to undergo clonal expansion. These predictions were based on the observation that HSC need to reside in the BM to retain their "infinite" stem potential, whereas HSC homing in the spleen are more restricted in their capacity to sustain hematopoiesis [42-44]. Although the idea of the niche was proposed for HSC, the first stem cell niches experimentally characterized were in *Drosophila* gonads and in *Caenorhabditis elegans*.

Today, stem cells and their niches have been identified in a number of mammalian tissues such as muscle, central nervous system, intestinal epithelium, bulge region of the hair follicle, interfollicular epidermis, testis and BM [45, 46].

HSC niches in BM consist of non-hematopoietic cells including endothelial cells, fibroblasts, adipocytes, macrophages and osteoblasts. Evidences suggest the existence of two types of BM niches: the "osteoblastic niche", located near the endosteum, and the vascular niche, located in the sinusoidal vasculature. Although the functional differences between these niches, and whether they are spatially distinct or not, have not been completely elucidated yet, experimental

evidences indicate that both types of niches participate in hematopoiesis, probably playing a complementary role [47-50]. In particular, the osteoblastic niche, which is a hypoxic niche, is best suited to maintain hematopoietic stem cells in a quiescent (slow cycling or G0) state, whereas the vascular niche, in better oxygenated areas, supports stem/progenitor cells which are actively proliferating [51]. Quiescence is critical for sustaining stem cell compartment. When it is disrupted, as occurs with p21cip1 deficiency, HSC cannot remain in G0 and the long-term repopulating ability is lost [52]. In addition, quiescent HSC are resistant to 5-fluorouracil (5-FU)-induced myelosuppression [48], suggesting that the quiescence of HSC is closely associated with the protection of the HSC pool from the various stresses induced by myelotoxic insults.

The osteoblast-HSC conjunction retains the HSC in a microenvironment rich in modulation factors as transforming Growth Factor-β (TGF-β), Angiopoietin-1 (Ang-1) and Fibroblast Growth Factor-1 (FGF-1). TGF-β is a negative regulator of HSC cycling, effect obtained by inhibiting cell surface expression of growth factor receptor molecules including c-Kit, Llt-3, Mpl and IL-6R. Ang-1 acts on tyrosine kinase receptor Tie-2, enhancing the adhesion of HSC to the niche and thus increasing the probability of HSC to remain in a quiescent state. Both Ang-1 and TGF-β act on the SMAD transcription complex that increases the expression of cell cycle inhibitors like p27 and p57 and reduces that of IL-6R and Flt3 (involved in the amplification of HSC), resulting in the maintenance of HSC quiescence. FGF-1 acts on FGF receptors (FGFR)-1, -2, -3, and -4, which, via MAPK, STAT and PI3K, activate signaling pathways leading (in mice) to HSC clonal expansion [53, 54].

HSC adhesion to osteoblasts is mediated by adhesion molecules such as the Very Late Antigens (VLA) VLA-4 and VLA-5, integrins (the Vascular Cell Adhesion Molecule-1; VCAM-1) and N-Cadherin. The N-Cadherin creates, on the inner face of plasma membrane, a heterodimeric complex with β-catenin, which is involved, together with Wnt, in cell cycle regulation. In the presence of Wnt (also secreted by osteoblasts), β-catenin is preserved from degradation and can migrate into the nucleus, where it binds to transcription factors such as Tcf/Lef. The complex β-catenin/Tcf regulates the expression of cell cycle genes like c-Myc and cyclin-D1 [55, 56]. The Wnt/β-catenin pathway has been shown involved in the maintenance and amplification of murine HSC [57, 58].

It has been hypothesized that, under certain conditions, some matrix metallo-proteinases (MMP), such as MMP9 and ADAM10, cleave the N-cadherin bound to the membrane, increasing the cytosolic portion of β-catenin that can interact with the Wnt pathway [59]. Probably, the accumulation of β-catenin in the nucleus is the hinge factor [60] that regulates the passage of stem cells from quiescence, when they are docked to the niche [61, 62], to the activated state [63, 64]. The integrins VLA-4 and VLA-5, in addiction to physically anchor HSC to the niche, are also effective activators of small GTPases RAC1 and RAC2, whose removal causes HSC mobilization. Moreover, the lack of VLA-4 expression reduces the self-renewal and the hematopoietic repopulating activity of HSC [65].

Osteoblasts also act on HSC through the Notch signaling pathway. The Notch pathway is activated by the interaction of iuxtacrine ligands (Jagged-1, Jagged-2, Delta-1, Delta-3, Delta-4) with a Notch receptor (4 types: -1 to -4). This fact determines the cleavage of an intracellular domain of Notch, that translocates to the nucleus where it forms a DNA binding complex with other transcriptional coactivators such as MAML, CSL, p300/CBP and ICN, thus leading to the expression of genes of the *Hes1* and *Hey2* families, involved in the control of cell fate (**Figure VI**).

Hypoxia has been shown to reinforce this signaling pathway by stabilizing the transcription complex associated with the intracellular domain of Notch. Chromatin immuno-precipitation (ChIP) experiments showed that, in cells exposed to low oxygen tensions, Hypoxia Inducible Factor (HIF)-1α, an important mediator of cell adaptation to hypoxia, is physically associated with the transcription complex of Notch, the C-terminus domain of HIF-1 directly interacting with p300/CBP.

In the stem cell niche, extracellular matrix (ECM) molecules have an active role in the maintenance of stemness. Osteopontin is very abundant in bone ECM and its deletion is sufficient to expand the number of HSC, an event that is accompanied by the increased stromal expression of Jagged-1 and Ang-1, factors that inhibit apoptosis in hematopoietic cells [66]. If osteoblasts are stimulated in the absence of osteopontin, HSC number increases. Thus the osteopontin/osteoblast axis is a factor that limits HSC amplification [67].

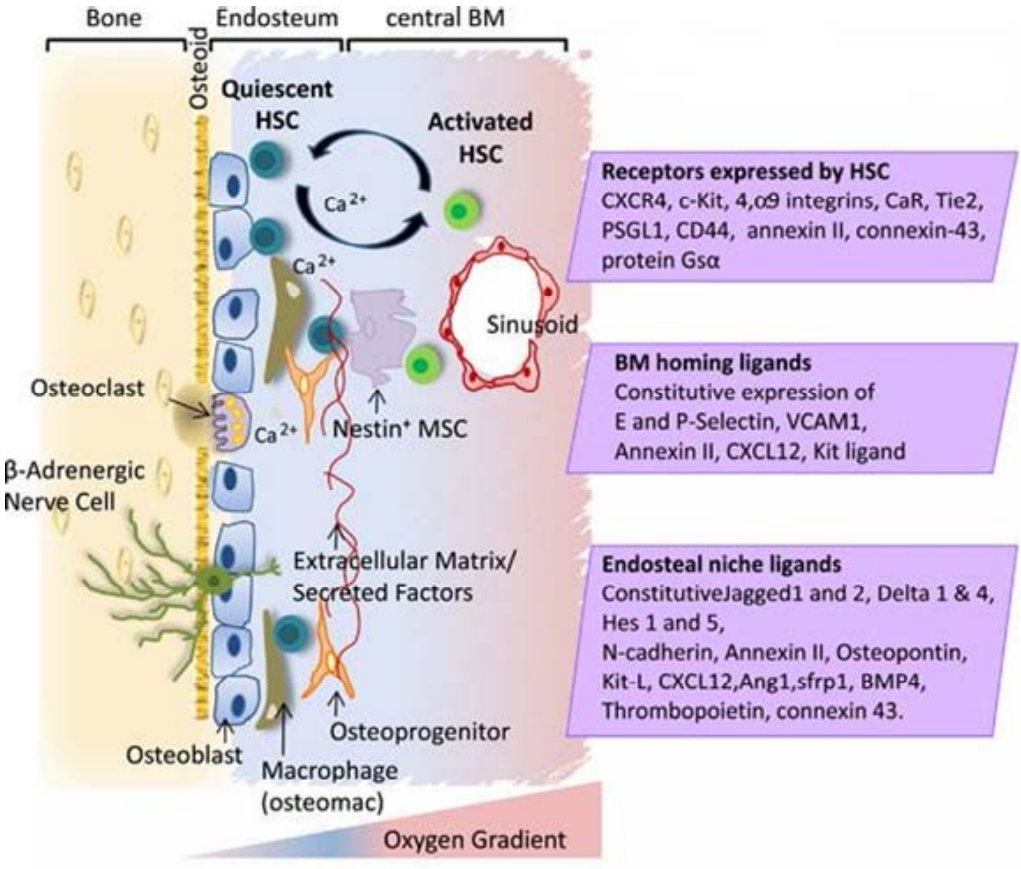


Figure VI. Molecules involved in HSC homing, lodgement and retention. Lévesque J.P. et al., Leukemia 2010; 24:1979-1992.

Two other important factors that govern the association between HSC and niche cells are Stem Cell Factor/Kit Ligand (SCF/KL) and Stromal cell Derived Factor-1 (SDF-1/CXCL12). KL exists in two forms: a membrane-bound form (mKL), expressed by niche cells, and a soluble form (sKL). The binding of mKL with its receptor c-Kit determines the maintenance of HSC within the niche [68]. As for sKL, it derives from mKL and is released following a cleavage operated by MMP9 [69]. This event results in the mobilization of HSC from the osteoblastic niche to the vascular niche. SDF-1 is a CXC chemokine (CXCL12) constitutively produced in BM by stromal cells [70]. SDF-1 is a potent chemoattractant for HSC which express its receptor CXCR4. SDF-1 regulates survival, cell cycle and especially the mobilization and homig/migration of HSC. Indeed, G-CSF, which mobilizes HSC from BM to peripheral blood, blocks the SDF-1/CXCR4 interaction. G-CSF, however, also inhibits osteoblast activity (the major producers of SDF-1 in the BM), reducing the mRNA levels of SDF-1 [71], and induces the proteolytic degradation of CXCR4 on HSC [72].

Recent observations on the HSC-niche association highlight a strong sensibility of HSC for the levels of extracellular Ca_{2+}, mediated by receptors with seven transmembrane domains, that is useful to maintain stem cells in proximity of endostium [73].

Role of hypoxia in stem cells niche

Despite the large number of blood vessels present in the osteo-muscular apparatus, the hematopoietic tissue is characterized by areas that are highly hypoxic. The presence of such zones is easily explained considering the peculiar vascular anatomy of BM: blood enters the long bones via the nutrient artery, which branches in small caliber vases only at the endostium level, which anastomose with branches of periosteal arteries. Blood then penetrates into the marrow, and crosses the hematopoietic tissue via the sinusoidal system; hence the venous blood leaves the medullary cavity through emissary vein. This implies that the hematopoietic tissue is mostly supplied by blood that has already supplied other tissues, and is therefore relatively oxygen-poor.

Measurements of medullary oxygen tension revealed that it does not exceed 40 mmHg. Such a scarce supply of oxygen is made worse by the fact that hematopoietic cells are extremely active in biosynthesis and they are high-density packed in an environment extremely poor of extracellular material. This situation highlights the competition among cellular phenotypes more or less resistant to hypoxia.

In vitro studies have demonstrated how the oxygen levels is a relevant factor for the physiological regulation of the hematopoietic system and the maintenance of stem cell potential. STR-HSC in murine and human hematopoietic populations are maintained in deep hypoxia (1% O_2) better that in normoxia. On the contrary, clonogenic progenitors such as Colony-Forming Cells (CFC) are completely suppressed in hypoxia. Thus, adaptation to hypoxia is higher, the higher the progenitor level in hematopoietic hierarchy. This fact led to the hypothesis that the peculiar resistance to hypoxia of HSC compared to less immature progenitors allows the maintenance of HSC in areas of the hematopoietic tissue characterized by particularly low oxygen tensions. In these areas, called “hypoxic stem cell niches”, hypoxia would help to maintain stem cell properties by inhibiting commitment to differentiation and the generation of line-restricted progenitors [74, 75] (**Figure VII**). Further studies confirmed that low oxygen tensions induce

apoptosis in cells of lower rank and simultaneously allow the maintenance of HSC capable of short-term hematopoietic reconstitution [76].

Hypoxia does not suppress HSC proliferation, rather modulating it in favor of the maintenance or amplification of the stem cell compartment. This is driven via the modulation operated by hypoxia on the effects of other components of microenvironment, such as cytokines. The most important are Interleukin-3 (IL-3) and Vascular-Endothelial Growth Factor (VEGF), the expression of the latter being controlled, in response to hypoxia, by HIF-α. Interestingly, VEGF is active directly on hematopoietic cells [77]. It has been shown in our laboratory that IL-3, when left uncontrolled, increases the percentage of CD34+ STR-HSC that replicate more than once and that therefore are committed to a rapid reduction of their self-renewal potential [78, 79]. Under hypoxic pressure, on the contrary, cycling of STR-HSC is limited to one cycle, which favors their maintenance. We also found stem cell potential is maintained in hypoxia even when VEGF is the only growth factor added to the system. This seem to indicate that a factor typically induced in hypoxia is capable to support HSC survival. Interestingly, the effect of VEGF is also inhibited by IL3, indicating that factors capable to stimulate clonal expansion antagonize those sustaining the maintenance of stem cell potential within the hypoxic niches.

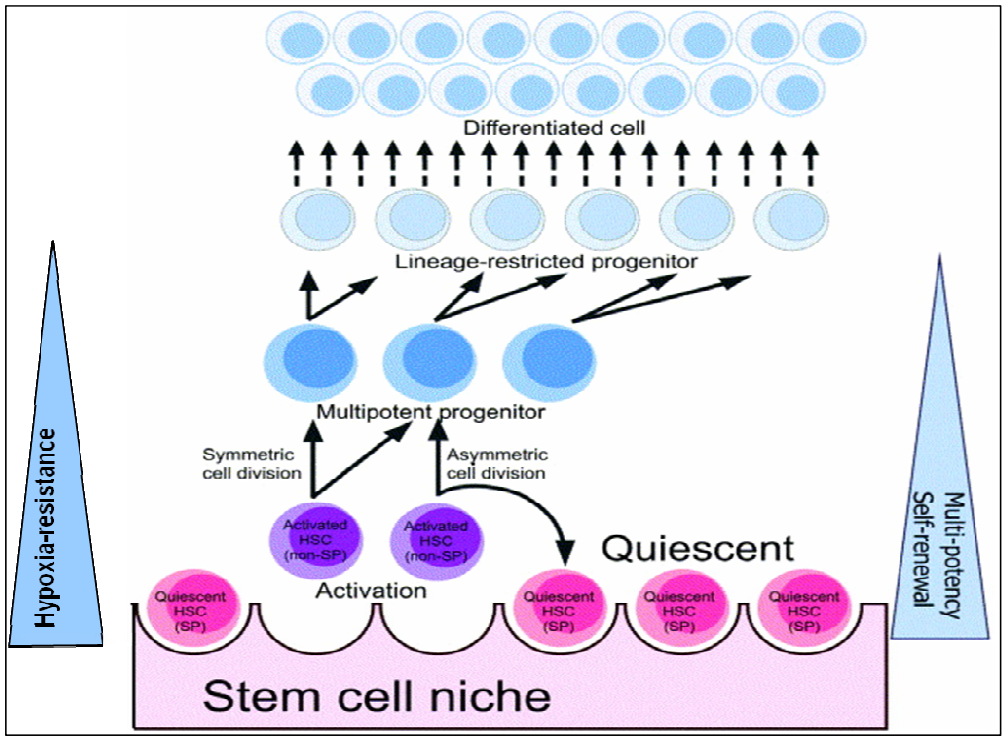


Figure VII. The HSC niche. Modified from Suda T. *et al.*, Trends Immunol. 2005; 26:426-433.

Role of hypoxia in leukemia

In the human body, physiologically, the cells are located within a few cell diameters of a blood vessel, to ensure an adequate delivery of oxygen and nutrients to tissue. This organization is often lost in solid tumors, due to the massive cancer cell proliferation, that increases the distance of cells from the closest blood vessel, leading to the development of hypoxic and acidic region within tumor mass [80-83]. Cancer cells undergo genetic, epigenetic and adaptive changes that allow them to survive and even proliferate in a hypoxic environment. These processes contribute to the malignant phenotype and to the aggressive tumor behavior.

It was first reported nearly 50 years ago that the growth of human tumors is organized around blood vessels [84]. Tissue areas located more than 180 μm away from blood vessel are necrotic ; this is similar to the calculated distance that oxygen diffuses as it passes from the capillary to cells before it is completely metabolized. Such an "anatomical" limit to oxygen diffusion was termed "diffusion-limited" or "chronic" hypoxia.

Another type of hypoxia, known as "acute" or "perfusion-limited" hypoxia, occurs when normal or aberrant blood vessels are rapidly shut off, which can also cause blood flow to be reversed [85]. Closed vessels can be reopened, leading to reperfusion of hypoxic tissue with oxygenated blood. This leads to an increase in free-radical concentrations, tissue damage and activation of stress-response genes, a process known as "reoxygenation injury" [86].

These two different states (chronic *vs.* acute hypoxia) generate different situations for cancer cells. In any case, the neoplastic population is continuously exposed to the effects of hypoxic/ischemic environments with the following consequences: (a) selection of hypoxia-resistant clones, either arising spontaneously in the course of neoplastic progression, or driven by hypoxia itself, which is capable of acting as a potent gene modulator and is a major source of genetic instability in tumor populations; (b) the creation of hypoxic niches, in which only cancer cells selected for their adaptation to hypoxia (but not on a genetic basis) can survive; to home in these niches, the stem cell pool is given an advantage by its resistance to hypoxia.

Adaptation to hypoxia is also one of the main aspects of the leukemogenesis. Oxygen tension in BM is constitutively much lower than in most other normal

tissues, due to both the peculiar structure of vascularization and the high level of cell crowding, factors which favor the onset of hypoxic areas within cell conglomerates. Oxygen availability in leukemic BM is possibly even lower, due to the a further increase of cell density determined by the unrestrained expansion of neoplastic clones. On the other hand, a number of studies carried out in our laboratory showed that LSC are able to adapt to severe hypoxia [26, 87-89], as they retain many characteristics of HSC. However, the relevance of hypoxia to leukemia progression is not as clear as in solid tumors.

Leukemias are often characterized by the expression of a peculiar oncogenetic protein responsible for the pathogenesis of disease, such as BCR/Abl in CML and AML1/ETO in AML, both derived from an aberrant chromosomal rearrangement. Other signaling molecules, however, contribute to the onset of disease, including HIF. HIF is overexpressed in many types of leukemias, such as ALL [90] and CML. Recent studies show that the chemokine receptor CXCR4 and its ligand SDF-1 are up-regulated in AML as well as in niche cells, suggesting that LSC of AML may be recruited to home in stem cell niches where HSC physiologically reside. As CXCR4 expression is regulated by HIF-1α, hypoxia appears to contribute to LSC maintenance, thereby predisposing disease to progression [91, 92]. VEGF is also involved in blood cell cancer, as it stimulates proliferation and the expression of anti-apoptotic proteins such BCL2.

Our laboratory showed not only that resistance to hypoxia is a common feature of HSC and LSC, but also that a hypoxia-selectable LSC subset is likely to exists within any type of leukemia, including stabilized clonal cells lines [26, 87-89]. In CML, either cell lines or CD34+ cells explanted from CML patients, hypoxia completely suppresses the expression of $BCR/Abl_{protein}$ [26, 93] but not that of BCR/Abl transcript, so that hypoxia-resistant cells, while remaining genetically leukemic, are independent of BCR/Abl signaling for their maintenance *in vitro* and resistant to treatment with IM. Thus, hypoxia-selectable LSC are refractory to IM due to the lack of its molecular target. This is very well in keeping with the notion that IM, despite its impressive efficacy as first-line therapy for patients with chronic-phase CML, induces a state of MRD, rather than cure, and that LSC are the source of MRD.

The Extracellular signal-Regulated Kinase 5

The Extracellular signal-Regulated Kinase 5 (ERK5, also referred to as BMK1, Big Mitogen activated Kinase 1) is a member of the Mitogen-Activated Protein Kinase (MAPK) family. It is expressed in many tissue types, but is particularly abundant in the heart, skeletal muscle, placenta, lung and kidney [94, 95]. ERK5 is also expressed in a number of different cell lines and is localized in both nuclear and cytoplasmic compartments [96].

Initially identified as a stress-activated MAPK activated by both oxidative and osmotic stresses [97], ERK5 was then shown to be stimulated by a plethora of extracellular stimuli, such as VEGF, epidermal growth factor (EGF), FGF-2 and platelet-derived growth factor [98-100]. ERK5 is also activated by trophic factors in neurons, namely brain-derived neurotrophic factor, nerve growth factor (NGF) and some inflammatory cytokines, such as IL-6 [101-104]. Furthermore, physiological and pathological conditions including laminar shear-stress, ischemia and hypoxia are also able to activate ERK5 [97, 105-107]. ERK5 is involved in cell survival/apoptosis, motility, differentiation and proliferation [108-111]. *In vivo* studies have indicated that ERK5 may support vascular endothelial viability in the adult animal [112] and critically participates in embryogenesis, probably due to its role in proliferation and angio/vasculogenesis [113-115].

Activation of ERK5 occurs by dual phosphorylation at the threonine (Thr218) and tyrosine (Tyr220) residues present in the conserved Threonine-Glutamic Acid-Tyrosine (TEY) motif of the activation loop [98]. Phosphorylation at these sites is carried out by the upstream MAPK *kinase 5* (MKK5, MEK5), that is specific for ERK5 [95]. How activated receptors couple to ERK5 is still unclear. Growth factor receptors may cause ERK5 activation through Ras in certain cell lines [116, 117], but not in others [98]. In addition, other intracellular kinases, such as MAPK kinase kinase 2/3 (MKKK2/3, MEKK2/3) [118], c-Cot [119] and c-Src [120] may also regulate the activation of ERK5 cascade.

The human *ERK5* gene (also called *MAPK7*) totals 5824 bases in length, with an open reading frame of 2451 bp that encodes for a protein of 816 amino acids. Gene analysis reveals that the mouse *ERK5 gene* is encoded for by six exons and five introns spanning ~5.6 kb. Alternative splicing across introns 1 and/or 2 generates three transcriptional variants (*mERK5-a*, *-b* and *–c*), the net effect of which is the translation of a peptide that lacks the kinase domain. It has been

demonstrated that the expression of *mERK5-b* and *-c* impinges on the function of the full-length mERK5 protein product via a dominant/negative effect [121]. A premature in-frame stop codon introduced by failure to splice intron 4 gives rise to another mouse splice variant, *mERK5-t*, which encodes for a protein truncated at the C-terminal end unable to translocate to the nucleus [122]. Together, these data suggest that the regulation of ERK5 signaling may be mediated, at least in part, at the level of RNA processing.

The ERK5 protein contains an N-terminal kinase domain and a large C-terminal extension (**Figure VIII**). The N-terminus (amino acids 1-406) begins with a region required for cytoplasmic targeting (a.a. 1-77), followed by a kinase domain (a.a. 78-406) which shares 66% sequence identity to the kinase domain of ERK2 [95]. The kinase domain can be further separated into a region essential for MEK5 interaction (a.a. 78-139) and for oligomerisation (a.a. 140-406) [121]. Like other MAPK, ERK5 contains a common docking (CD) domain, consisting of a short sequence of negatively-charged amino acid residues (a.a. 350-358), which allows ERK5 to form associations with certain docking (D)-domain-containing substrates [123].

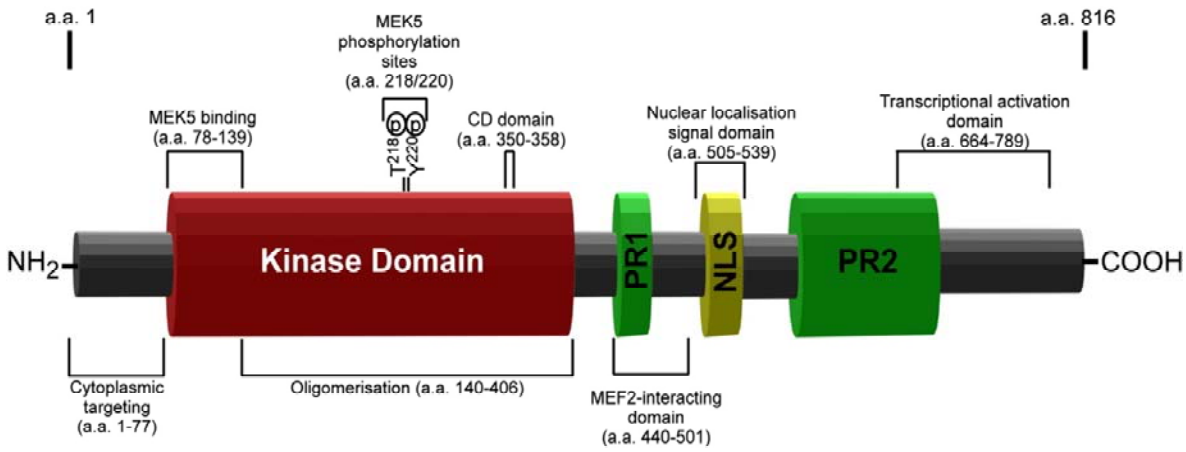


Figure VIII. Structure and functional domains of ERK5. Gopika *et al.*, Cellular Signalling 2012, 24:2187-2196.

Compared to the "classical" MAPK (namely ERK1 and 2, p38 and JNK) ERK5 has a unique, extended C-terminal tail of 410 amino acids, giving it a molecular weight of approximately 102 kDa, which makes it more than twice the molecular weight of many other MAPK [95]. The C-terminal domain includes an NLS

domain (a.a. 505-539), important for ERK5 nuclear targeting [96, 121], two proline-rich (PR) domains termed PR1 (a.a. 434-465) and PR2 (a.a. 578-701), which are considered to be potential binding sites for Src-homology 3 (SH3)-domain-containing proteins [95, 121], and a myocyte enhancer factor 2 (MEF2)-interacting region (a.a. 440-501) [121]. Recently, a potent transcriptional activation domain (a.a. 664-789) was identified [124], which undergoes autophosphorylation, thus enabling ERK5 to directly regulate gene transcription, which is a unique ability of ERK5 amongst MAPK [125]. Furthermore, it has been shown that truncation of this C-terminal tail gives rise to increased kinase activity of ERK5, suggesting that the tail has an auto-inhibitory function [96].

Nucleo/cytoplasmic transport of signaling molecules is essential for the regulation of eukaryotic cellular processes such as cell cycle progression, differentiation, and circadian clocks [126, 127]. In the context of MAPK signaling, relocalization of MAPK from cytosol to nucleus is required for the phosphorylation and activation of transcription factors, that exclusively reside in nuclear compartments [128, 129]. The analysis of several cell lines indicated that endogenous ERK5 is localized to cytoplasmic as well as nuclear compartments. In the murine myoblast cell line C2C12 or breast cancer cell line MCF7, ERK5 localizes in the cytoplasm as a result of serum deprivation and translocates to the nucleus in response to FGF or neuregulin. In HeLa cells, ERK5 is localized in the nucleus even in the absence of stimulation, but treatment with EGF causes an increase of ERK5 nuclear localization [130]. When ERK5 is overexpressed in COS-7 cells, ERK5-specific staining is predominantly cytoplasmic; however, deletion of the last hundred amino acids resulted in nuclear accumulation of ERK5. Further deletions of most or all of the ERK5 tail suppresses the predominance of nuclear localization, resulting in an equal distribution in cytosol and nucleus. The nuclear localization of truncated ERK5 is MEK5-dependent, because mutations of the two phosphorylation sites for MEK5 resulted in relocalization of ERK5 to the cytosolic compartment. The distribution of the mutants can be explained by the presence of functional nuclear import and export sequences in the tail region.

Yan *et al.* identified a functional NLS between amino acids 505 and 539 [121]. Sensitivity of wild-type ERK5 to leptomycin B, which is lost in the 409 mutant, further shows that the tail is required for a NES-dependent export mechanism. This result further indicates that wild-type ERK5 is continuously shuttling between nucleus and cytosol.

A simplified model put forward by Bushbeck and Ullrich predicts that ERK5 exists in "closed" and "open" conformations [131] (**Figure IX**). Under steady-state conditions and in the absence of any factor, the majority of ERK5 is assumed to adopt the closed conformation. Under this condition, ERK5 is predominantly cytosolic and its activation and phosphorylation by MEK5 is hampered. This is related to the masking of the strong NLS and the exposure of the weak NES (or coexport with an NES-containing protein). The open conformation of ERK5, on the other hand, facilitates its activation and is stabilized by activating phosphorylations. In this cases, the exposure of the strong NLS overrides that of the weak NES, resulting inERK5 nuclear accumulation. Dephosphorylation of ERK5 by phosphatases triggers the return to the closed/inactive/cytosolic state. Later, it was shown that the closed conformation is stabilized by the interaction between the C-terminal and N-terminal tails; the dissociation of the two ends, induced by activating phosphorylation by MEK5 [130], in fact, promotes the nuclear translocation of ERK5 and inhibits its export from the nucleus, in keeping with the model of Figure IX. For the active nuclear export of ERK5, therefore, interaction is required between the two ends of this MAPK that can work from their own NES or create the docking site for other proteins that contain a NES.

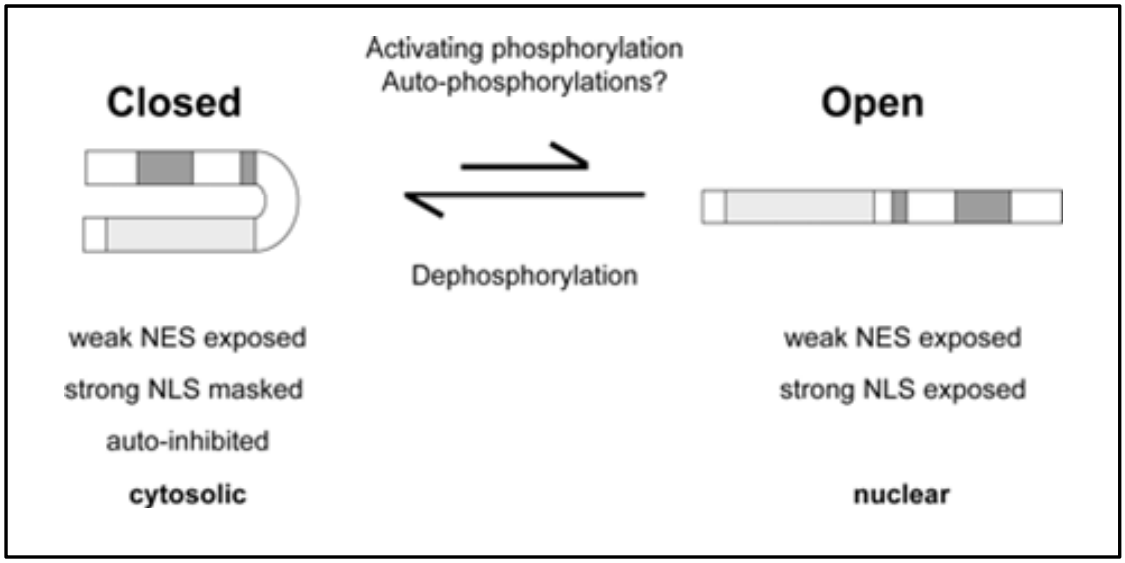


Figure IX. Two hypothetical conformations of ERK5. Buschbeck M, Ullrich A, J Biol Chem 2005, 280:2659-2667.

Once ERK5 has been imported into the nucleus, it associates with, phosphorylates and activates many transcriptional factors of which the MEF-2 family members (MEF2A, C and D) are the best characterized [132-134]. ERK5

phosphorylates MEF2C on Ser^{387}, which in turn increases MEF2C transcriptional activity, subsequently increasing gene expression of c-Jun [130]. It has been shown that MEF2D is an ERK5-specific substrate [133, 134], whereas the activities of MEF2A and C are controlled by both ERK5 and the p38 MAPK [135, 136]. As previously mentioned, ERK5 contains a MEF2-interacting region and a transcriptional activation domain in its C-terminal tail, both of which are vital for regulating MEF2 activity [124], as a truncated C-terminal ERK5 mutant lacks the ability to stimulate MEF2 activity [121].

In addition to the MEF2 family of substrates, ERK5 is also able to directly enhance the transcription of c-Myc, CREB and Sap1a [117, 137], the latter of which occurs via a serum response element that may also be involved in activating the c-Fos promoter. Furthermore, it was shown that, while both ERK5 and ERK1/2 are capable of phosphorylating c-Fos on Ser^{387}, ERK5 activation leads to phosphorylation of alternative sites on c-Fos, leading to maximal c-Fos transactivation activity; phosphorylation of these sites in c-Fos requires the C-terminal tail of ERK5 [138].

Like other MAPK, ERK5 phosphorylates substrates on Ser/Thr residues immediately preceding a Pro residue. Intriguingly, residue Thr^{28} in the ERK5 N-terminal domain and residues Ser^{421}, Ser^{433}, Ser^{496}, Ser^{731} and Thr^{733} in the C-terminal tail are not followed by a Pro residue, but undergo autophosphorylation. Furthermore, ERK5 is capable of phosphorylating MEK5 at specific non-proline directed residues Ser^{129}, Ser^{137}, Ser^{142} and Ser^{149} [139]. Taken together, these findings suggest that the substrate specificity of ERK5 may differ from that of other MAPK family members.

ERK5 is also required to mediate the survival response of endothelial cells (EC) to shear stress [140]. Over-expression of constitutively-activated MEK5 restricts caspase-3 activity and inhibits apoptosis of EC induced by serum deprivation or inflammatory stimuli, while inhibition of ERK5 activity with a dominant/negative mutant or following gene deletion causes EC death. The mechanism by which ERK5 protects EC from apoptosis implicates the phosphorylation of Bad at Ser136 and Ser112 independently of Protein Kinase B (PKB), since dominant/negative ERK5 does not inhibit the phosphorylation of PKB in response to fluid shear stress [140]. However, some studies have shown that the ERK5 signaling pathway promotes the survival of fibroblasts by down-regulating the expression of *Fas ligand* (FasL) via the PKB-dependent inhibition

of Foxo3a activity [110] (**Figure X**). ERK5 then induces the phosphorylation of Bad and Foxo3a by PKB-independent and dependent mechanisms, respectively. In both cases, phosphorylation provides a mechanism by which ERK5 sequesters the proteins in the cytoplasm by promoting their interaction with 14-3-3, thereby blocking their apoptotic effect.

According to other studies, however, the anti-apoptotic action of ERK5 could be mediated by its nuclear accumulation; in HeLa cells, the expression of an ERK5 mutant protein ($ERK5^{\Delta 570}$), that constitutively resides in the nucleus, reduces apoptotic cell death in response to TRAIL receptor activation [141].

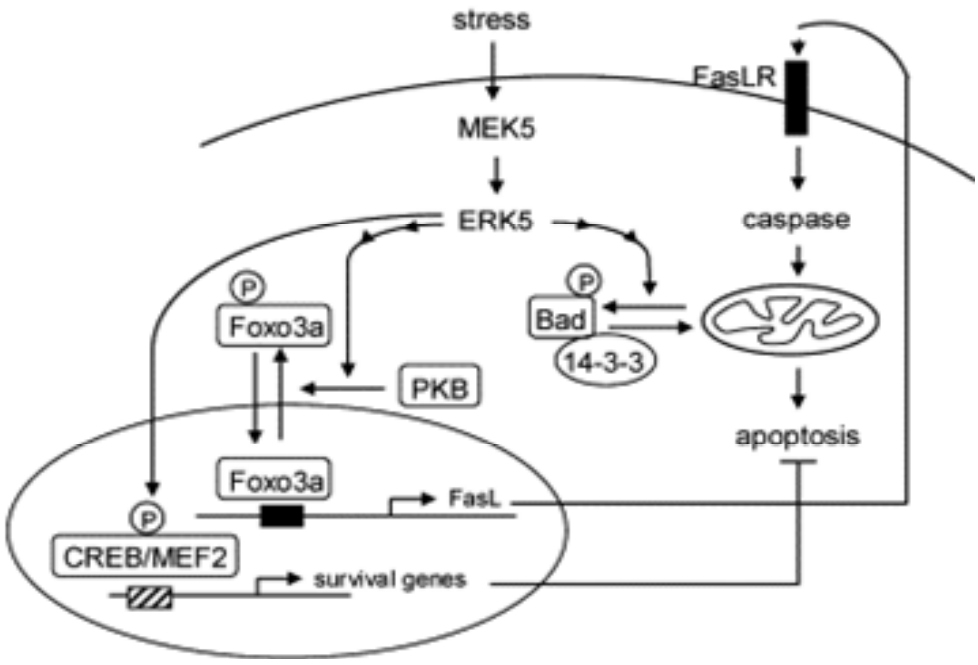


Figure X. Regulation of cell survival by the ERK5 cascade. Wang X and Tournier C, Cell Signal 2006, 18:753-760.

The ERK5 signaling pathway is important for promoting or regulating the proliferation of some cell types under certain conditions. ERK5 regulates the activation of SGK, a protein kinase that is closely linked to the G1/S transition of cell cycle [142]. The phosphorylation of SGK by ERK5 at serine 78 is required for mediating SGK activation and for promoting the entry of cells into S phase of cell cycle in response to growth factors [143]. The transcriptional activation of the *cyclin D1* gene, a key cell proliferation checkpoint, the deregulation of which is frequently associated with neoplastic transformation, has also been shown to be regulated by the ERK5 cascade. The expression of a kinase-dead mutant of ERK5 blocks the synthesis of endogenous *cyclin D1* protein induced by serum in a number of breast cancer cell lines [144]. It has also been reported that ERK5

might have a role in the regulation of G2/M transition and timely entry into mitosis. This function requires ERK5 activation of Nuclear Factor kB via Ribosomal S6 Kinase 2 [145].

ERK5 in cancer

Cancer represents a progression from normal cellular homeostasis to a neoplastic condition with the cellular acquisition of a number of defined hallmarks: sustained proliferative signaling, evasion from the control of suppressors, resistance to induction of apoptosis, onset of replicative immortality, induction of angiogenesis, acquisition of the capacity of invasion and metastasis, reprogramming of energy metabolism and resistance to immune destruction [146]. The role of MAPK, particularly ERK1/2, in cancer progression has been the focus of intense research over the last 20 years [147] and attention is now also turning to the potential role of ERK5 in cancer development and progression [148].

Oncogenic signals such as Her2, Ras, STAT3, Cot and Src are potent stimuli in activating ERK5, thereby transmitting signals leading to malignancy, including uncontrolled proliferation, transformation, antiapoptotic signals, and actin reorganization in tumor cells [105, 116, 117, 120, 137, 149-156]. For example, Cot potently stimulates the activity of the *c-jun* promoter via JNK, p38 MAPK and ERK5 to induce neoplastic transformation in NHI3T3 cells [119]. Src is involved in the formation of a functional Lad/MEKK2/MEK5 complex that regulates MEF2 activity [150]. ERK5 synergizes with ERK1/2 to promote Src-dependent cell proliferation [120, 151].

As previously mentioned, ERK5 regulates cell cycle by promoting the G1/S or G2/M transitions [144, 145]. The increased activation of ERK5 in tumor cells, therefore, promotes uncontrolled proliferation also through the overcoming of these two cell cycle checkpoints. It has also been shown that ERK5 is able to suppress the promyelocytic leukaemia protein (PML) in the PML nuclear body by direct phosphorylation on Ser^{403} and Thr^{409}, consequently preventing the upregulation of p21 expression, an important proliferation modulator [157]. Additionally, this suppression enables the cells to overcome the G1/S transition checkpoint in cell cycle progression, thereby reducing the inhibition of tumor cell proliferation [158].

In oral squamous cell carcinoma cells, high ERK5 expression was associated with an advanced tumor stage and the presence of lymph node metastases [159]. In addition, the ERK5 pathway was found constitutively active in Hodgkin lymphoma (HL) cells lines, and an upregulated ERK5 was shown to be responsible for both proliferative and antiapoptotic signals of HL cells through deregulating HOXB9 expression [160]. ERK5 activity is also important for the survival of leukemic T cells *in vivo*, as ERK5 knockdown in leukemic T cells decreases nuclear accumulation of the NFκB p65 subunit and suppresses the induction of tumors in mice [161]. Additionally, it has been shown that the ERK5 activation by the Hepatocyte Growth Factor/Scatter Factor (HGF/SF) is critical for cell proliferation of human mesothelioma (MM) cells [162]. Experimental results suggest that ERK5 takes part in the increase of MM cell viability via the upregulation of Fos-related antigen 1 (Fra-1), which is commonly overexpressed in epithelial tumors and involved in cancer invasiveness.

Several publications have shown that cancer features can be acquired by increasing the expression of MEK5. In breast cancer cells, the activated oncogene STAT3 binds to the promoter regions of MEK5 and induces transcription of MEK5, conferring a critical survival signal [153]. In metastatic prostate cancer, strong MEK5 expression is correlated with bony metastases, and less favorable prognosis is caused by upregulated ERK5-induced Activator Protein-1 activity, a consequent induction of a high level of MMP-9 and augmented invasive potential [163]. ERK5 also plays a role in tumor metastasis. For example, ERK5 is capable of promoting integrin-mediated cell adhesion and motility in cancer cells through regulating FAK signaling [164]. ERK5 activity is also known to be critical for forming Src-induced invasive adhesions, podosomes, in tumor cells by inducing RhoGAP7 and consequently limiting Rho activation [165]. Additionally, in breast tumor cells, the ERK5 pathway mediatees HGF-induced cell migration through the MET receptor/*Breast tumor kinase* (Brk) signaling module, indicating that the ERK5 cascade contributing to breast cancer progression to metastasis is activated via the HGF/MET/Brk pathway [166].

In addition to its contribution to the malignancy of tumor cells, genetic evidence that ERK5 is involved in tumor-associated angiogenesis was provided by analyzing the effect of the deletion of the *ERK5 gene* in the development of vasculature in melanoma and carcinoma xenografts [167]. The number of large blood vessels was greatly diminished in tumors growing in the flank region of the *erk5-/-* mice. The results indicate that ERK5 is involved in VEGF-mediated

survival and tubular morphogenesis of human endothelial cells, through mediation of VEGF-induced phosphorylation of both AKT and proapoptotic protein BAD, as well as by VEGF-induced increased expression of the antiapoptotic protein BCL2 [168].

Receptor tyrosine kinases of the ErbB family are activated following interaction with members of the EGF family of ligands. Over-expression or mutations of these receptors in cancer is associated with poor prognosis, shorter disease-free intervals, increased risk of metastasis, and resistance to chemotherapy. The analysis of ERK5 in human-derived tumor cell lines has demonstrated a link between the presence of constitutively-activated ERK5 and activated forms of ErbB2, ErbB3 and ErbB4 [169]. The ability of a dominant/negative mutant forms of ERK5 to decrease the growth rate of human breast cancer cells suggests that the ERK5 pathway delivers growth-promoting signals via an ErbB-dependent mechanism [169]. The activity of STAT3 is regulated by ErbB. Increased MEK5 expression in breast cancer cell lines expressing an activated form of STAT3 [153] provides a mechanism by which mutations in the *ErbB* genes may indirectly activate ERK5.

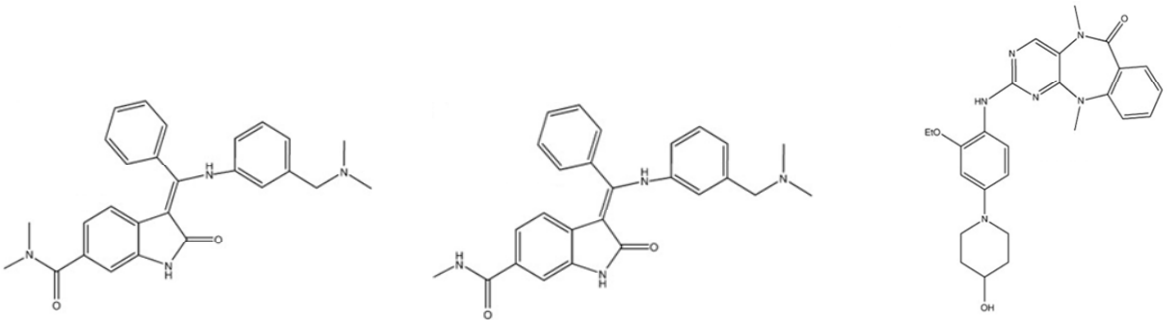
In multiple myeloma, ERK5 can be activated by cytokines, such as IL-6, produced by the stromal compartment, and which support MM cell proliferation and survival [170]. Expression of a dominant/negative form of ERK5 results in impaired proliferation of MM cells, and potentiates the antimyeloma action of drugs used for MM therapy [171].

Pharmacological inhibitors of the MEK5/ERK5 pathway

The search for small-molecule inhibitors of the MEK5/ERK5 pathway led to identify two indolinone-6-carboxamides (developed by Boehringer Ingelheim) that selectively inhibit MEK5 over MEK1/2 [172]. *In vitro*, these inhibitors, BIX02188 and BIX02189, are able to block MEK5 activity with IC50 of 4.3 nmol/L and 1.5 nmol/L, respectively. In HeLa and HEK293 cells, both osmotic stress-induced ERK5 and MEF2C activation are inhibited by treatment with this compounds [172]. In PC12 cells, the use of these compounds led to establish the involvement of MEK5/ERK5 in NGF-induced neurite outgrowth and stabilisation of tyrosine hydroxylase [173]. No data are currently available with respect to the effect of these inhibitors on growth factor-induced activation of ERK5, or to their

pharmacodynamics or pharmacokinetics. Although MEK5 is the only upstream kinase to directly phosphorylate ERK5, MEK5-independent phosphorylation of ERK5 by CDK occurs during mitosis [174], so that the MEK5-targeting with the above inhibitors may prove insufficient in inhibiting ERK5 signaling within mitotic tumor cells.

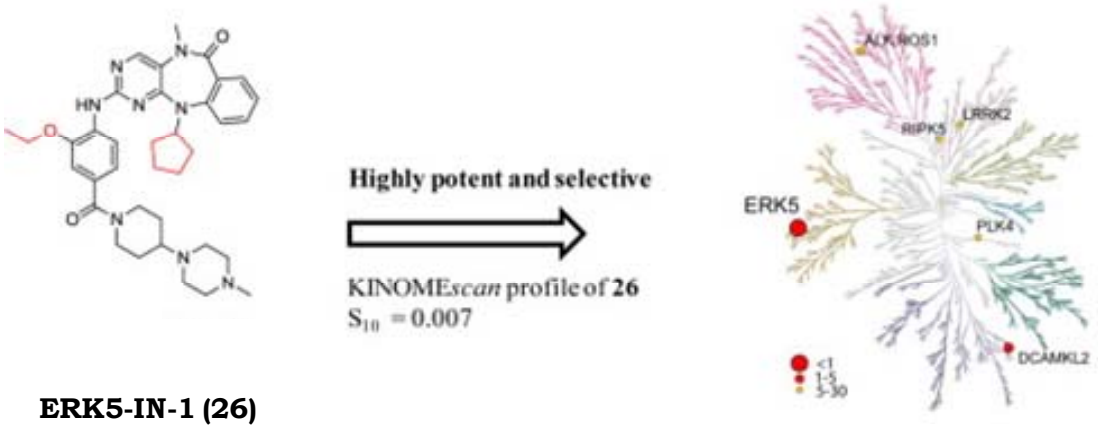
A possibility to overcome this problem may, however, come from the use of ERK5 inhibitors. XMD8-92 is a highly selective inhibitor of ERK5 activity synthesized by modifying the Polo kinase inhibitor BI-2536 [157]. XMD8-92 reduces transactivation of MEF2C, a known substrate of ERK5. It has been also demonstrated that the inhibition of ERK5 activation by XMD8-92 was indistinguishable from that operated by the expression of a dominant/negative form of ERK5 in HeLa cells. XMD8-92 also exhibits a high selectivity for ERK5. When XMD8-92 was profiled against all kinases expressed in HeLa cells, it was found to be at least 10-fold more selective for ERK5 than any other kinase studied, with an IC50 on ERK5 of 1.5 μM. In addition, XMD8-92 did not block the growth factor-induced activation of ERK 1/2 and did not significantly inhibit MEK5. The use of XMD8-92 allowed to demonstrate that ERK5 interacts with the tumor suppressor PML and to characterize the impact that this interaction has on the induction of p21, a known downstream effector of PML which modulates cell proliferation [157, 175, 176]. It was shown that ERK5 forms a complex with PML which suppresses expression of p21, thus blocking PML's anti-proliferative function. Treatment with XMD8-92 induces the expression of p21 which in turn suppresses cancer cell proliferation (**Figure XI**).



BIX02188 **BIX02189** **XMD8-92**

Figure XI. Chemical structures of MEK5/ERK5 inhibitors.

More recently, ERK5-IN-1 (26) was discovered as the most selective and potent ERK5 inhibitor reported to date [177] (**Figure XII**). It inhibits ERK5 biochemically with an IC50 of 0.162 ± 0.006 μM. Furthermore, 26 displays excellent selectivity over other kinases with a KINOMEscan selectivity score of 0.007, and exhibits exceptional bioavailability of 90% in mice. 26 therefore will serve as a valuable tool compound to investigate the ERK5 signaling pathway and as a starting point for developing an ERK5 directed therapeutic agent.



ERK5-IN-1 (26)

Figure XII. Chemical structure of ERK5-IN-1 (26), a novel ERK5 inhibitor.

Materials and Methods

Cells and culture conditions

K562, KCL22 and primary CML cells were routinely cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 4 mM glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin (all from Euro-Clone, Paington, U.K.) and incubated at 37 °C in a water-saturated atmosphere containing 5% CO_2 and 95% air. K562 [178] and KCL22 [179] are immortalized BCR/Abl-positive CML cell lines derived from patients in blast crisis. Primary cells were collected through a collaboration with the Division of Hematology of AOUC from CML patients. Informed consent for *in vitro* research and approval of the Ethics Committee of the Azienda Ospedaliero/Universitaria Careggi (AOUC; University Hospital) had been previously obtained. Mononuclear cells were isolated/recovered by centrifugation in a Ficoll-Hypaque gradient (Cedarlane Laboratories, Ontario, Canada) and cultured in the presence of Flt-3 ligand (50 ng/ml), TPo (20 ng/ml), SCF (50 ng/ml) and IL-3 (10 ng/ml) in LC1 and of SCF (50 ng/ml), G-CSF (100 ng/ml), IL-6 (20 ng/ml), SCF (50 ng/ml) and IL-3 (10 ng/ml) in LC2 (all from PeproTech, Rocky Hill, NJ, U.S.A.).

Experiments were performed with exponentially-growing cells plated at 3×10^5/ml and incubated as above (normoxia; 21% O_2 and 5% CO_2) or in a Concept 400 anaerobic incubator (Ruskinn Technology Ltd., Bridgend, U.K.) flushed with a water-saturated preformed gas mixture containing 0.1% O_2, 5% CO_2 and ≈95% N_2 (hypoxia).

Kinase inhibitors

The ERK5-specific inhibitor XMD8-92 [157, 158], obtained through an ongoing collaboration with Dr. N. Gray (Harvard University, Boston, MA, U.S.A.), and the MEK5-specific inhibitors BIX02188 and BIX02189 [172], kind gift of Dr. R. Tatake (Department of Cardiovascular Disease, Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, CT, U.S.A.), were added to cultures at 10 μM for the indicated times.

Imatinib-mesylate (STI571; Gleevec©; Glivec©; Novartis Pharma, Basel, Switzerland) was added to cultures at 1 μM for the indicates times.

Kinase assay

For kinase activity measurement of endogenous ERK5, KCL22 and K562 cells ($5x10^6$) were incubated in the presence or the absence of 10 µM XMD8-92 for 5 and 24 hours. Cells were centrifuged at 1300 rpm for 5 minutes at 4 °C and washed twice with ice-cold phosphate-buffered saline (PBS) containing 100 μM orthovanadate. Cell pellet was lysed in 1 ml of lysis buffer (20 mM Tris-Cl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% NP40, 50 mM Sodium Fluoride (NaF), 30 mM Sodium Pyrophosphate (NaPP), 1 mM orthovanadate (Na3VO4), 1 mM phenyl-methyl-sulphonyl-fluoride (PMSF), 1 μg/ml aprotinin) for 30 minutes on ice and extracted by pipetting 20 times, followed by centrifugation at 13000 rpm for 30 minutes at 4 °C. The supernatant was incubated with 20 μl of Protein A/G PLUS-Agarose (sc-2003, Santa Cruz Biotechnology Inc., Dallas, TX, U.S.A.) and 0.4 μg of a goat anti-ERK5 antibody (C-20; sc-1284, Santa Cruz) for 16-18 hours at 4 °C under constant agitation. After three washes with ice-cold washing IP MAPK buffer (20 mM Tris-Cl, pH 7.4, 1 mM Dithiothreitol (DTT), 1 mM Na3VO4, 1 mM PMSF), the immune complex was used for ERK5 *in vitro*-kinase assay using a nonradioactive MAP Kinase/ERK Assay Kit (#17-191, Merck Millipore, Billerica, MA, USA), following the manufacturer's instruction. Briefly, 10 µl of MAP Kinase Substrate Cocktail (#20-166), 10 µl of MAP Kinase Inhibitor Cocktail (#20-116) and 10 µl of Assay Dilution Buffer I (ADBI) (#20-108) are added to immune complex. The reaction starts by adding 10 µl of Magnesium/ATP Cocktail (#20-113) and incubating shaking at 30°C for 30 minutes. Aliquots of reaction mixture were boiled for 10 minutes in the presence of 100 mM 2-mercaptoethanol and Laemmli buffer (Tris/HCl 62.5 mM, pH 6.8, 10% glycerol, 0.005% blue bromophenol, 2% SDS). Kinase activity of immune-precipitated ERK5 was measured on the basis of its ability to phosphorylate myelin basic protein (MBP). The extent of phosphorylated MBP was evaluated by western blotting with a specific antibody (see below).

Quantitative Real-Time PCR (Q-PCR)

RNA extraction was performed with TRIzol® reagent (no. 15596-026; Invitrogen, Carlsbad, CA), as specified by the manufacturer. Briefly, $5x10^6$ cells were homogenized with 1 ml of TRIzol® and incubated for 5 minutes at RT. Subsequently 0.2 ml of chloroform was added and immediately the sample was shaken vigorously by hand for 15 seconds and incubated for 3 minutes at RT.

After incubation centrifugation was performed at 12,000 g for 15 minutes at 4 C°. The mixture separates into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase where RNA exclusively remains. The aqueous phase was placed into a new tube and 0.5 ml of 100% isopropanol was added. The sample was incubated for 10 minutes at RT and after centrifuged at 12,000 g for 10 minutes at 4 C°. The supernatant was removed, leaving only the RNA pellet. The pellet was washed with 75% ethanol and left air dry. RNA pellet was resuspended in 30-50 µl RNase-free water and incubated in a heat block set at 55-60 C° for 10 minutes. RNA quality was evaluated by visualizing 1 µg RNA on a 1% agarose gel (to exclude the presence of RNA degradation the absence of smears was considered necessary. Moreover two bright discrete bands that represent the 28S and 18S ribosomal species should be shapely visible) and by spectrophotometry (a ratio of the absorbance (A) at 260 nm and 280 nm (A260/A280 ratio) of 1.8-2.1 was considered a good quality of RNA). Total RNA (1.5 µg/sample) was subjected to reverse transcription with SuperScriptVILO-Reverse Transcriptase (Invitrogen) by incubating for 10 minutes at 25°C, 1 hour at 42°C and 5 minutes at 85°C, utilizing 50 pmol hexameric random primers. Reverse Transcription was performed with a MasterCycler (Eppendorf; Milano, Italy). 1 µl of cDNA was subjected to Q-PCR and each sample was done in triplicate. Q-PCR (2 minutes at 50°C, 5 minutes at 95°C, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute) was performed with the ABI Prism 7500 Sequence Detection System (Applied Biosystems; Carlsbad, CA, U.S.A.) using Power SYBR® Green PCR master mix (Applied Biosystems). A melting curve analysis was performed to discriminate between specific and non-specific PCR products. The relative expression of ERK5 mRNA was evaluated using the following primers:

Gene	Primer FW	Primer RV
ERK51hC-ter	5'-GGCTACGGTGTTGGCTTTGA-3'	5'-CACGCCCATGTCGAAAGAC-3'
ERK531hintra	5'-TGCCCCACCAAAGAAAGATG-3'	5'-AAGACTTGAGCAGGGCAGCTT-3'

All primers were used at 0.2 µM. The relative expression of ERK5 mRNA was calculated by using a comparative threshold cycle method and the formula $2^{(-\Delta\Delta Ct)}$ [180].

GAPDH mRNA and 18S (for primers see the table below) were used for normalization:

Gene	Primer FW	Primer RV
GAPDH	5'-AACAGCCTCAAGATCAT CAGCAA-3'	5'-CAGTCTGGGTGGCAGTGAT-3'
18S	5'-CGGTACCACATCCAAGGAA-3'	5'-GCTGGAATTACCGCGGCT-3'

Measurement of cell viability, apoptosis and cell cycle phases

Cell viability was measured by the trypan blue (0.2 gm Trypan Blue from Sigma-Aldrich (St. Louis, MO, U.S.A., #F-7378), 99.8 ml Distilled Water, 0.2% Sodium Azide) exclusion test, by counting trypan-negative cells in a haemocytometer. To quantify apoptosis, cells were centrifuged, resuspended in antibody-binding buffer (HEPES-buffered saline solution with 2.5 mM $CaCl_2$) and incubated with FITC-labeled Annexin-V (Roche Diagnostics, Basel, Switzerland) and propidium iodide (PI) for 15 minutes at room temperature (RT) in the dark. Flow-cytometry was performed using a FACSCanto (Beckton& Dickinson, San Josè, CA, U.S.A.). Annexin-V+/PI- cells were considered early apoptotic and annexin-V+/PI+ cells late apoptotic, whereas Annexin-V+ cells defined total apoptosis. To determine cell cycle phase distribution, cells were centrifuged and pellets resuspended in 500 μL of PI solution (50 μg/ml PI, 0.1% trisodium citrate, 0.1% NP40). After 30 min of incubation in the dark, nuclei were analyzed by flow-cytometry.

The Culture-Repopulating Ability (CRA) assays

This assay estimates the culture-repopulating power of normal [37, 181-184] or leukemic [26, 87, 93] hematopoietic cells that underwent a selection treatment (e.g. hypoxia) in a primary liquid culture (LC1) by means of their wash and transfer in fresh medium to non-selective conditions (e.g. normoxia) in a secondary culture (LC2) and following a further incubation therein. In LC2, the expansion of cell population selected in LC1 is unrestrained, allowing to estimate the stem cell potential of this population on the basis of entity and kinetics of LC2 repopulation. In the experiments reported here, the CRA assays estimated the content of culture-repopulating cells within the hypoxia-resistant cell subsets rescued at the end of LC1. The CRA assays are non-clonogenic assays validated

as capable of revealing *in vitro* different types of normal hematopoietic stem or progenitor cells endowed with marrow-repopulating ability (MRA) *in vivo* [37]. The CRA assays were later adapted to determine the content of stem or progenitor cells within leukemia cell populations [87]. The standard version of CRA assay used for leukemia studies is shown in Figure XIII.

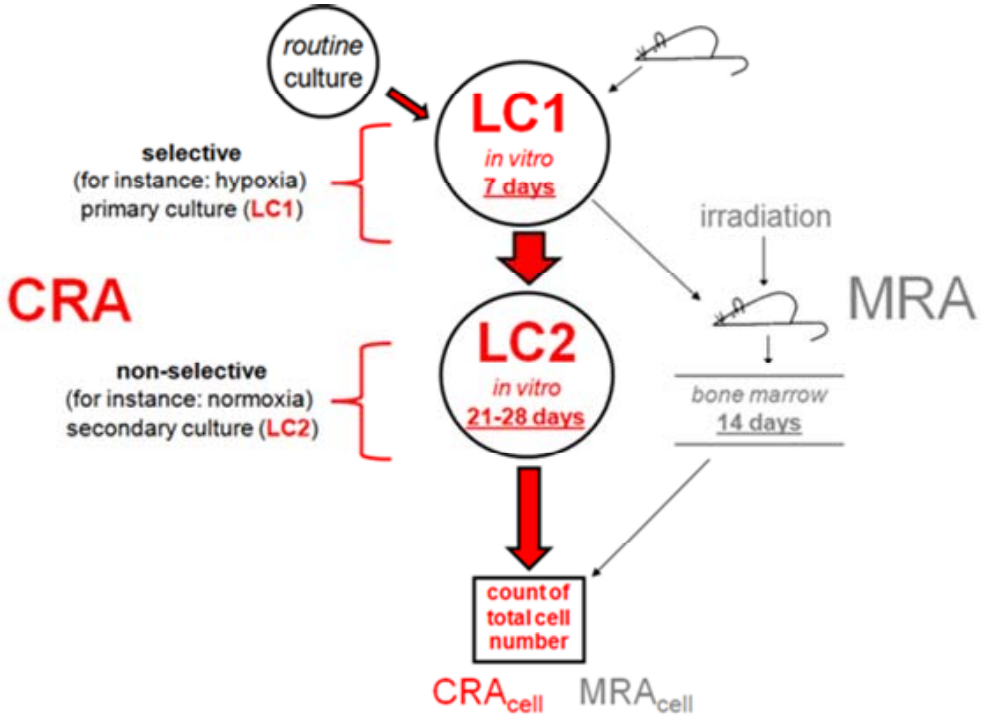


Figure XIII. The Culture-Repopulating Ability (CRA) and Marrow-Repopulating Ability (MRA) assays. The LC1 and LC2 incubation times indicated are just an example, as they are subjected to change according to the cell population studied.

Cell lysis and western blotting

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) containing 100 μ Orthovanadate and solubilized by incubating for 10 minutes at 95 °C in Laemmli buffer. Lysates were then clarified by centrifugation (20 000 × *g*, 10 minutes, RT) and protein concentration in supernatants was determined by the BCA method. Aliquots (30 μg protein/sample) were boiled for 10 minutes in the presence of 100 mM 2-mercaptoethanol, subjected to SDS-PAGE in 9–15% polyacrylamide gel and then transferred onto polyvinylidene fluoride membranes (Millipore) by electroblotting.

Membranes were incubated for 1 hour at RT in Odyssey Blocking Buffer (LI-COR® Biosciences, Lincoln, NE, U.S.A.) diluted 1 : 1 with PBS, and then for 16–18 h at 4 °C in PBS containing 0.1% Tween-20 and the primary antibody. Primary

antibodies used were: rabbit polyclonal anti-phospho(Tyr245)c-Abl (#2862), rabbit polyclonal anti-c-Abl(#2861), rabbit polyclonal anti-phospho(thr218/tyr220)ERK5 (#3371), rabbit polyclonal anti-ERK5 (#3372), rabbit polyclonal anti-phospho(Thr202/Tyr204)p44/42 MAPK(ERK1/2) (#9101), rabbit polyclonal anti-c-Myc (#9402), rabbit polyclonal anti-p27Kip1 (#2552), all from Cell Signaling Technology (Danvers, MA, U.S.A.); mouse monoclonal anti-vinculin (V9131), from Sigma-Aldrich (St. Louis, MO, U.S.A.); mouse monoclonal anti-Klf4 (cat. 09-0021) and rabbit polyclonal anti-Nanog (cat. 09-0020), from Miltenyi Biotech S.r.l. (Milano, Italy); goat polyclonal anti-GAPDH (sc-20357), rabbit polyclonal anti-ERK1 (sc-93), mouse monoclonal anti-Oct3/4(C-10) (sc-5279) and rabbit polyclonal anti-p21(C-19) (sc-397), from Santa Cruz Biotechnology (S. Cruz, CA, U.S.A.). Anti-phospho c-Abl, anti-c-Abl, anti-phospho ERK5 and anti-ERK5 antibodies were diluted 1:500 in PBS containing 0.1% Tween-20. Other antibodies were diluted 1:1000 in the same buffer mentioned before.

After extensive washing with 0.1% Tween-20 in PBS, membranes were incubated for 1 hour at 4 °C in Odyssey Blocking Buffer diluted 1 : 1 with PBS containing an IRDye800CW- or IRDye680-conjugated secondary antibody (LI-COR®). Mouse and rabbit IRDye800CW- conjugated secondary antibodies were diluted 1:20000, goat IRDye680-conjugated secondary antibodies 1:20000, mouse/rabbit/goat IRDye680-conjugated secondary antibody 1:30000.

Antibody-coated protein bands were visualized by the Odyssey Infrared Imaging System Densitometry (LI-COR®), images were recorded as TIFF files.

RNA interference by lentiviral vectors

Stable knockdown of ERK5 in K562 cells was performed using lentivirus. 2 x10^6 human embryonic kidney cell-line 293T (HEK293T) cells [185] were seeded in 100 mm Ø dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, and 2 mM glutamine without antibiotics (complete medium). After 24 hours (50-70% confluency) medium was replaced with fresh complete medium. The plasmid mixture for each sample was prepared as follows: 8 µg lentiviral vectors coding for shRNA (TRC1.5-pLKO.1-puro vector containing non-targeting or human MAPK7-targeting sequences, see the table above for sh sequences), 4 µg pRSV-Rev, 4µg pMDLg/pRRR, 4µg pMDG.1-VSV and 150 mM NaCl, to a final volume of 250 µl. The plasmid mixture was vortexed for 10

seconds and incubated for 5 minutes at room temperature. Then, 40 µl jetPEI™ reagent (Polypus Transfection™, Euroclone, Milano, Italy) was the added to the DNA solution and vortexed for 10 seconds before spinning down. DNA/transfection reagents mixtures were incubated for 30 minutes at room temperature and then gently and quickly dropped onto cells. Plates were then moved forward and backward and side to side for three times in order to distribute DNA. 24 hours after transfection the medium was replaced with fresh complete medium. The following day (48 hours after transfection), cell supernatant was collected and fresh complete medium added to the cells. Harvested medium was centrifuged at 1500 rpm for 5 minutes to remove cells and debris, filtered through a 0.45 µm filter and added to k562 cells or stored at 4 °C for 24 hours. This procedure was repeated the following day. In case collected supernatants were left they were frozen at -80 °C for subsequent use. K562 to be infected were seeded the day after HEK293T transfection at $2x10^5$/ml (4 ml). Normally, 1 ml virus-containing supernatant was added after dilution 1:1 with complete medium in the presence of 5 µg/ml polybrene and the flasks were swirled gently to mix. Alternatively, in case knock down was not optimal, an appropriate amount of viral particles at a suitable Multiplicity of Infection (MoI) was added after virus titration (see below). In some experiments, K562 cells were infected using frozen medium containing lentivirus, following the same procedure. Infected K562 cells were selected with 2 µg/ml puromycin for at least 72 hours. ERK5 knock down was verified by Q-PCR and/or western blotting.

Clone number	Region	Sequence
control vector TRC1.5-pLKO.1		5'-CCGGCAACAAGATGAAGAGCACCAACTC-GAGTTGGTGCTCTTCATCTTGTTGTTTTT-3'
TRCN0000010261	CDS	5'-CCGGCCGCCCTTTGACTTTGCCTTTCTCGAG AAAGGCAAAGTCAAAGGGCGGTTTTT-3'
TRCN0000010262	CDS	5'-CCGGGCTGCCCTGCTCAAGTCTTTGCTCGA GCAAAGACTTGAGCAGGGCAGCTTTTT-3'
TRCN0000010271	CDS	5'-CCGGCCAGTCCAACCTACCAGTCCTCTCGA GAGGACTGGTAGGTTGGACTGGTTTTT-3'
TRCN0000010275	CDS	5'-CCGGGCCAAGTACCATGATCCTGATCTCGA GATCAGGATCATGGTACTTGGCTTTTT-3'

Titration of lentivirus amount

It is recommended that the pseudoviral stock is titrered to control the number of copies of viral construct per target cell. Virus titration was performed using A375 human melanoma cells [186]. 24 hours before viral infection, A375 cells were seeded in 6-well plates, at $2x10^4$ cells in 2 ml of DMEM supplemented with 10% FBS and 2 mM glutamine without antibiotics (complete medium) for each well. After 24 hours medium was removed and cells were infected by adding 900 µl of 10-fold diluted viral stock into the first well, 900 µl of 100-fold diluted viral stock into the second well, 900 µl of 1,000-fold diluted viral stock into the third well, 900 µl of 10,000-fold diluted viral stock into the fourth well, 900 µl of 100,000-fold diluted viral stock into the fifth well and 900 µl of 1,000,000-fold diluted viral stock into the sixth well. Lentivirus were diluted in DMEM supplemented with 10% FBS and 8 µg/ml polybrene. Virus-infected cells were incubated at 37°C and the plate was rocked every 10-15 minutes for 1 hour. 1.1 ml of complete medium was then added to each well. After 48 hours, culture medium was replaced with 2 ml of fresh complete medium with a low dose of puromycin (1 µg/ml). The following day, culture medium was replaced with 2 ml of fresh complete medium with a standard dose of puromycin (2 µg/ml). 7 days after viral infection, the medium was removed and wells were washed twice with PBS. Crista violet staining of cells was then performed by adding a fixing/staining solution (0.5% crystal violet, 3% formaldehyde, 30% ethanol) to the cells and incubating for 10 minutes at RT. The solution was removed and wells were washed with tap water until there was no visible background staining. Blue colonies were counted and the titer of lentivirus stock was calculated. The titer of the original suspension was defined as the number of infectious units per unit volume of the preparation. MoI is a frequently used term in virology which refers to the number of virions that are added per cell during infection. To calculate MoI: (number of cells seed) x (desired MoI) = plaque-forming units (PFU).

Total ml of lentiviral particles to add = (PFU) / (number of colonies x dilution factor).

Flow-cytometry

To analyze CD34 and CD38 cell surface markers, 10^5 cells in PBS were stained with 5 µl of anti-human CD34-FITC (#21270343; Immuno Tools, JPT

Peptide Technologies GmbH, Berlin, Germany) and 5 µl of anti-human CD38-APC (#21270386; Immuno Tools) for 15 minutes at RT in the dark. After washing with PBS, the expression of CD34 and CD38 was measured in a FACSAria flow-cytometer (Becton-Dickinson; Franklin Lakes, NJ, U.S.A.). Background signal was established in the same populations by staining with 5 µl of a matched isotype control: Mouse IgG1 control FITC- (#21275513) and APC- (#21275516) conjugated from Immuno Tools.

To investigate intracellular protein expression, 10^5 cells were permeabilized with PBS containing 0.5% Tween-20 for 15 minutes at RT. Cells were then incubated for 1 hour at 4 °C in PBS containing 0.5% Tween-20 and the primary antibody diluted 1:100. After extensive washing with PBS, cells were incubated for 30 minutes at 4 °C in PBS containing 0.5% Tween-20 and FITC- or APC-conjugated secondary antibody (Chemicon International Inc., Temecula, CA, U.S.A.) diluited 1:600. Background signal was determined in the same populations by staining with a matched isotype control. Intracellular MFI was measured in a FACSAria and all data were reported as $MFI_{sample}/MFI_{isotype}$.

Statistical Analysis

All experiments were performed in triplicate or higher numbers of repeats. Statistical significance was evaluated by Student's *t*-test for paired samples; p values < 0.05 (two-sided) were considered statistically significant.

Results and Discussion

Effects of pharmacological inhibition of ERK5 pathway on survival and growth of CML cells.

The ERK5 pathway is the most recently discovered and least-studied mammalian MAPK cascade. This pathway has been shown to be involved in many cellular functions, including survival, anti-apoptotic signaling, angiogenesis, cell motility, differentiation and proliferation. However, there is still much to learn regarding the potential activators of this pathway and many of the intermediary molecules involved in signaling both upstream and downstream ERK5 activation. Recent studies have shown that ERK5 is over-expressed with respect to healthy cells or constitutively activated in a number of cancers. Mitogens and oncogenic signals strongly activate this MAPK pathway, thereby passing down proliferative, survival, chemo-resistance, invasive and angiogenic signals in tumor cells.

Importantly, the recent development of a pharmacologic agents able to specifically inhibit the ERK5 signaling pathway will allow to further delineate the regulation and physiological role of the ERK5 signaling cascade and, more importantly, to test the translatability of experimental findings to a preclinical setting.

The work reported here was directed to study the importance of the ERK5 pathway in the survival of CML cells, including leukemia stem and progenitor cells. To this end, we started to evaluate the effects of the ERK5-specific inhibitor XMD8-92 and of two MEK5-specific inhibitors, BIX02188 and BIX02189, in cells of the KCL22 and K562 stabilized CML lines. In fact, ERK5 is constitutively activated in KCL22 and K562 cells, suggesting that strategies leading to ERK5 inactivation may affect survival and proliferation of CML cells (see below).

The effectiveness of the three above-mentioned inhibitors was preliminarily evaluated in cells of the human hepatocellular carcinoma (HCC) Huh-7 (**Figure 1A).** Cells were serum-deprived for 24 hours and then treated or not with a single dose of 5 or 10 μM XMD8-92, or 10 μM BIX02188 or BIX02189, for 1 hour before exposure to 100 ng/ml EGF for 10 minutes. Cells were then lysed and ERK5 expression was evaluated by Western Blotting. All three drugs at the concentration of 10 μM prevented ERK5 band shift which occurs after its

phosphorylation induced by EGF treatment. In **Figure 1B**, KCL22 and K562 cells were incubated in the presence or the absence of 10 μM XMD8-92 for 5 and 24 hours and lysed. After cell lysis, ERK5 was immunoprecipitated and ERK5 kinase activity *in vitro* was determined. XMD8-92 treatment impaired ERK5 ability to phosphorylate its substrate myelin binding protein (MBP). ERK5 inhibition did not induce compensatory activation of ERK1/2. The effects of the other inhibitors on ERK5 activation were similar (data not shown).

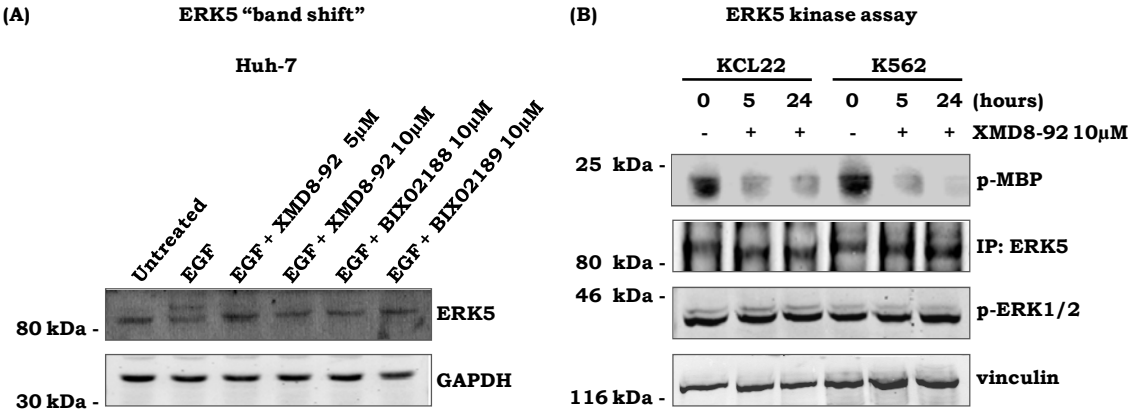


Figure 1. Pharmacological inhibition of ERK5 activity. (A) Huh-7 cells were serum-deprived for 24 hours and then treated or not with the indicated inhibitors for 1 hour before exposure to 100 ng/ml EGF for 10 minutes. Total cell lysates were analyzed by Western Blotting using the indicated antibodies. An anti-GAPDH antibody was used to verify equalization of protein loading. **(B)** KCL22 and K562 cells were incubated in the presence or the absence of XMD8-92 at the indicated concentrations for the indicated times. Cell lysates were used for ERK5 *in vitro* kinase assay using a non-radioactive MAP Kinase assay kit. Kinase activity of immunoprecipitated ERK5 was determined on the basis of the ability to phosphorylate myelin basic protein (MBP). Vinculin are used to verify equalization of protein loading. In (**A**) and (**B**) migration of molecular weight markers is indicated on the left.

To determine the effects of inhibitors of ERK5 pathway on survival, growth and cycling of CML cells, exponentially-growing KCL22 and K562 cultures were replated in fresh medium at $3x10^5$ cells/ml and incubated for 7 days. Inhibitors were added or not, at the dose of 10 μM at the beginning of incubation and again, at a half dose (5 μM), at day 3. We treated again cells with half dose in order to maintain constant concentration of the inhibitors throughout incubation. Growth kinetics was determined by counting viable cells via Trypan-blue exclusion at days 2, 3 and 7 of incubation. The number of viable cells increased about 2-fold in KCL22 and 2.6-fold in K562 cells over the first 3 days of incubation, to decrease thereafter, possibly as an effect of culture overcrowding, especially in the case of K562 cells. The treatment with inhibitors did not affect growth kinetics significantly (**Figure 2**).

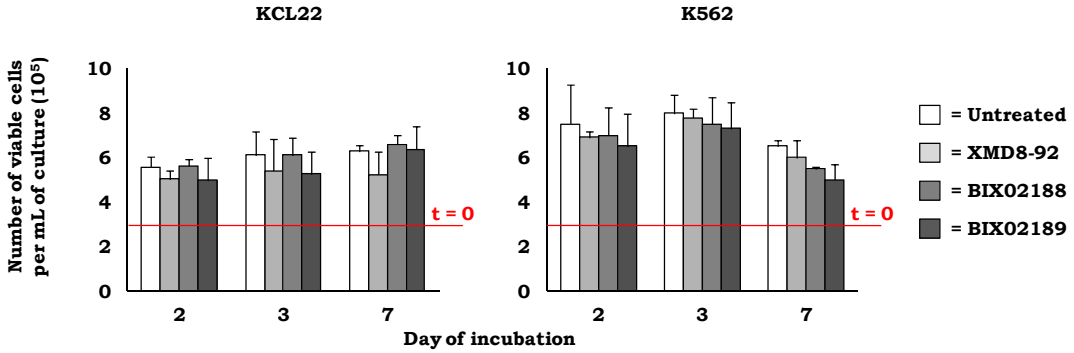


Figure 2. Effects of pharmacological inhibition of ERK5 pathway on growth kinetics of CML cell lines. Exponentially-growing KCL22 and K562 cells from routine cultures were replated in fresh medium at $3x10^5$ cells/ml and incubated for the indicated times. Cultures were treated or not, at the beginning of incubation with a 10 μM dose and again, at day 3, with a 5 μM dose. Trypan blue-negative cells were counted. Values represent means ± S.E.M. of data from 3 independent experiments.

Several publications have shown that the ERK5 signaling pathway is important for regulating cell cycle progression of some cell types [98, 145, 183]. Although the number of viable cells was not altered, we decided to test whether or not the pharmacological inhibition of ERK5 pathway interferes with cell cycle progression by flow-cytometry **(Figure 3)**. Cells were treated as described above. XMD8-92 determined an appreciable increase of the percentage of KCL22 cells in G0/G1 phase after 48 hours and 72 hours of incubation; this increase was paralleled by a decrease of the percentage of cells in S phase. In K562 cells, XMD8-92 determined a slight, not significant effect. BIX02188 and BIX02189 did not any appreciable effect on cell cycle phase distribution in either cell lines.

Molecular aspects of the effects of ERK5 inhibitors on CML cell cycling are shown in **Figure 4**. XMD8-92, but not BIX02188 or BIX02189, determined an increase of p27kip expression, a critical negative regulator of cell cycle progression, typically expressed in G0/G1-arrested cells. Several studies indicated that ERK5 activity contributes to down-regulate p27. Indeed, ERK5 phosphorylates p27 at Ser10, a site known to be involved in nuclear export and possibly in cytoplasmic degradation of p27 in G1 [187]. p27 expression was also reported to be increased in macrophages [155] and in AML cells displaying a reduced ERK5 activity [188]. More recently, the treatment of Huh-7 or HepG2 HCC cells with XMD8-92 or with ERK5 siRNA decreased cell proliferation and increased the percentage of cells in G0/G1 phase, increasing in parallel p27Kip expression.

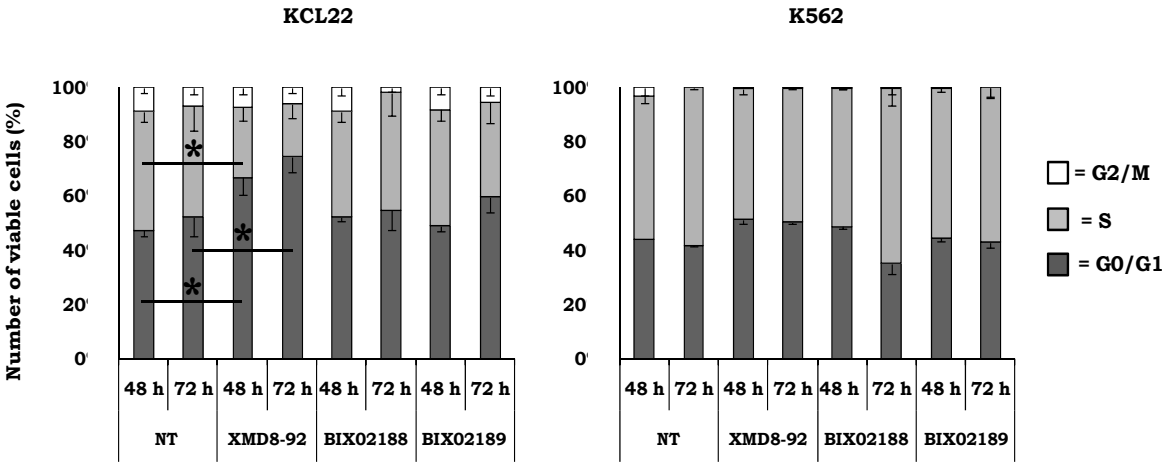


Figure 3. Effects of pharmacological inhibition of ERK5 pathway on cell cycle phase distribution of CML cell lines. KCL22 and K562 cells were treated with the indicated drugs (10 μM at time 0 and, again, 5 μM at day 3) and cell distribution through mitotic cycle determined by flow-cytometry following propidium iodide staining of nuclei. Values represent means ± S.E.M. of data from 3 independent experiments. *p<0.05 vs untreated control (NT).

We also monitored the possible pro-apoptotic effects of pharmacological inhibition of the ERK5 pathway (**Figure 5**). MEK5 inhibitors did not determine significant effects in either cell line. The ERK5 inhibitor XMD8-92 reduced the basal apoptosis occurring in untreated K562 cell cultures.

The dose/response of ERK5 inhibition with XMD8-92 on survival and proliferation of CML cell lines was then determined (**Figure 6**). Exponentially-growing KCL22 and K562 cells from routine cultures were replated in fresh medium at $3x10^5$ cells/ml and incubated in normoxia. Cells were treated or not at the beginning of incubation (treatment at time 0) with XMD8-92 at the concentrations 5, 10, 25, 50 or 100 μM. Cells were counted daily for 4 days following Trypan-blue staining. The 5 and 10 μM doses, as well as the XMD8-92 solvent DMSO, were ineffective. Increasing the doses, from the 25 μM on, the number of viable cells was dose-dependently reduced.

Mammalian BM has been shown to be relatively hypoxic compared to other tissues, and primitive hematopoietic cells, including HSC, are thought to localize to the most hypoxic microenvironments in the BM. Oxygen availability in leukemic BM is possibly even lower, due to the increase of cell density determined by the unrestrained expansion of neoplastic clones. Very low oxygen tensions are therefore believed to protect immature leukemia cell subsets, including LSC, the most likely candidates to sustain treatment-resistant MDR [189]. We previously demonstrated that severe hypoxia (~0.1% 0_2) interferes with

intracellular signaling pathways relevant to leukemia cell survival and growth in murine Friend's erythroleukemia (MEL) and K562 cells, leading to early and massive apoptosis and cell cycle arrest of hypoxia-surviving cells [26, 87]. However, the response of LSC to severe hypoxia is different from that of the bulk of leukemia cell bulk.

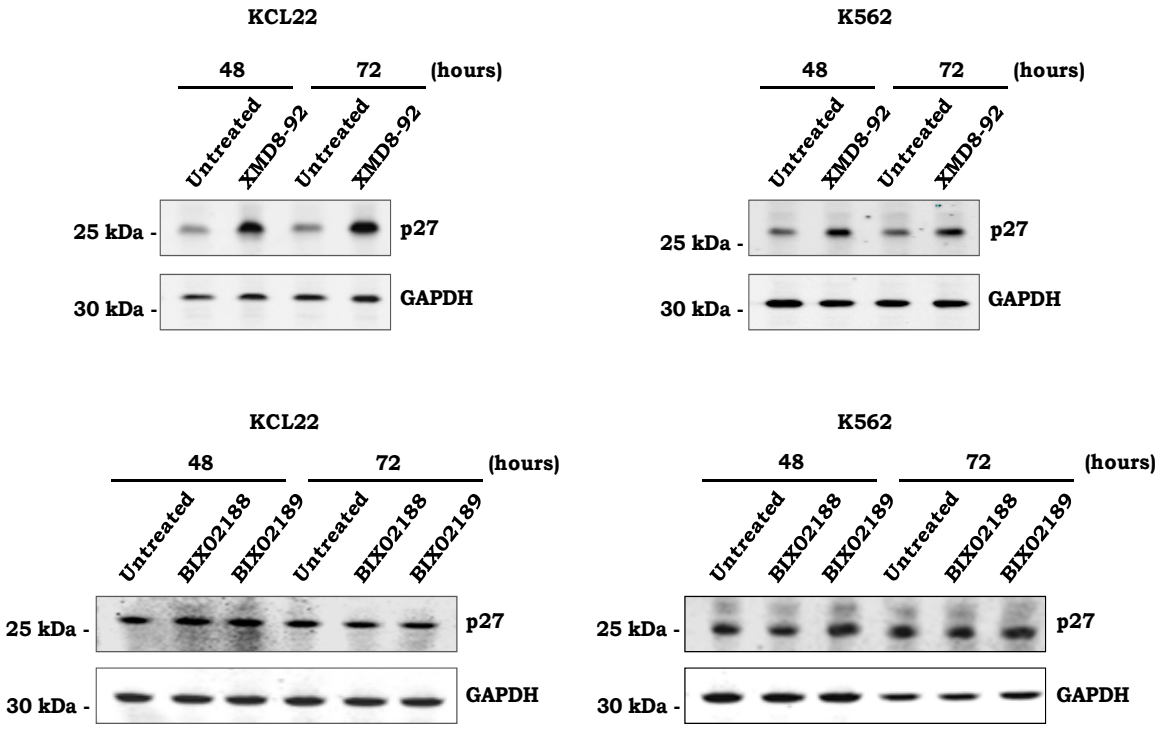


Figure 4. Effects of pharmacological inhibition of ERK5 pathway on p27 protein expression in CML cell lines. Total lysates of KCL22 and K562 cells, treated or not with 10 μM XMD8-92, BIX02188 or BIX02189 for the indicated times, were subjected to Western Blotting with the indicated antibodies. An anti-GAPDH antibody was used to verify equalization of protein loading. Migration of molecular weight markers is indicated on the left.

On the other hand, the involvement of ERK5 in the response to hypoxia is emerging. Several studies have shown indeed that HIF-1α, a critical mediator in the cellular response to hypoxia and angiogenesis, is regulated by several MAPK including ERK5 [190-192]. One of the proposed mechanisms involves ubiquitin-dependent degradation of HIF-1α mediated by ERK5. Interestingly, gene profiling studies demonstrated that there is a large overlapping between the gene expression patterns regulated by ERK5 and HIF-1α, with 82% of the genes specifically regulated by ERK5 being modulated in response to hypoxia through HIF-1α [193].

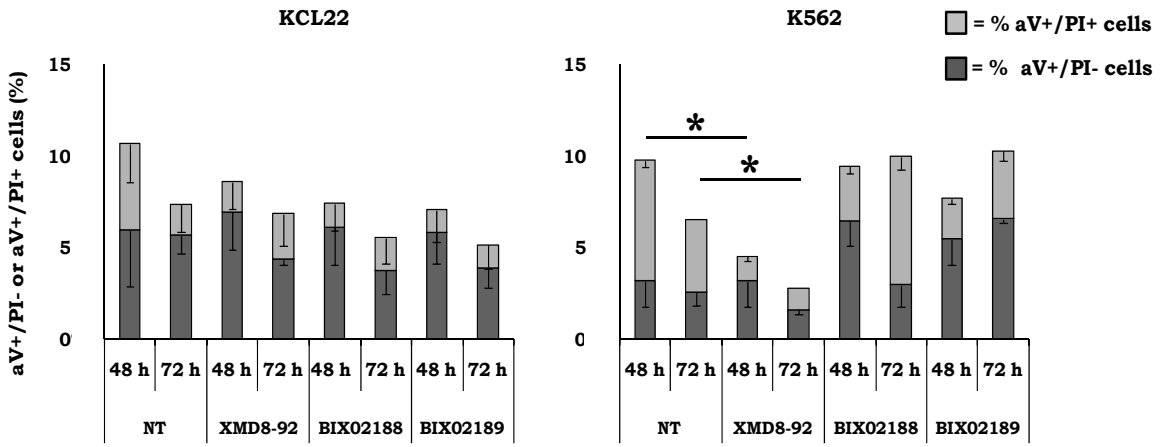


Figure 5. Effects of pharmacological inhibition of ERK5 pathway on apoptosis of CML cell lines. KCL22 and K562 cells were treated with the indicated drugs (10 μM at time 0 and, again, 5 μM at day 3) for the indicated times and the percentage of apoptotic cells was measured by the annexin V test and flow-cytometry. Values represent means ± S.E.M. of data from 3 independent experiments. *p<0.05 vs untreated control (NT).

In our experimental system, severe hypoxia (~0.1% 0_2) suppresses the constitutive activation of ERK5 occurring in CML cells (**Figure 7)**. This suppression occurs beyond days 2 or 3 of incubation, depending on the cell line. ERK1/2 activity was increased at days 3 or 1 of incubation, to be later strongly suppressed. We also observed a decrease of the level of ERK5 protein after 6/7 days of incubation in hypoxia in K562 cells; this decrease is possibly due to sub-loading of samples. Alternatively, the decrease of ERK5 protein observed could be driven via the ubiquitin-proteosoma machinery [194-196].

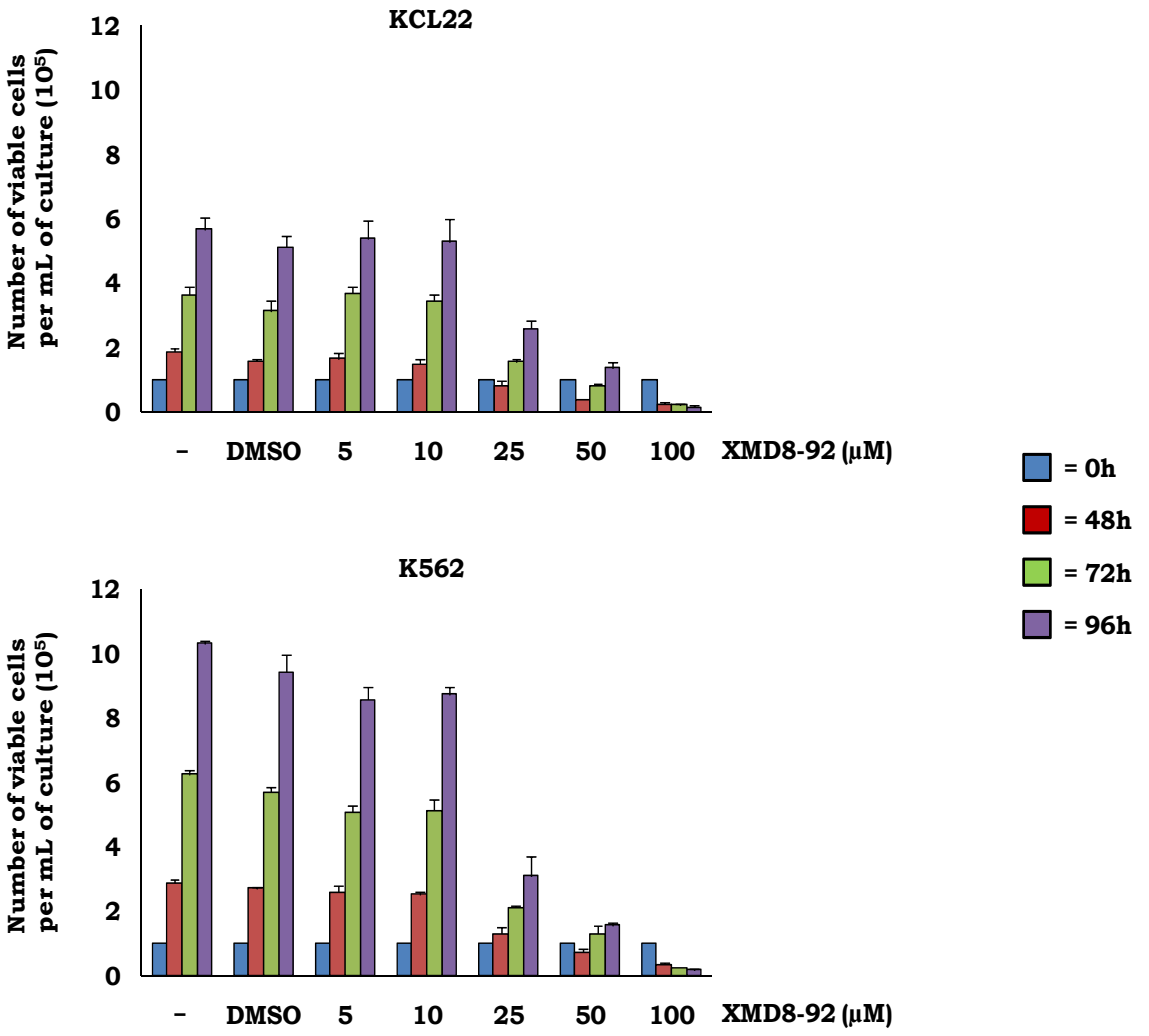


Figure 6. Dose/response of pharmacological inhibition of ERK5 on survival and growth of CML cell lines. K562 and KCL22 cells were incubated in normoxia for the indicated times and treated or not at time 0 with XMD8-92 at the indicated doses. Trypan blue-negative cells were counted. DMSO, used as XMD8-92 solvent was tested as an additional control. Values represent means ± S.D. of data from one experiment performed in triplicate.

On the other hand, prolonged hypoxia increased ERK5 mRNA in both CML cell lines (**Figure 8**). However, other mechanisms in addition to those mediated by pVHL, such as those involving c-Abl [197], could be considered to understand the molecular basis of ERK5 expression levels and function. Buschbeck *et al.* [197] suggested that oncogenic Abl kinase activity was able to regulate the cellular amount of ERK5 by stabilizing the protein. Furthermore, basal activity of ERK5, but not necessarily its activation, was sufficient to mediate survival of BCR/Abl-positive leukemia cells. We previously reported that severe hypoxia suppresses the expression of $BCR/Abl_{protein}$ [26, 93]. Therefore the possible

decrease in the expression of ERK5 (**Figure 7**) is consistent with the presumed role of Abl kinases in regulating ERK5 expression in leukemia cells.

To establish the role of ERK5 in the survival and proliferation of CML cells in hypoxia, KCL22 and K562 cells were incubated in hypoxia in the presence or the absence of the three above-mentioned inhibitors of ERK5 pathway. Exponentially-growing KCL22 and K562 cells from routine cultures were replated in fresh medium at $3x10^5$ cells/ml and incubated in the presence or absence of XMD8-92, BIX02188 or BIX02189, added at the beginning of incubation (10 μM) and again at day 3 (5 μM).

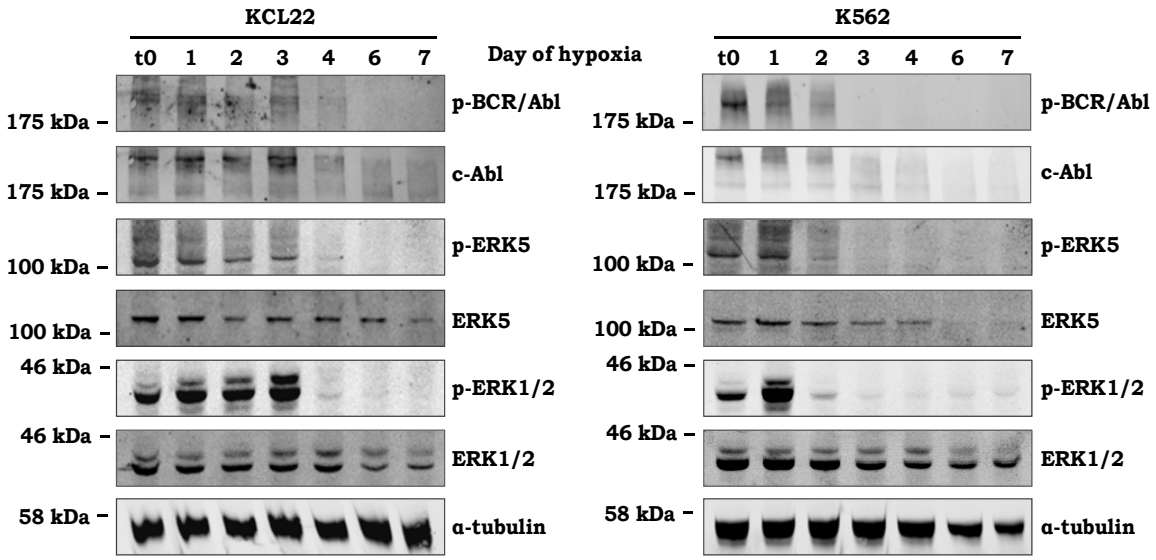


Figure 7. Hypoxia suppresses the ERK5 constitutive activation. KCL22 and K562 cells were incubated in hypoxia (~0.1% 0_2) for the indicated times. Total cell lysates were subjected to Western Blotting with the indicated antibodies. An anti-α-tubulin antibody was used to verify equalization of protein loading. Migration of molecular weight markers is indicated on the left.

The effects of pharmacological inhibition of the ERK5 pathway on growth kinetics of CML cell lines are shown in **Figure 9**. In control cultures incubated in hypoxia, the number of viable cells underwent a modest and transient (over the first 2-3 days of incubation) increase, followed by a progressive decrease to reach levels below those of time zero. BIX02188 or BIX02189 did not affect the cell number significantly in either CML cell line. XMD8-92 treatment, on the contrary, resulted in a significant, although not marked, increase of viable cell number. This increase was transient (day 3) for KCL22 cells and sustained

throughout the incubation for K562 cells. Overall, the results obtained in hypoxia are comparable to those in normoxia (**Figure 2**).

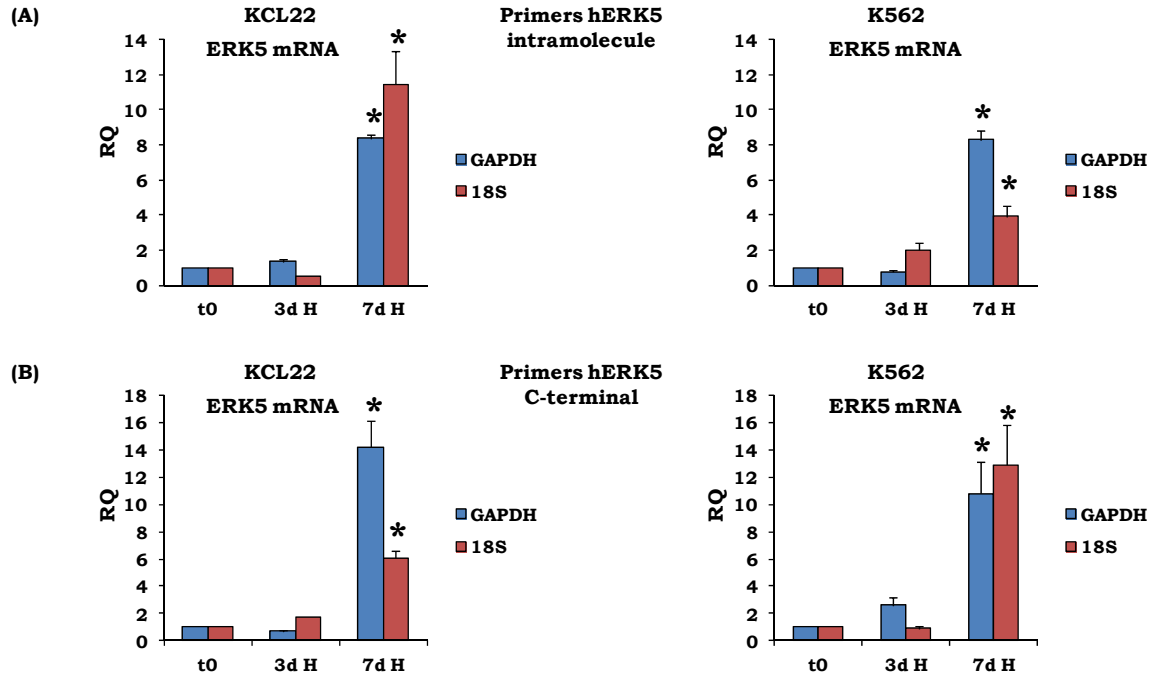


Figure 8. Hypoxia increases ERK5 mRNA in CML cell lines. Exponentially-growing KCL22 and K562 cells from routine cultures were replated in fresh medium at $3x10^5$ cells/ml and incubated in hypoxia (~0.1% 0_2) for the indicated times. Total mRNA was harvested and the relative expression of ERK5 estimated by QPCR using two set of primers. The GAPDH mRNA and 18S were used for normalization. Time 0 (t0) values were used as normalizator in order to calculate the fold-change (RQ). Values represent means ± S.D. of data from one experiment performed in triplicate. *p<0.01 vs time 0.

The effects of pharmacological inhibition of the ERK5 pathway on cell cycle distribution of CML cells incubated in hypoxia is shown in **Figure 10**. While MEK5 inhibitors were ineffective, the treatment with XMD8-92 resulted in a reduction of the percentage of cells in S phase and a concomitant increase of that of cells in G0/G1 phase, after 48 h and especially after 72 h of incubation in hypoxia. The percentage of G2/M-phase cells showed no evident changes. Thus, XMD8-92 determined an increase of G0/G1 phase/decrease of S phase while the number of cells in culture was not affected (**Figure 9**).

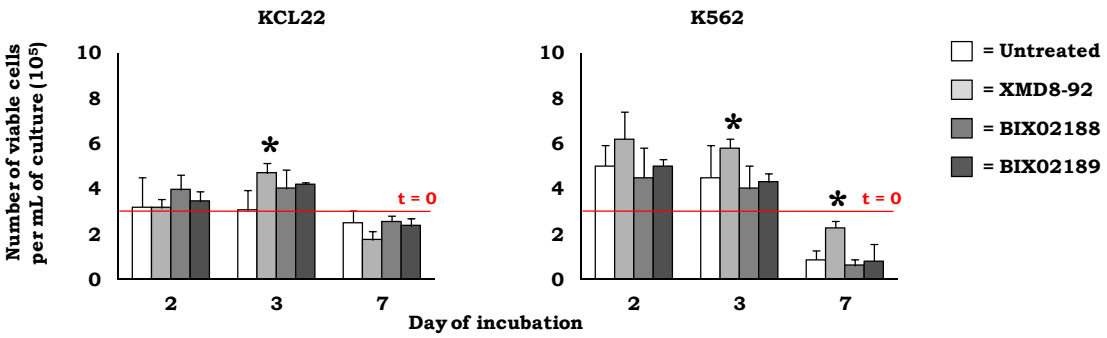


Figure 9. Effects of pharmacological inhibition of ERK5 pathway on growth kinetics of CML cell lines in hypoxia. Exponentially-growing KCL22 and K562 cells from routine cultures were replated in fresh medium at $3x10^5$ cells/ml and incubated in hypoxia (~0.1% 0_2) for the indicated times. Cultures were treated or not at the beginning of incubation with a 10 μM dose and again, at day 3, with a 5 μM dose. Trypan blue-negative cells were counted. Values represent means ± S.E.M. of data from 3 independent experiments. *p<0.05 vs untreated control.

The block of progression of CML cells to the S phase operated by XMD8-92 was paralleled by an increase of p27Kip (**Figure 11**). Consistently, BIX02188 and BIX02189, which did not induce a detectable G0/G1 accumulation, neither increased the expression of p27Kip. Taken together with the data obtained in normoxia (**Figure 3** and **Figure 4**), these results point to a cytostatic, rather than cytotoxic, effect of ERK5-specific inhibition.

We also monitored the possible apoptogenic/cytotoxic effects of pharmacological inhibition of ERK5 pathway in hypoxia **(Figure 12)**. The treatment with XMD8-92, but not with BIX02188 and BIX02189, resulted in a significant reduction of the hypoxia-induced apoptosis, after 48 h and 72 h of incubation. Taken together with the data obtained in normoxia (**Figure 5**), these results suggest that in hypoxia the protective effect of XMD8-92 against basal apoptosis occurring in control cultures is enhanced.

Effects of pharmacological ERK5 pathway inhibition on the maintenance of immature CML cell subsets in hypoxia.

The effects of pharmacological MEK5/ERK5 inhibitors were evaluated at this point on cell subsets exhibiting properties of leukemic progenitor cells (LPC) or leukemia stem cells (LSC).

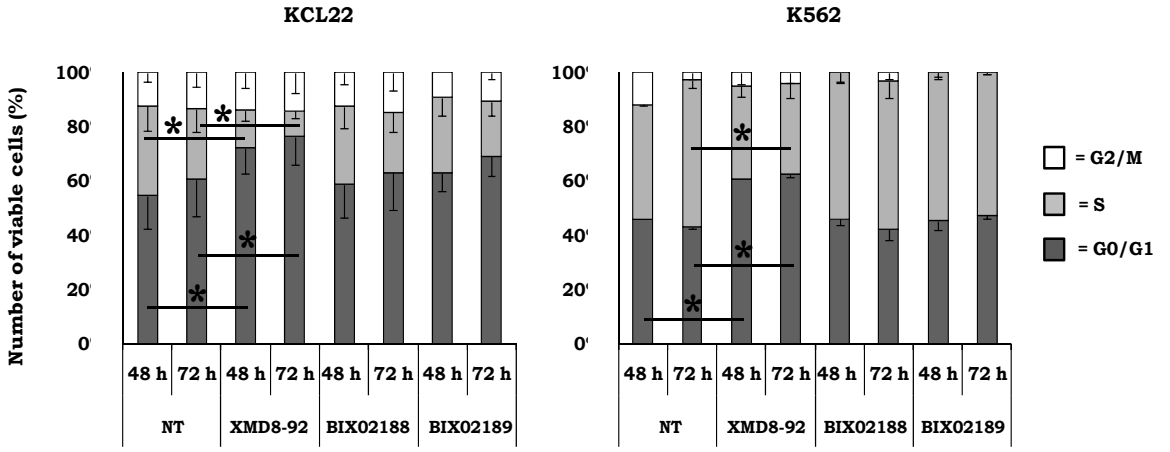


Figure 10. Effects of pharmacological inhibition of ERK5 pathway on cell cycle phase distribution of CML cell lines in hypoxia. KCL22 and K562 cells were incubated in hypoxia (~0.1% 0_2) in the presence or absence of the indicated drugs (10 μM at time 0 and, again, 5 μM at day 3) and cell distribution through mitotic cycle determined by flow-cytometry following propidium iodide staining of nuclei. Values represent means ± S.E.M. of data from 3 independent experiments. *p<0.05 vs untreated control (NT).

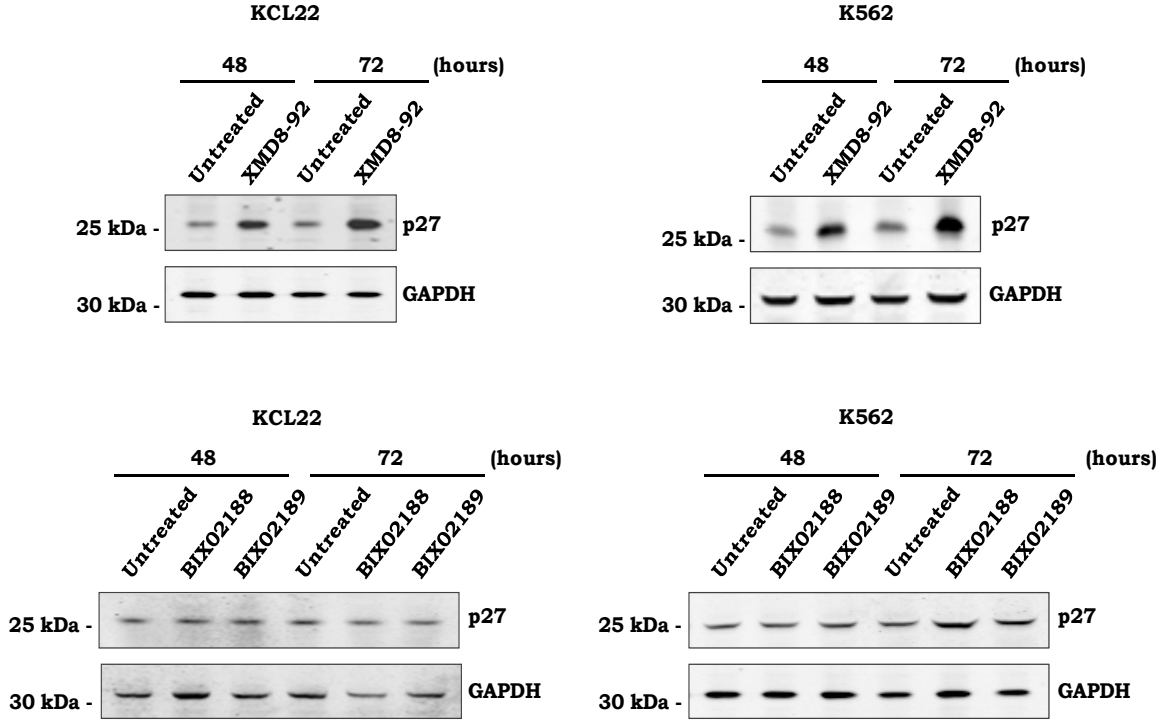


Figure 11. Effects of pharmacological inhibition of ERK5 pathway on p27 protein expression in CML cell lines incubated in hypoxia. Total lysates of KCL22 and K562 cells incubated in hypoxia (~0.1% 0_2) and treated or not with 10 μM XMD8-92, BIX02188 and BIX02189 for the indicated times were subjected to Western Blotting with the indicated antibodies. An anti-GAPDH antibody was used to verify equalization of protein loading. Migration of molecular weight markers is indicated on the left.

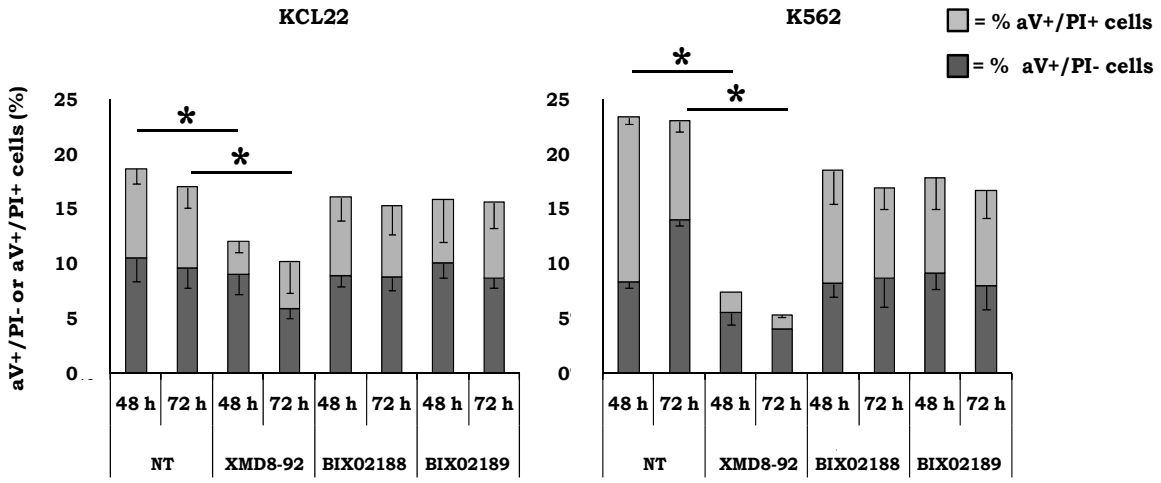


Figure 12. Effects of pharmacological inhibition of ERK5 pathway on apoptosis of CML cell lines incubated in hypoxia. KCL22 and K562 cells were incubated in hypoxia (~0.1% 0_2) for the indicated times and treated or not with the indicated drugs (10 μM at time 0 and, again, 5 μM at day 3). The percentage of apoptotic cells was measured by Annexin V test and flow-cytometry. Values represent means ± S.E.M. of data from 3 independent experiments. *p<0.05 vs untreated control (NT)

Cells were incubated in primary hypoxic or normoxic cultures (LC1) for 7 days and treated or not from time zero with MEK5/ERK5 inhibitors. At day 7 of incubation in LC1, cells were washed free of drug and transferred at a density of $3x10^4$ cells/ml to drug-free, growth-permissive LC2, always incubated in normoxia (CRA assay). In cells incubated in hypoxia for 7 days, $BCR/Abl_{protein}$-positive cells are suppressed, while the $BCR/Abl_{protein}$-negative cell subset, which the LPC/LSC belong to, is maintained [26, 88].

Cells transferred to LC2 from control untreated LC1 incubated in normoxia rapidly repopulated LC2, as appropriate for cells expressing $BCR/Abl_{protein}$ at the time of transfer. Cell number increased starting from day 3, to generate a relatively early peak (day 7-14), a consequence of the growth-promoting stimuli induced by $BCR/Abl_{protein}$ immediately upon transfer to LC2 (**Figure 13A**). On the other hand, LC2 growth curves obtained following transfer of cells control untreated LC1 incubated in hypoxia exhibited the kinetics typical of LC2 repopulation sustained by $BCR/Abl_{protein}$-negative LPC/LSC. Repopulation was characterized, indeed, by an initial 7-day lag-phase, due to the time necessary for the generation in LC2 of cell expressing $BCR/Abl_{protein}$ and the successive BCR/Ab-dependent clonal expansion; in this case, the peak of repopulation was reached relatively late, at day 21-24 (**Figure 13B**). In either case, beyond the peak cell number in culture gradually decreased, surely due to the exhaustion of nutrients.

The treatment of KCL22 and K562 cells with BIX02188 or BIX02189 in normoxic LC1 reduced the ability to repopulate LC2, while that with XMD8-92 almost completely suppressed LC2 repopulation **(Figure 13A)**. On the other hand, when hypoxic LC1 were treated with BIX02188 or BIX02189, LC2 repopulation was almost immediate, without lag-phase, i.e., differently from that of control untreated cultures, followed the kinetics typical of $BCR/Abl_{protein}$–expressing cells. Treatment with XMD8-92 completely suppressed LC2 repopulation **(Figure 13B)**. These results indicate that MEK5 inhibitors have a modest effect on CRA of $BCR/Abl_{protein}$-expressing CML, actually accelerating $BCR/Abl_{protein}$ re-expression in cells rescued from hypoxia. On the contrary, both $BCR/Abl_{protein}$-expressing and $BCR/Abl_{protein}$-negative cell subsets were markedly sensitive to the treatment with XMD8-92. Thus, XMD8-92 was inactive on the bulk of CML cell population (**Figure 9**), but capable to suppress completely the hypoxia-selected, $BCR/Abl_{protein}$-negative cell subsets.

Effects of genetic ERK5 inhibition on growth kinetics in hypoxic cultures and Culture-Repopulating Ability of hypoxia-selected K562 cells.

To confirm the above results while overcoming problems of interpretation of data due to possible off-target effects of ERK5-inhibiting drugs, K562 cells were infected with lentiviral vectors expressing shRNA against ERK5 (shERK5) or non-targeting shRNA (shNT), used as a control.

This strategy achieved an effective and long-lasting silencing of ERK5 (**Figure 14**). We obtained 90-100% inhibition of the expression of ERK5 protein with two lentiviral vectors expressing 271 and 275 shRNA against ERK5, and a slight decrease of protein levels with 261 and 262 shRNA.

The biological effects of genetic inhibition of ERK5 in cells incubated in hypoxia are shown in **Figure 15**. ERK5 knockdown in sh271 and sh275 cells significantly reduced the number of viable cells in cultures incubated in hypoxia compared to the treatment with non-targeting shRNA (shNT). No differences of growth kinetics were observed for sh261 and sh262 cells, where ERK5 silencing had not been obtained (**Figure 15A**). Thus, genetic knockdown of ERK5 was more effective than its pharmacological inhibition on the survival and proliferation of CML cells (**Figure 2** and **Figure 9**). These differences can be explained by the well known property of ERK5 to regulate a number of genes by

direct interaction, independently of its kinase activity. This makes the consequences of downregulation of the whole molecule different from those of kinase inhibition.

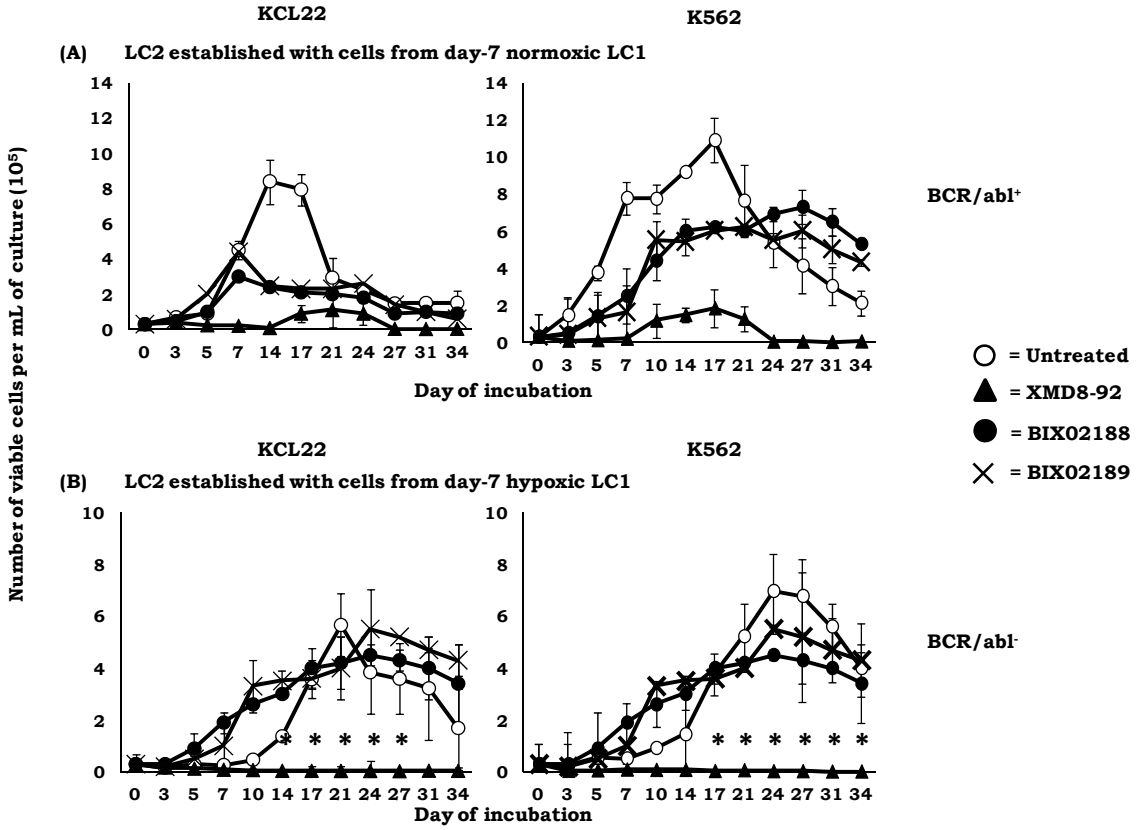


Figure 13. Effects of pharmacological inhibition of ERK5 pathway on Culture-Repopulating Ability of CML cells selected or not in hypoxia. KCL22 and K562 cells were treated as indicated in normoxic **(A)** or hypoxic **(B)** LC1 (established with $3x10^5$ viable cells/ml) and transferred at day 7 into normoxic LC2 (established with $3x10^4$ viable cells/ml). Trypan blue-negative cells were counted at the indicated times of incubation in LC2. Values represent means ± S.E.M. of data from 3 independent experiments. *$p<0.05$ vs untreated.

The effects of genetic inhibition of ERK5 in hypoxia-selected K562 cells were then evaluated (**Figure 15B**). At day 7 of incubation in hypoxic LC1, cells were transferred into growth-permissive normoxic LC2 and the kinetics of LC2 repopulation was determined (CRA assay). ERK5 knockdown suppressed LC2 repopulation driven by hypoxia-selected cells as much the inhibition of ERK5 enzymatic activity by XMD8-92 (**Figure 13B**). Taken together, our results suggest that while the presence of ERK5 molecule (but not its activity) contributes to the survival of CML cells in hypoxia, ERK5 expression and activity are important for the maintenance of hypoxia-selected cells expressing the LSC phenotype.

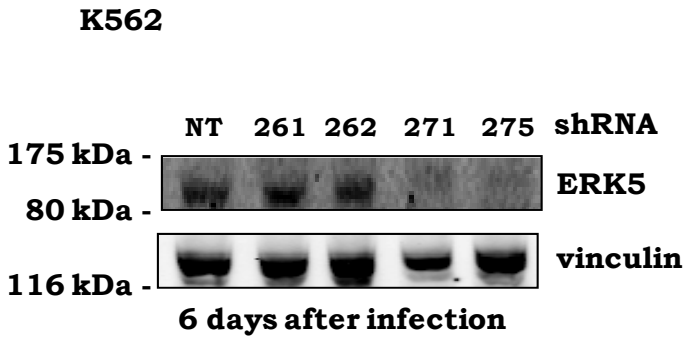


Figure 14. Genetic ERK5 inhibition in CML cells. K562 cells were infected with lentiviral vectors expressing shRNA against ERK5 (shERK5) or non-targeting shRNA (shNT) and selected with 2 µg/ml puromycin for 72 hours. Cells were harvested, lysed and lysates subjected to Western Blotting with the indicated antibodies. An anti-vinculin antibody was used to verify equalization of protein loading.

Effects of pharmacological ERK5 inhibition together with Imatinib treatment on CML cells.

Treatment of patients with the Abl-specific kinase inhibitor Imatinib induces remission in the early chronic leukemic phase as well as in the late blast crisis [198]. Successful therapy is unfortunately hampered by the refractoriness of some cell subsets to therapy (primary resistance) or by the development of secondary drug resistance, particularly in patients with advanced CML. Now, it is accepted that IM-refractory malignant cells are typically LSC [199, 200] and recent studies indicated that LSC of CML are independent of BCR-Abl kinase activity [26, 88, 89, 201, 202]. Therefore, combination therapy protocols adding to IM (active on CML cell bulk) the targeting of BCR/Abl-independent survival pathways (to hit LSC) appear a promising strategy for the prevention of relapse of disease [203]. Since we found that XMD8-92 does not inhibit the overall survival of CML cells, but suppresses hypoxia-selected LSC-like cell subsets, we tested the effects of the XMD8-92/IM combination.

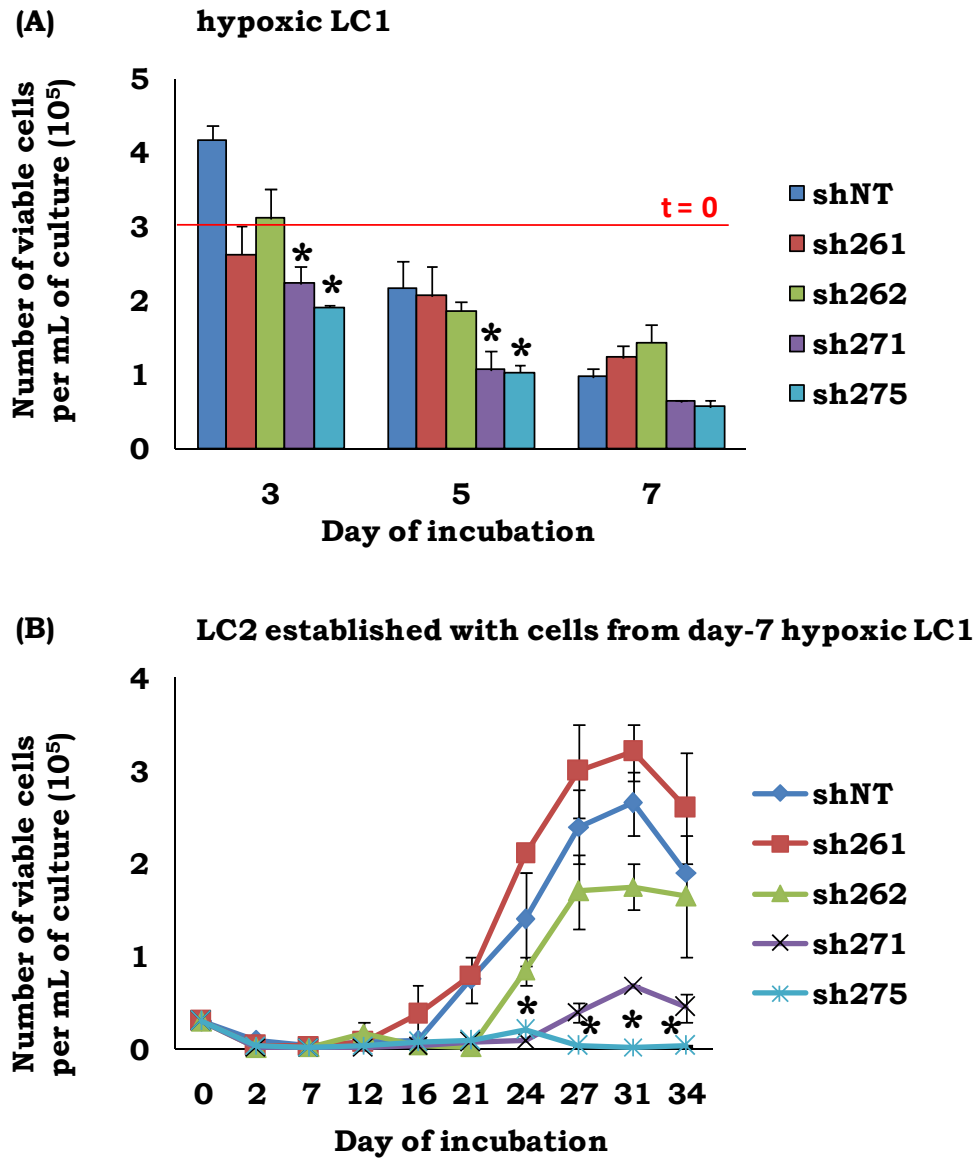


Figure 15. Effects of genetic ERK5 inhibition on CML cell bulk in hypoxia and on Culture-Repopulating Ability of cells selected in hypoxia. (A) K562 cells were infected with non-targeting (NT) or ERK5-targeting shRNA. Cultures were incubated in hypoxia (~0.1% 0_2) for the indicated times and Trypan blue-negative cells counted. **(B)** At day 7 of hypoxic LC1 (established at $3x10^5$ viable cells/ml), cells were transferred into normoxic LC2 (established at $3x10^4$ viable cells/ml). Trypan blue-negative cells were counted at the indicated times of incubation in LC2. **(A)** and **(B)** values represent means ± S.E.M. of data from 3 independent experiments. *p<0.05 vs non-targeting (NT).

CML cells were incubated in hypoxia (~0.1% 0_2) or normoxia (used as a markedly IM-sensitive control) for 7 days and treated or not at the beginning of incubation at the concentration of 10 μM XMD8-92 and/or 1 μM Imatinib and again, at half dose for either drugs, at day 3. The effects on K562 or KCL22 cell bulk are shown in **Figure 16.** IM suppressed the cell population expansion occurring in normoxia as well as in hypoxia and then determined a marked

reduction of the number of viable cells with respect to time 0. XMD8-92 addition to cultures at time 0, instead, did not markedly affect the number of viable cells throughout incubation in normoxic and hypoxic LC1, for both cell lines, producing results comparable to those shown previously (**Figure 2** and **Figure 9**). The combination with XMD8-92 determined a marginal, if any, enhancement of the inhibitory effect of IM.

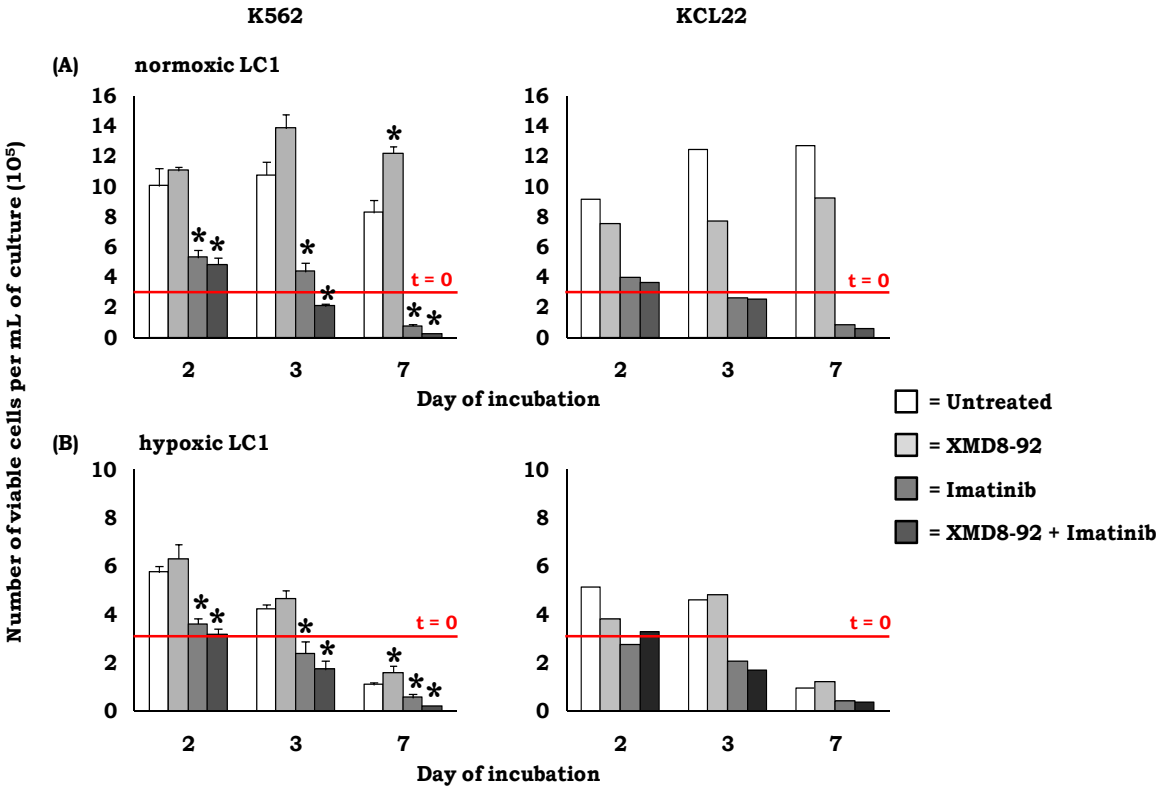


Figure 16. Effects of XMD8-92, alone or with Imatinib, on CML cell bulk in normoxia or hypoxia. Exponentially-growing K562 and KCL22 cells from routine cultures were replated in fresh medium at $3x10^5$ cells/ml and incubated in normoxia (**A**) or hypoxia (**B**) (~0.1% 0_2) for the indicated times. Cultures were treated or not, at the beginning of incubation with 10 μM XMD8-92 and/or 1 μM Imatinib and again, at half dose for either drugs, at day 3. Trypan blue-negative cells were counted. (**A**) values represent means ± S.E.M. of data from 3 independent experiments. (**B**) values represent one experiment. *p<0.05 vs untreated control.

The CRA of hypoxia-resistant cells was then tested for its sensitivity to the combined treatment of XMD8-92 with Imatinib **(Figure 17)**. To this end, cells were recovered from hypoxic or normoxic LC1 after 7 days of incubation and transferred into growth-permissive normoxic LC2 (CRA assay).

LC2 repopulation was reduced, but not abolished, by IM treatment of normoxic LC1, despite the fact that in normoxia BCR/$Abl_{protein}$ is fully expressed (**Figure 17A)**. XMD8-92, in agreement with the data shown in **Figure 13A**, markedly reduced LC2 repopulation and was thereby confirmed highly effective on BCR/$Abl_{protein}$–expressing cells. The combination of two drugs suppressed LC2 repopulation completely. Likewise, **Figure 17B** shows results comparable to those of **Figure 13B**. IM treatment of hypoxic LC1 reduced the repopulation of LC2 established with cells rescued at day 7 from LC1, in agreement with the fact that drug was added to LC1 when BCR/$Abl_{protein}$ was fully expressed. However, cells rescued from hypoxic LC1 are in large part refractory to IM. This finding is irrelevant with respect to the effects of XMD8-92, which alone is sufficient to completely suppress LC2 repopulation.

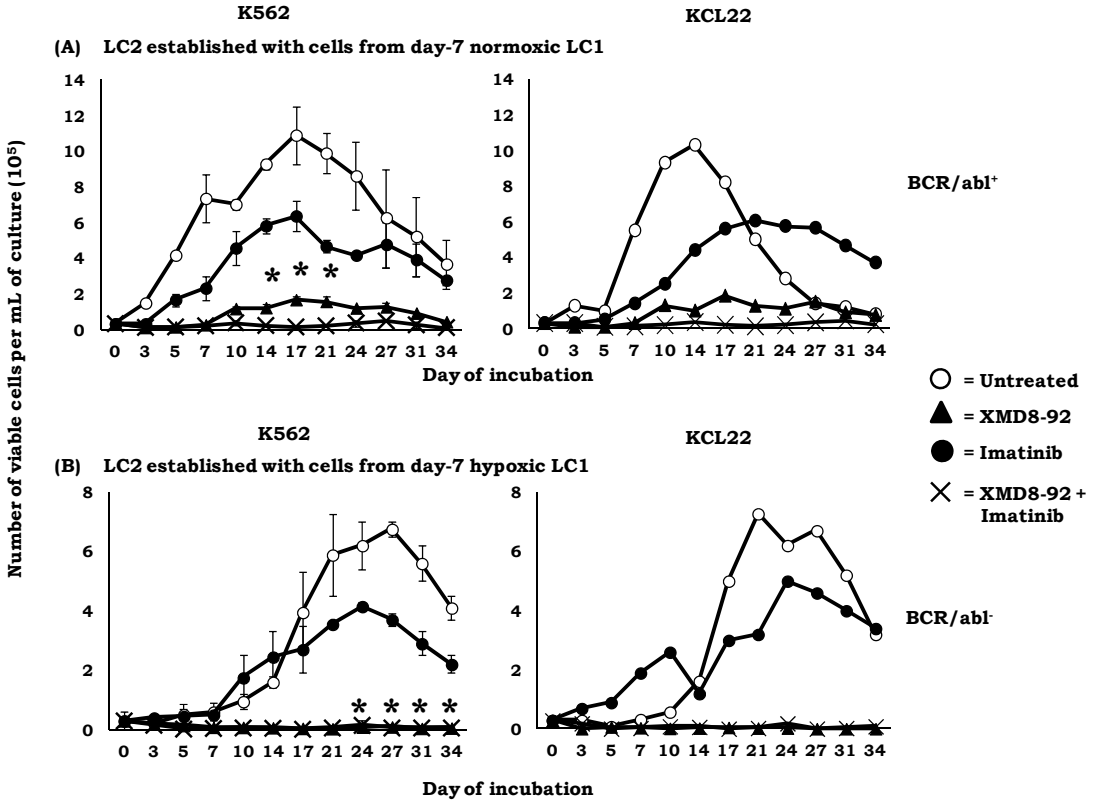


Figure 17. Effects of XMD8-92, alone or with Imatinib, on Culture-Repopulating Ability of CML cells selected or not in hypoxia. K562 and KCL22 cells were treated as indicated in normoxic (**A**) or hypoxic (**B**) LC1 (established with $3x10^5$ viable cells/ml) and transferred at day 7 into normoxic LC2 (established with $3x10^4$ viable cells/ml). Trypan blue-negative cells were counted at the indicated times of incubation in LC2. (**A**) values represent means ± S.E.M. of data from 3 independent experiments. (**B**) values represent one experiment. *p<0.05 vs untreated control.

Effects of XMD8-92, alone or with Imatinib, on hypoxia-selected cells of CML patients.

The effectiveness of XMD8-92 demonstrated on hypoxia-selected cell subsets of stabilized CML lines was also tested on primary cells explanted from CML patients (**Figure 18**). In keeping with what observed for cell lines, IM treatment of cells selected in hypoxic LC1 reduced but not suppressed LC2 repopulation. Likewise, XMD8-92, alone or in combination with IM, completely suppressed repopulation. DMSO used as XMD8-92 solvent did not have any effect. These results confirm that the combined treatment of XMD8-92 with IM may be an useful approach to try to eradicate CML, being XMD8-92 active on LPC/LSC but not cell bulk, and, *viceversa*, IM unable to suppress LPC/LSC but very active on the bulk of CML population.

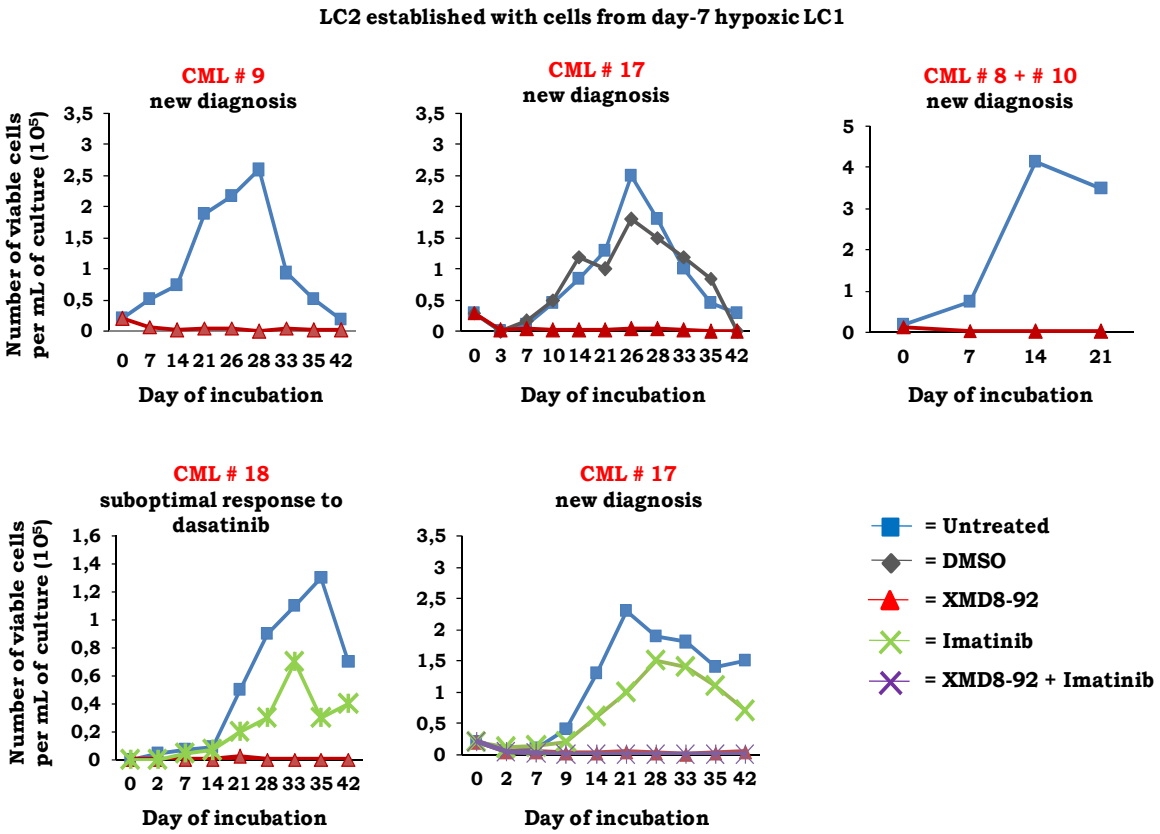


Figure 18. Effects of XMD8-92, alone or with Imatinib, on Culture-Repopulating Ability of hypoxia-selected cells of CML patients. Primary cells explanted from CML patients were treated as indicated in hypoxic LC1 (established with $3x10^5$ viable cells/ml) and transferred at day 7 into normoxic LC2 (established with $3x10^4$ viable cells/ml). Trypan blue-negative cells were counted at the indicated times of incubation in LC2. DMSO, used as XMD8-92 solvent, was tested as an additional control.

The LPC/LSC hierarchy is similar to the normal HPC/HSC hierarchy. It is known that LSC and HSC express similar surface immune-phenotypes and are, indeed, CD34+CD38- [204-206]. On this basis, we determined the effects of ERK5 inhibition (either genetic or pharmacological) on the CD34/CD38 profile of the KCL22 and K562 cell lines. Exponentially-growing cells were replated into cultures incubated in normoxia or hypoxia for 7 days and FACS analysis of CD34 and CD38 antigens was performed at the end of incubation. As shown in **Figure 19**, both cell lines resulted CD34-negative. K562 cells were also CD38-negative, while KCL22 cells included a small subpopulation of CD38+ cells. Kasumi-1 cells were used as a CD34-positive control. These results indicated that the classical molecular markers of HSC/LSC are not suitable for the study of the effects of ERK5 inhibition on the LPC/LSC subsets of CML cell lines

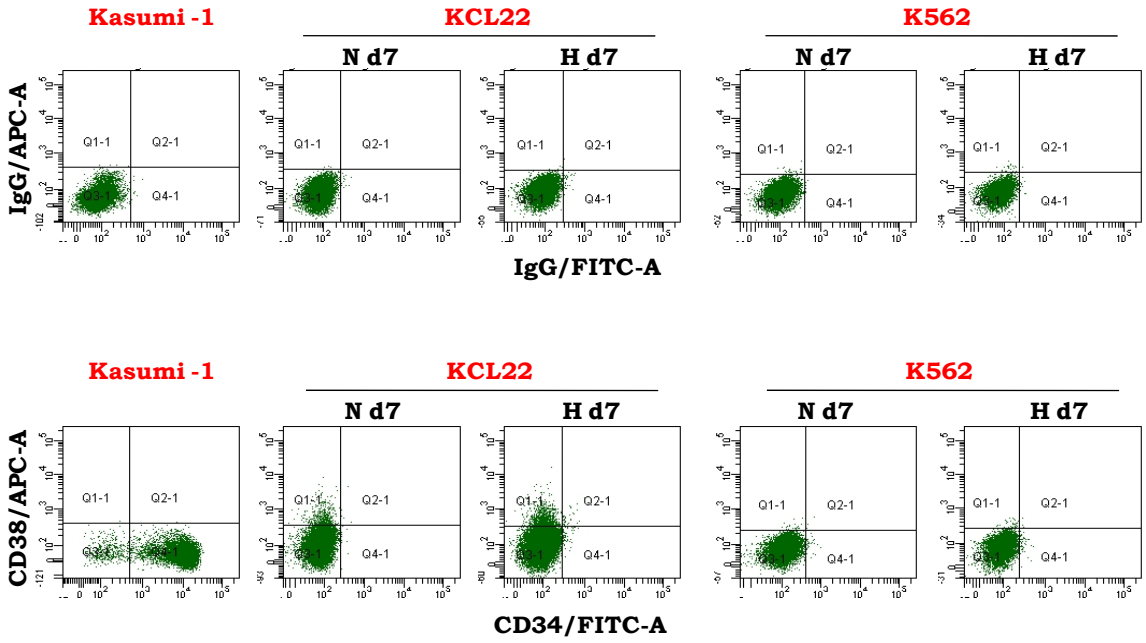


Figure 19. FACS analysis of CD34 and CD38 cell surface markers on Kasumi-1, KCL22 and K562 cells. Exponentially-growing KCL22 and K562 cells from routine cultures were replated in fresh medium at $3x10^5$ cells/ml and incubated in normoxia or hypoxia (~0.1% 0_2) for the indicated times. Flow cytometry analysis of cells stained with anti-CD34:FITC antibody and anti-CD38:APC antibody were performed. Kasumi-1 cells were used as a CD34+ control. Background signal was established in the same populations by staining with a matched isotype control. Data represent one typical experiment that was performed three times with consistent results. Calculation of Mean Fluorescence Intensity confirmed the absence of expression of the CD34 antigen in either KCL22 and K562 cells.

Based on the results described above, we evaluated by Western Blotting the expression of several stem cell markers in total lysates of KCL22 and K562 cells incubated in hypoxia and treated or not with XMD8-92, alone or in combination with Imatinib (**Figure 20**). p21Cip1/Waf1 (p21) and p27kip1 (p27) are members of the cip/kip cyclin-dependent kinase inhibitor (CDKi) family of proteins, which suppress cell cycle progression by repressing the activities of the cyclin-E/CDK2 complex in late G1 phase and cyclin-A/CDK2 complex in early S phase. p21 and p27 have been shown to play critical roles in normal hematopoiesis by restricting the proliferation of HSC and HPC, respectively [207-209]. On the other hand, either CDKis known to be modulated by ERK5 [155, 183-185]. In **Figure 20**, the trend of the expression of p21 is different in the two CML cell lines, while XMD8-92 or IM treatments alone determined an increase of p27 throughout incubation in hypoxia. This increase seems greater in KCL22 cells treated with XMD8-92 alone, especially at 2 and 3 days of incubation. However, treatment with either drugs determined a low 27 and low p21 expression status at variance with single treatments were either CDK are higher with respect to control cells.

The quiescence of stem cells has been conjectured to be of critical biologic importance in protecting the stem cell compartment. When it is disrupted, as occurs in the absence of p21, HSC cannot remain in G0, exhaust prematurely and display reduced reconstitution capacity [52, 210]. These studies may explain why the treatment with either drugs, that suppresses the expression of p21 throughout incubation in hypoxia especially in KCL22, impairs culture repopulation driven by hypoxia-selected LSC. XMD8-92, while being active on LSC like the combinatorial treatment with IM, on the contrary, does not downmodulate p21 but indeed overexpresses it. The same speculations can be made about p27.

We then determined the effects of ERK5 inhibition on the so-called “Yamanaka factors”, namely Oct3/4, Sox2, Klf4, c-myc. These genes have been described as the genes responsible, by themselves, for self-renewal [211-214] and pluripotent differentiation in both mouse and human somatic cells [215, 216]. Therefore, we wanted to investigate possible changes in Oct3/4, Klf4, c-Myc protein expression in order to evaluate whether XMD8-92, alone or in combination, affects the expression of genes responsible for LSC maintenance.

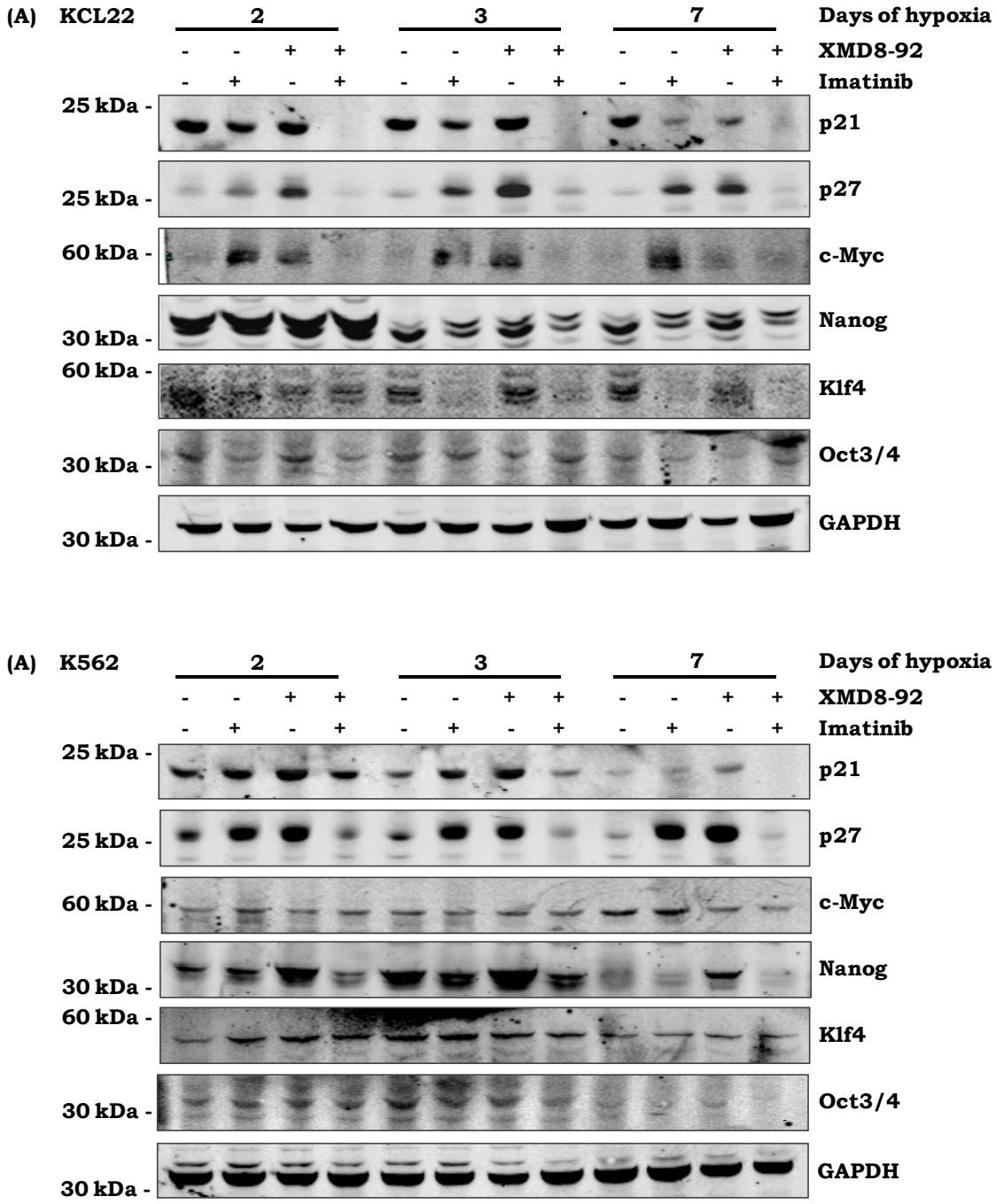


Figure 20. Effects of XMD8-92, alone or with Imatinib, on protein expression in KCL22 and K562 cell bulks in hypoxia. Total lysates of KCL22 **(A)** and K562 **(B)** cells incubated in hypoxia (~0.1% 0_2) and treated or not with 10 μM XMD8-92 and/or 1 μM Imatinib for the indicated times were subjected to Western Blotting with the indicated antibodies. An anti-GAPDH antibody was used to verify equalization of protein loading. Migration of molecular weight markers is indicated on the left.

We were not able to obtain results about Sox-2 due to technical problems (apparently the antibody available did not work). On the other hand, the pattern of expression of Klf4, c-Myc and Oct3/4 was not clear and reproducible and, in any case, did not give us molecular details enough to understand how the ERK5 pathway is involved in the maintenance of CML stem cells. Of note, these results are preliminary and deserve further experiments in order to reach more clarity.

(A) KCL22 **7days of hypoxia**

Treatment	MFI Oct3/4	MFI Nanog	MFI Klf4	MFI c-Myc
Untreated	1	1	1	1
Imatinib	0.7	0.5	1	0.9
XMD8-92	0.9	1.2	1	1.3
Imatinib + XMD8-92	1	0.2	0.8	0.7

(B) K562 **7days of hypoxia**

Treatment	MFI Oct3/4	MFI Nanog	MFI Klf4	MFI c-Myc
Untreated	1	1	1	1
Imatinib	1.1	1.1	1	1.3
XMD8-92	1.6	1.1	0.7	1.7
Imatinib + XMD8-92	0.9	0.6	0.8	0.9

Figure 21. Intracellular protein MFI in KCL22 and K562 cell bulks treated with XMD8-92, alone or with Imatinib, in hypoxia. Exponentially-growing KCL22 (**A**) and K562 (**B**) cells from routine cultures were replated in fresh medium at $3x10^5$ cells/ml and incubated in hypoxia (~0.1% 0_2) for the indicated times. Cultures were treated or not at the beginning of incubation with 10 μM XMD8-92 and/or 1 μM Imatinib and again, at half dose, at day 3. Oct3/4, Nanog, Klf4 and c-Myc MFI was measured on a FACSAria. All data are reported as $MFI_{sample}/MFI_{isotype}$.

As Yamanaka factors, the homeodomain-containing transcription factor Nanog is an integral part of the pluripotency network [217-219]. Although Nanog expression is not required for the maintenance of pluripotent stem cells (PSC), it has been reported to be essential for the establishment of both ESC from blastocysts and induced PSC from somatic cells [220, 221]. In our experiments, XMD8-92 treatment increased Nanog expression throughout the whole incubation period in hypoxia, especially in K562 cells.

We also wanted to investigate possible changes in Oct3/4, Nanog, Klf4 and c-Myc protein expression by flow cytometry (**Figure 21**). XMD8-92 and Imatinib in combination determined a decrease in Nanog MFI relative to untreated cells but also to cells treated with Imatinib alone. Thus, the combinatorial treatment impairs the expression of *Nanog* gene, especially in KCL22.

Oct3/4, Klf4 and c-Myc did not show appreciable differences in MFI values. These data should be considered "preliminary" because many more experiments must be performed prior to confirming the results.

Conclusions

During the last decade, CML has been a model disease in targeted cancer therapy. The understanding of molecular mechanism underlying the pathogenesis of disease (generation of Philadelphia chromosome and *BCR/abl* fusion gene) made possible to design tyrosine kinase inhibitors (TKI) active on BCR/Abl kinase. IM (Glivec®), the first-generation TKI, has been in clinical use for over ten years, and more potent second-generation TKI, such as Dasatinib®, Nilotinib®, Bosutinib®, are now all approved for the treatment of CML [222-226]. TKI therapy has improved the prognosis significantly [227], and deaths due to CML are nowadays rare.However, some patients may develop resistance due to mutationsof the Abl kinase domain, which led to the development of third-generation TKI, such as Ponatinib® [228]. However, overall, current CML treatment protocols are still not considered to be curative, although treatment results in CML are far better than in most other cancers [229]. TKI therapy eradicates very efficiently the majority of leukemic cells, but not the most primitive quiescent LSC [230-232], the best candidates to sustain MRD of CML, so that relapses occur if treatment is discontinued. Therefore, there is great interest in designing novel therapies capable to target LSC.

Cell adaptation to hypoxia is a very relevant aspect of stem cell biology, especially in the field of hematopoiesis. Oxygen tension in BM is constitutively much lower than in most other normal tissues, due to both the peculiar structure of vascularization and the high level of cell crowding, factors which favor the onset of hypoxic areas within cell conglomerates. Oxygen availability in leukaemic BM is possibly even lower, due to the a further increase of cell density determined by the unrestrained expansion of neoplastic clones.We previously demonstratedthat resistance to hypoxia is a common feature of HSC and LSC, but also that a hypoxia-selectable LSC subset is likely to exists within any type of leukemia, including stabilized clonal cells lines [26, 87-89]. Moreover, severe hypoxia suppresses the expression of $BCR/Abl_{protein}$but not that of BCR/Abl transcript [26, 93], so that hypoxia-selected stem cells, while remaining leukemic, are independent of BCR/Abl signaling and therebyrefractory to IM .This is very well in keeping with the notions that: (i) IM, despite its impressive efficacy as first-line therapy for patients with chronic-phase CML, induces a state of MRD, rather than cure; (ii) LSC most likely sustain MRD and are thereby

responsible for the late relapses of CML; (iii) tissue hypoxia provides optimal conditions for the homing of normal and neoplastic stem cells.

The ERK5 is a member of the MAPK family involved in the control of cell survival and proliferation, as well as the pathogenesis of a number of cancers. Studies performed in hematopoietic cancers have demonstrated that active ERK5 is essential for growth of leukemias and Hodgkin lymphoma and is involved in BCR/Abl-dependent CML cell survival [160, 198, 233]. Moreover, ERK5 is activated by cytokines regulating stem cell compartments [101-104] and participates to cell response to hypoxia [107, 191, 193]. The work carried out in relation to this thesis led to obtain strong evidence *in vitro* that ERK5 is important for the maintenance ofstem and progenitor cells of CML.

Using two well-established CML cell models, our *in vitro*data indicate that the pharmacological inhibition of ERK5 pathway does not affects growth kinetics of CML cells incubated in normoxia or hypoxia. On the other hand, the treatment with the ERK5-specific inhibitor XMD8-92 resulted in a significant reduction of the hypoxia-induced apoptosis. On the other hand, ERK5 genetic inhibition impaired CML cell survival. These differences are likely due to the well-known property of ERK5 to regulate a number of genes by direct interaction, independently of its kinase activity, making the consequences of downregulation of the whole molecule different from those of kinase inhibition. In addition, XMD8-92 determined, especially in hypoxia, the block of progression of CML cells tothe S phase and the increase of p27Kip, a cell cycle inhibitor recentlyreported by our group to be regulated by ERK5 in macrophages [155] and confirmed by others indifferent types of cancer [188]. These results point to a cytostatic, rather than cytotoxic, effect of ERK5-specific inhibition on the bulk of CML cell population.

Besides the effects of pharmacological MEK5/ERK5 inhibitors on the bulk of CML cell population, we evaluated those on cell subsets exhibiting properties of LPC or LSC. We found that XMD8-92, unlike MEK5-specific inhibitors, was capable to suppress the BCR/$Abl_{protein}$-positive LPC and, more importantly, the BCR/$Abl_{protein}$-negative LSC which are selected following an extended incubation in hypoxia [26, 88]. Taken together with the data on growth kinetics of CML cell bulk, these results suggest that, while the presence of ERK5 molecule (but not its activity) contributes to cell survival, ERK5 expression and activity are important for the maintenance of cells expressing the LSC phenotype.

Based on the results described above, we tested the effects of the combined treatment of XMD8-92 with IM, to mimic a potentially clinical setting. As expected, IM markedly reduced the number of viable cells in normoxia as well as in hypoxia and the combination with XMD8-92 determined a marginal, if any, enhancement of this inhibitory effect. The above-summarized suppression of the LSC subset following XMD8-92 treatment was confirmed when the drug was administered in combination with IM. The fact that the effects of XMD8-92 and IM do not interfere with each other represents the most relevant result of the study reported here, as IM cannot be eliminated from clinical therapeutic protocols, although completely inactive on hypoxia-selected, $BCR/Abl_{protein}$-negative LSC [26, 88]. Thus, our results indicate that the combined treatment of XMD8-92 with IM may be an useful approach to try to eradicate CML together with the induction of remission.

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UNIVERSITÀ
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Dottorato di Ricerca in SCIENZE BIOMEDICHE
sede amministrativa: Dipartimento di Scienze Biomediche Sperimentali e Cliniche
coordinatore: Prof. Persio Dello Sbarba

Dottorato di Ricerca in Scienze Biomediche

Presentazione del candidato : Ignazia Tusa
Curriculum: Oncologia Sperimentale e Clinica
Ciclo: XXVI
Titolo della tesi: Role of ERK5/BMK1 in the survival, proliferation and stemness of chronic myeloid leukemia cells in hypoxia

A conclusione del corso triennale del XXVI° Ciclo del Dottorato di Ricerca in Scienze Biomediche (curriculum Oncologia Sperimentale e Clinica), il Collegio dei Docenti, facendo propria la relazione presentata dal Prof. Persio Dello Sbarba, in qualità di tutor, circa l'attività di ricerca, l'operosità e l'assiduità del candidato, rilascia con parere unanime il seguente giudizio da presentare alla Commissione Giudicatrice ai fini dell'espletamento dell'esame finale.
Il Dr. Ignazia Tusa, nata a Palermo il 14/03/1983, laureata in Scienze Biologiche il 30/09/2008, discutendo una tesi dal titolo "Analisi di danno spontaneo e bleomicina-indotto in soggetti affetti da patologie tiroidee" con la votazione di 110/110 e lode, è stata ammessa, a partire da 01/01/2011, al Dottorato di Ricerca in Scienze Biomediche curriculum Oncologia Sperimentale e Clinica (XXVI° Ciclo), svolgendo la propria attività di ricerca presso il Dipartimento di Scienze Biomediche, Sperimentali e Cliniche, Università di Firenze, sotto il tutoraggio del Prof Persio Dello Sbarba.

Descrizione dell'attività di ricerca/Risultati ottenuti:

Abbiamo precedentemente dimostrato che la resistenza all'ipossia è una caratteristica comune delle cellule staminali ematopoietiche (HSC) e delle cellule staminali leucemiche (LSC), ma anche che un sottoinsieme di LSC selezionabile in ipossia è probabile che esista all'interno di qualsiasi tipo di leucemia. Nella Leucemia Mieloide Cronica (CML), così come nelle linee cellulari che in cellule CD34+ espiantate da pazienti affetti da CML, l'ipossia sopprime l'espressione e fosforilazione della proteina BCR/Abl ma non quella del corrispondente mRNA. Ciò indica che le LSC, pur rimanendo geneticamente leucemiche, sono indipendenti da BCR/Abl per il loro mantenimento in vitro, risultando infatti del tutto insensibili al trattamento con Imatinib-mesilato (IM), caratteristica

peculiare delle cellule di CML responsabili della malattia minima residua (MRD). La proteinchinasi ERK5 appartiene alla famiglia delle MAP-chinasi ed è coinvolta nel controllo della sopravvivenza e della proliferazione cellulare, così come nella patogenesi di diversi tipi di tumore, compreso la CML. ERK5 è attivato da citochine coinvolte nella regolazione dei compartimenti di cellule staminali e partecipa alle risposte all'ipossia.
I principali obiettivi del lavoro sperimentale di questa tesi sono stati i seguenti: 1) esplorare il ruolo di ERK5 nel mantenimento della cellule progenitrici leucemiche (LPC) e delle LSC; 2) valutare gli effetti di differenti inibitori farmacologici della via di segnalazione MEK5/ERK5 sulla sopravvivenza e la proliferazione delle cellule di CML; 3) raccogliere informazioni utili per identificare un trattamento (combinatorio) innovativo in grado di eliminare le LSC refrattarie all'IM e quindi la MDR.
La selezione delle LPC/LSC, così come gli effetti dell'inibizione farmacologica della via di ERK5 sulle LPC/LSC selezionate in ipossia, è stato valutato mediante il Culture-Repopulating Ability (CRA) assay, messo a punto nel nostro laboratorio per la misura del potenziale staminale di popolazioni ematopoietiche normali e leucemiche. Sulla base della entità e della cinetica di ripopolazione di colture secondarie (LC2) in cui la crescita delle cellule è incontrollata, è possibile stimare il contenuto di popolazioni di LSC o LPC sottoposte a un trattamento selettivo in colture primarie (LC1). Negli esperimenti riportati qui, il saggio CRA è stato utilizzato per la stima del contenuto di popolazioni di cellule di CML ipossia-resistenti con caratteristiche di LSC/LPC e per testare gli effetti dell'inibizione genetica o farmacologica della via di ERK5 su questi sottogruppi cellulari. Abbiamo utilizzato l'inibitore ERK5-specifico XMD8-92 e gli inibitori MEK5-specifici BIX02188 e BIX02189. L'inibizione genetica di ERK5 è stata ottenuta utilizzando vettori lentivirali che esprimono short hairpin RNA specifici per ERK5. Gli effetti dei trattamenti inibenti sono stati testati su due linee cellulari umane stabilizzate di CML, KCL22 e K562, dove la proteina ERK5 è attivata costitutivamente.
I risultati ottenuti indicano che tutti e tre gli inibitori erano inattivi sul bulk della popolazione cellulare di CML rispetto al numero di cellule di controllo. D'altra parte, XMD8-92 determina un sensibile aumento della percentuale di cellule KCL22, ma di K562 , in fase G0/G1 del ciclo cellulare e una diminuzione della percentuale di cellule in fase S. BIX02188 e BIX02189 non mostrano alcun effetto sul ciclo cellulare sia nelle K562 che nelle KCL22 . XMD8 - 92 , ma non BIX02188 o BIX02189 , determinava inoltre un aumento di espressione di p27 in entrambe le linee cellulari e una riduzione dell'apoptosi basale presente nelle colture cellulari di K562 non trattate. In ipossia, dove l'attivazione costitutiva di ERK5 è soppressa, BIX02188 e BIX02189 non hanno influenzato il numero di cellule vitali in modo significativo in entrambe le linee cellulari. Il trattamento con XMD8-92, al contrario, ha determinato un significativo, anche se non marcato, aumento del numero di cellule vitali. Un blocco di progressione delle cellule di CML in fase S e l'aumento di p27 determinati da XMD8-92 sono stati osservati in entrambe le linee cellulari. Coerentemente BIX02188 o BIX02189, che non inducono un accumulo di cellule in fase G0/G1 provocano un aumento dell'espressione di p27. Presi insieme con i dati ottenuti in normossia, questi risultati indicano un effetto citostatico, piuttosto che citotossico, dell' inibizione specifica di ERK5. Inoltre, il trattamento con XMD8-92, ma non con BIX02188 o BIX02189, ha determinato una significativa riduzione dell'apoptosi indotta dall' ipossia. Questi risultati, insieme a quelli ottenuti in normossia, suggeriscono che l'effetto protettivo di XMD8-92 nei confronti dell'apoptosi basale presente nelle colture di controllo in normossia è aumentato in ipossia. Il trattamento delle cellule KCL22 e K562 con BIX02188 o BIX02189 in LC1 normossica riduce la capacità di ripopolazione delle LC2, mentre XMD8-92 sopprime quasi

completamente la ripopolazione delle LC2. D'altra parte, quando le LC1 ipossiche sono state trattate con BIX02188 o BIX02189, la ripopolazione LC2 è stata quasi immediata. Al contrario, il trattamento con XMD8-92 sopprime completamente la ripopolazione delle LC2. Questi risultati indicano che gli inibitori MEK5 hanno un modesto effetto sulla CRA di cellule di CML esprimenti la proteina BCR/Abl, accelerando in realtà la riespressione di BCR/Abl nelle cellule recuperate dall' ipossia. Al contrario, sia le cellue BCR/Abl positive che BCR/Abl negative erano marcatamente sensibili al trattamento con XMD8-92.
XMD8 -92 risulta essere quindi inattivo sul bulk della popolazione di CML, ma è in grado di sopprimere completamente le LPC BCR/Abl positive e le LPC/LSC selezionate in ipossia , BCR/Abl negative.
Per confermare i risultati di cui sopra ed escludere problemi di interpretazione dei dati a causa di possibili effetti off-target dei farmaci utilizzati, cellule di K562 sono state infettate con vettori lentivirali che esprimono shRNA diretti contro ERK5 (shERK5) . In ipossia, il knockdown genetico di ERK5, a differenza della sua inibizione farmacologica, riduce la sopravvivenza delle cellule di CML. Queste differenze sono probabilmente dovute alla ben nota proprietà di ERK5 nel regolare un gran numero di geni mediante interazione diretta, indipendentemente dalla sua attività chinasica. D'altra parte, il knockdown di ERK5 sopprime la ripopolazione delle LC2 guidato dalle cellule selezionate in ipossia tanto quanto l'inibizione dell'attività enzimatica di ERK5 tramite XMD8-92.
Avendo osservato che l'inibitore XMD8-92 non inibisce la sopravvivenza delle cellule di CML, ma sopprime le LSC selezionate in ipossia, abbiamo testato gli effetti del trattamento combinatorio XMD8-92/IM . IM riduce notevolmente il numero di cellule vitali sia in normossia che in ipossia. La combinazione con XMD8 -92 ha determinato un aumento discreto dell'effetto inibitorio dell'IM . La ripopolazione delle LC2 è stata ridotta, ma non abolita, dal trattamento con IM delle LC1 normossiche e ipossiche. Importante, XMD8-92, da solo o in combinazione con IM, sopprime completamente la ripopolazione delle LC2. L'efficacia del farmaco XMD8-92 sulle cellule selezione in ipossia di linee di CML stabilizzate è stata testata anche su cellule primarie espiantate da pazienti affetti da CML . In linea con quanto osservato sulle linee cellulari , XMD8-92 da solo o in combinazione con IM, alterata la ripopolazione cellulare guidata dalle LSC selezionate in ipossia da pazienti affetti da CML.
Questi risultati indicano che il trattamento combinato di XMD8-92 con IM può essere un utile approccio per cercare di sradicare la CML insieme con l'induzione della remissione, essendo XMD8-92 attivo sulle LPC/LSC, ma non sul bulk, e ,viceversa, essendo IM molto attivo sul bulk, ma non in grado di sopprimere i progenitori della CML.

Pubblicazioni:

Chromatin-associated CSF-1R binds to the promoter of proliferation-related genes in breast cancer cells.
Barbetti V, Morandi A, Tusa I, Digiacomo G, Riverso M, Marzi I, Cipolleschi MG, Bessi S, Giannini A, Di Leo A, Dello Sbarba P, Rovida E.
Oncogene. 2013.

AML1/ETO sensitizes via TRAIL acute myeloid leukemia cells to the pro-apoptotic effects of hypoxia.
Barbetti V, Tusa I, Cipolleschi MG, Rovida E, Dello Sbarba P.
Cell Death Dis. 2013; 4:e536.

Non-thermal effects of 2.45 GHz microwaves on spindle assembly, mitotic cells and viability of Chinese hamster V-79 cells.
Ballardin M, Tusa I, Fontana N, Monorchio A, Pelletti C, Rogovich A, Barale R, Scarpato R.
Mutat Res. 2011; 716:1-9.

Spontaneous and bleomycin-induced chromosome damage in non cancer thyroid patients.
Scarpato R, Tusa I, Antonelli A, Fallhai P, Sbrana I.
Eur J Clin Invest. 2009 ; 39:1091-1097

Presentazione dati a congressi nazionali e internazionali:

Rovida E, Tusa I, Cheloni G, Gray NS, Deng X, , Dello Sbarba P. ERK5 inhibition as a novel approach to target chronic myeloid leukaemia stem cells. 54th Annual Meeting of the Italian Cancer Society, Bologna, October 1-4, 2012. Abstract book p46.

Tusa I, Cheloni G, Gray NS, Deng X, Rovida E, Dello Sbarba P. ERK5-inhibition as a novel approach to target CML stem cells. XII Congress of the Italian Society of Experimental Haematology, Roma, October 17-19, 2012. Haematologica, 2012; 97(s2):S73.

Rovida E, Di Maira G, Navari N, Rombouts K, Cannito S, Tusa I, Dello Sbarba P, Parola M, Marra F. ERK5 modulates the phenotype of hepatocellular carcinoma cells and tumor development. Biochemistry, Biology and pathology of MAP Kinases. 14-18 October 2012, Maale Hachamisha, Jerusalem Hills, Jerusalem, Israel. Abstract book p75.

Rovida E, Tusa I, Cheloni G, Gray NS, Deng X, Dello Sbarba P. ERK5-inhibition as a novel approach to target chronic myeloid leukemia stem cells. Biochemistry, Biology and pathology of MAP Kinases. 14-18 October 2012, Maale Hachamisha, Jerusalem Hills, Jerusalem, Israel. Abstract book p107.

Durante il corso di dottorato, il candidato ha seguito con il massimo impegno il programma didattico stabilito dal Collegio dei Docenti ed ha portato avanti con entusiasmo e determinazione le sue ricerche, dando prova di grande inventiva ed intraprendenza, nonché di notevole elasticità nella elaborazione dei dati sperimentali. Nel corso del triennio, il candidato ha inoltre maturato una buona cultura di base ed una vasta esperienza diretta in metodiche sperimentali.

Per quanto sopra, il Collegio dei Docenti unanime ritiene che la Dr.ssa Ignazia Tusa possa meritatamente aspirare a conseguire il titolo di Dottore di Ricerca.

Firenze, 14/02/2014

Il Coordinatore del Corso

Prof. Persio Dello Sbarba