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**Chronic Myeloid Leukaemia Stem Cells are  
sensitive to the pharmacological inhibition of  
Hypoxia Inducible Factor-1 $\alpha$ .**

Settore Scientifico Disciplinare MED/04

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# Abbreviations

AGM	Aorta-Gonad Mesonephros
ALL	Acute Lymphoblastic Leukaemia
AML	Acute myeloid leukaemia
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
BCR	Breakpoint Cluster Region
BHLH	Helix-Loop-Helix
BM	Bone Marrow
CA	Carbonic Anidrase
CaLB	Calcium-dependent lipid binding
CAR	CXCL-12 Abundant Reticular (cells)
CFC	Colony Forming Cell
CML	Chronic Myeloid Leukaemia
CSC	Cancer Stem Cell
CXCL	CXC Chemokine Ligand
CXCR	Chemokine CXC-motif Receptor
DA	Dorsal Aorta
DD	Dimerization Domain
DMSO	Dimethylsulfoxide
ECM	Extra Cellular Matrix
EHT	Endothelial-Haematopoietic Transition
EMT	Epithelial-to-Mesenchymal Transition
EPAS	Endothelial PAS-domain protein
EPo	Erythropoietin
ES	Embryonic Stem
5-FU	5-fluorouracil
G-CSF	Granulocyte-Colony Stimulating Factor
GEF	Guanidine Exchange Factors
GLUT	GLUcose Transporter

hES	human Embryonic Stem
HIF	Hypoxia Inducible Factor
HPC	Haematopoietic Progenitors Cells
HRE	Hypoxia Responsive Elements
HSC	Haematopoietic Stem Cells
IL	Interleukin
IM	Imatinib-Mesylate
iPS	induced Pluripotent Stem
IR	Ionizing Radiation
kDa	Kilodalton
LDH	Lactate De-Hydrogenase
LOX	Lysyl Oxidase
LPC	Leukemic Progenitor Cell
LSC	Leukaemia Stem Cells
LTR	Short-Term Repopulating
MMP	Matrix Metalloproteinases
MRD	Minimal Residual Disease
NLS	Nuclear Localization Signals
NSC	Neural Stem Cells
NOD-SCID	Non Obese Diabetic-Severe Combined Immuno-Deficient
ODD	Oxygen Dependent Degradation
PARP	Poly ADP-Ribose Polymerase
PAS	<u>P</u> ER- <u>A</u> RNT- <u>S</u> IM
PBS	Phosphate-Buffered Saline
Ph	Philadelphia chromosome
PDK	Pyruvate Dehydrogenase Kinase
PDGF	Platelet-Derived Growth Factor
PTH	Parathyroid Hormone
PPR	Parathyroid hormone-related Peptide Receptor
PxxP	Proline-rich regions
RAC-GAP	Rac-GTPase
ROS	Reactive Oxygen Species
SC	Stem Cells

SCF	Stem Cell Factor
SDF	Stromal cell-Derived Factor
SH	Src-Homology
SLAM	Stimulated Lymphocyte Activating Molecule
SNO	N-cadherin-positive osteoblasts
STR	Short-Term Repopulating
TKi	Tyrosine Kinase inhibitors
TGF	Transforming Growth Factor
TPo	Thrombopoietin
VEGF	Vascular Endothelial Growth Factor
vHL	von Hippel-Lindau

# Abstract

Chronic Myeloid Leukaemia (CML) is a clonal Haematopoietic Stem Cells (HSC) disorder due to the 9;22 reciprocal chromosomal translocation, which results in the generation of *BCR/abl* “fusion” oncogene and the expression of the BCR/ABL oncoprotein, a constitutively-active tyrosine kinase. Chronic-phase CML patients are treated with Tyrosine Kinase inhibitors (TKi), such as Imatinib-Mesylate (IM). However, rather than definitively curing CML, TKi induce a state of Minimal Residual Disease (MRD), apparently due to the persistence of Leukaemia Stem Cells (LSC). We proposed that the expression of BCR/Abl is suppressed in LSC, so that LSC are independent of BCR/Abl signaling and refractory to TKi. This suppression is facilitated in the hypoxic environment of stem cell niches where HSC as well as LSC reside in bone marrow. Hypoxia-Inducible Factor (HIF) -1 $\alpha$  is a key regulator of cell adaptation to hypoxia and of HSC and LSC maintenance. The aim of this study was to address the *in vitro* and *in vivo* effects of the pharmacological inhibition of HIF-1 $\alpha$  on LSC maintenance.

We compared three different putative HIF-1 $\alpha$  inhibitors, identifying Compound X as a very effective inhibitor of CML cell survival and growth in hypoxia. Compound X also inhibited the colony-formation ability of primary human or murine CML cells. Using our *in vitro* LSC assay, Culture-Repopulation Ability (CRA) assay, we demonstrated that HIF-1 $\alpha$  pharmacological inhibition in CML cell lines or primary cells incubated in hypoxia suppressed CRA, indicating that Compound X suppressed LSC maintenance. This result was confirmed using HIF-1 $\alpha$  shRNA-mediated knockdown. CRA, on the contrary, was not affected by IM treatment.

We tested the effects of the simultaneous combination of Compound X with TKi, thinking that such a protocol better approaches a potential clinical setting, to exclude antagonist effects of IM with respect to Compound X. We found that, in fact, the two drugs had an additive effect as far as the total number of viable cells

in hypoxic cultures is concerned. On the other hand, IM did not interfere with the detrimental effect of Compound X on LSC maintenance.

Compound X treatment of *BCR/abl*-transduced mice decreased the number and the percentage of the LSC-containing LSK (lineage<sup>-</sup>/Sca-1<sup>+</sup>/c-Kit<sup>+</sup>) cell subset in the BM, without affecting the HSC.

In conclusion, using two CML cell lines, primary CML cells and a mouse model of CML, we demonstrated that IM-resistant LSC are instead sensitive to the pharmacological inhibition of HIF-1 $\alpha$ . On this basis, we propose the Compound X/IM combination as a novel therapeutic approach targeting CML cell bulk as well as LSC in order to, at one time, induce remission and prevent MRD.



# Introduction

## Chronic Myeloid Leukaemia

Leukaemias are clonal disorders of haematopoiesis where a normal haematopoietic cell acquires mutations that confer the capacity for unlimited self-renewal, enhanced proliferation and impaired differentiation.

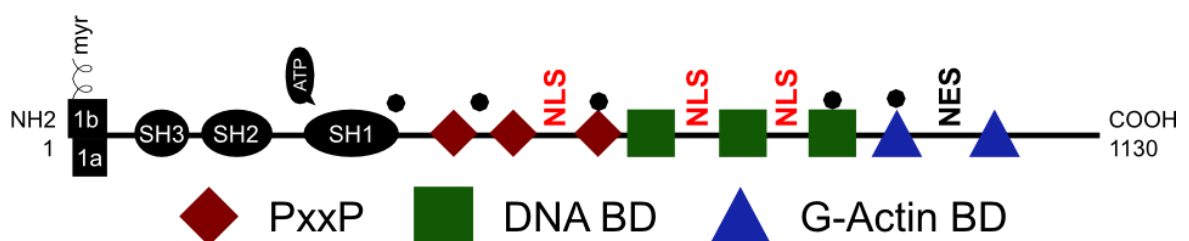
CML is characterized by myeloid hyperplasia of the Bone Marrow (BM) and increase of myeloid cells in peripheral blood. The clinical signs often include granulocytosis, splenomegaly and enlargement of the liver; nonspecific symptoms, such as fatigue and weight loss, may occur; about 40% of patients are asymptomatic. In the latter case the diagnosis is driven by an elevated white blood cell count [1].

The natural history of CML involves three different stages: chronic phase, accelerated phase and blast crisis. At diagnosis, 90% of patients are in chronic phase, which normally lasts from three to eight years. In this phase, blood cells retain a full ability to differentiate. When the disease progresses to the accelerated phase, which lasts about a year, myeloid cells, mainly of the neutrophil lineage, lose the capacity to differentiate and immature cells (“blasts”) appear in the blood. Finally, the disease progresses to the blast crisis, defined by the presence in the blood of 30% blasts over the total white blood cell number. During this stage, extra-medullary infiltrates of blast are often observed. The survival of patients after the onset of blast crisis is reduced to months and even weeks [2].

CML is characterized by an aberrant chromosomal rearrangement leading to the generation of the “Philadelphia chromosome” (Ph). Ph is a shortened chromosome 22 originated from the reciprocal translocation between the long arms of chromosomes 9 and 22 [t (9; 22)] that causes the addition of 3' segments of the proto-oncogene *c-abl* (9q34) to 5' segments of the BCR (*breakpoint cluster region*) gene (22q11). The translocation gives rise to the BCR/*abl* fusion gene that

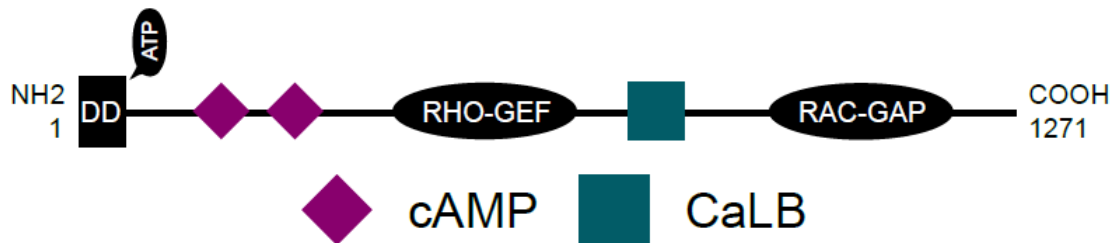
transcribes a chimeric mRNA of 8.5 kb. This mRNA is translated into the BCR/Abl fusion protein, a constitutively active tyrosine kinase [2]. Ph is found in more than 95% of CML patients; complex rearrangements that may involve one or more chromosomes in addition to 9 and 22 are also present in 5-10% of CML patients [3]. The events that favor the translocation are still unclear; however, ionizing radiation (IR) is a risk factor for CML and in vitro exposure of haematopoietic cells to IR induces BCR/*abl* fusion transcript [4, 5]. Although Ph was the first chromosomal abnormality found consistently associated with a specific type of leukaemia, aberrant chromosomal rearrangement are present in other leukaemias; e.g., a subset of Acute Myeloid Leukaemia (AML) is characterized by the expression of the AML1/ETO fusion protein.

The human *c-abl* gene, homologous of the viral *v-abl* oncogene of Abelson murine Leukaemia, encodes for a 145 kDa non-receptor tyrosine kinase expressed in particular in haematopoietic cells [6] and neurons [7] and localized mainly at the cytoplasmic level [6] (Figure 1). Abl is involved in the regulation of the cell cycle, response to genotoxic stress and in integrin-mediated intracellular signaling [8]. Under physiological conditions, the Abl tyrosine kinase activity is tightly regulated. Deletion or mutation of SH3 (Src-homology) domain activates the kinase, indicating that the domain plays a critical role in this process. Several proteins are able to bind Abl SH3 domain activating the inhibitory functions [9-11]. The fusion of BCR sequence to the Abl SH3 domain, probably, abrogates the physiological suppression of kinase activity. Other studies proposed that the SH3 domain binds the proline-rich (PxxP) region in the center of Abl protein causing a conformational change that inhibits the interaction with substrates [12].



**Figure 1. Structure of ABL protein.** Src-homology domains: SH; note three SH domains at the NH<sub>2</sub> terminus. Proline-rich regions: PxxP bind the SH3 domain inhibiting the kinase activity. Nuclear Localization Signal: NLS. Nuclear Export Signal: NES. DNA-Binding Domain: DNA BD. Actin-Binding Domain: Actin BD. The Abl isoform 1b, containing a Myristoylation site (myr) for attachment to the plasma membrane, is larger than the 1a isoform.

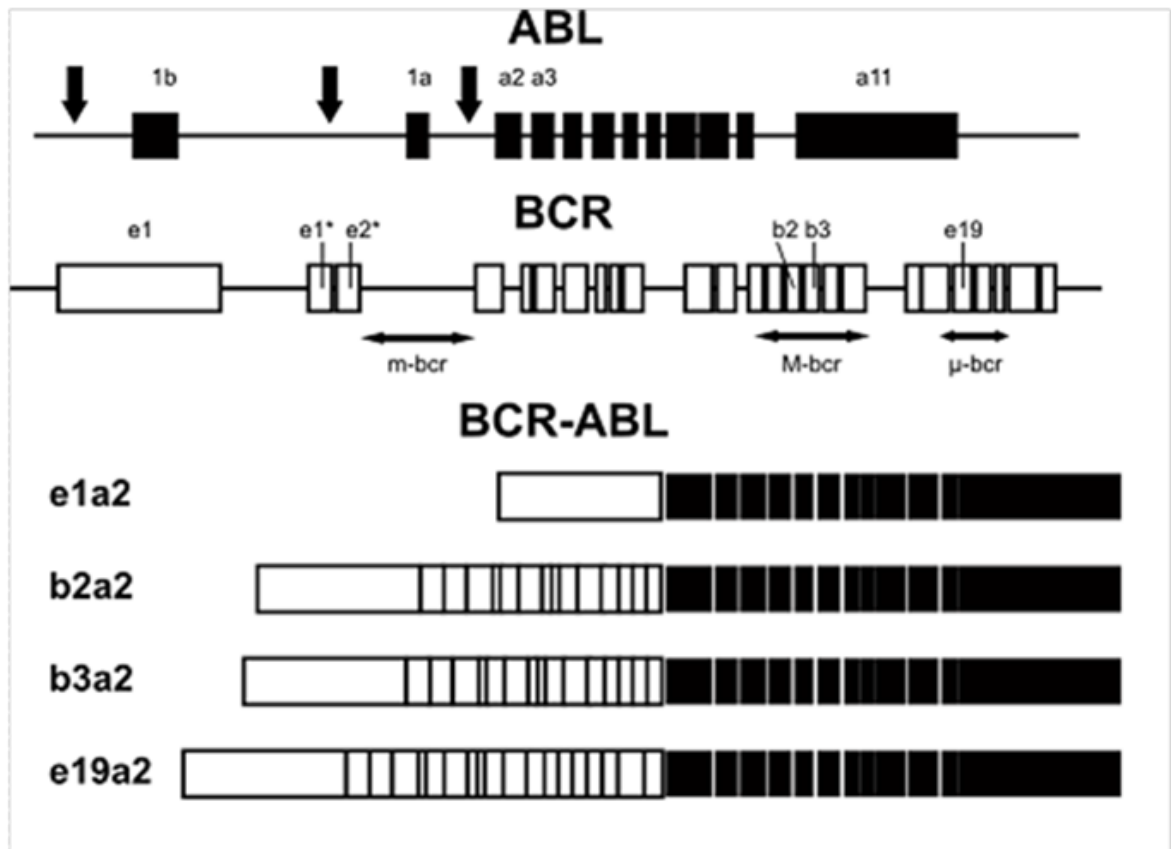
The normal human *BCR* gene encodes for a 160 kDa serine/threonine-kinase predominantly expressed in hematopoietic cells and neurons (Figure2). It localizes mainly at the cytoplasmic level, where it is involved in several signaling pathways, especially those regulated by G proteins [13-15].



**Figure 2. Structure of BCR protein.** Dimerization Domain: DD. Note the Y117 autophosphorylation site crucial for binding to Grb-2. Rho-Guanidine Exchange Factors domain: RHO-GEF. Calcium-dependent lipid binding domain: CaLB. Rac-GTPase-activating protein domain: RAC-GAP.

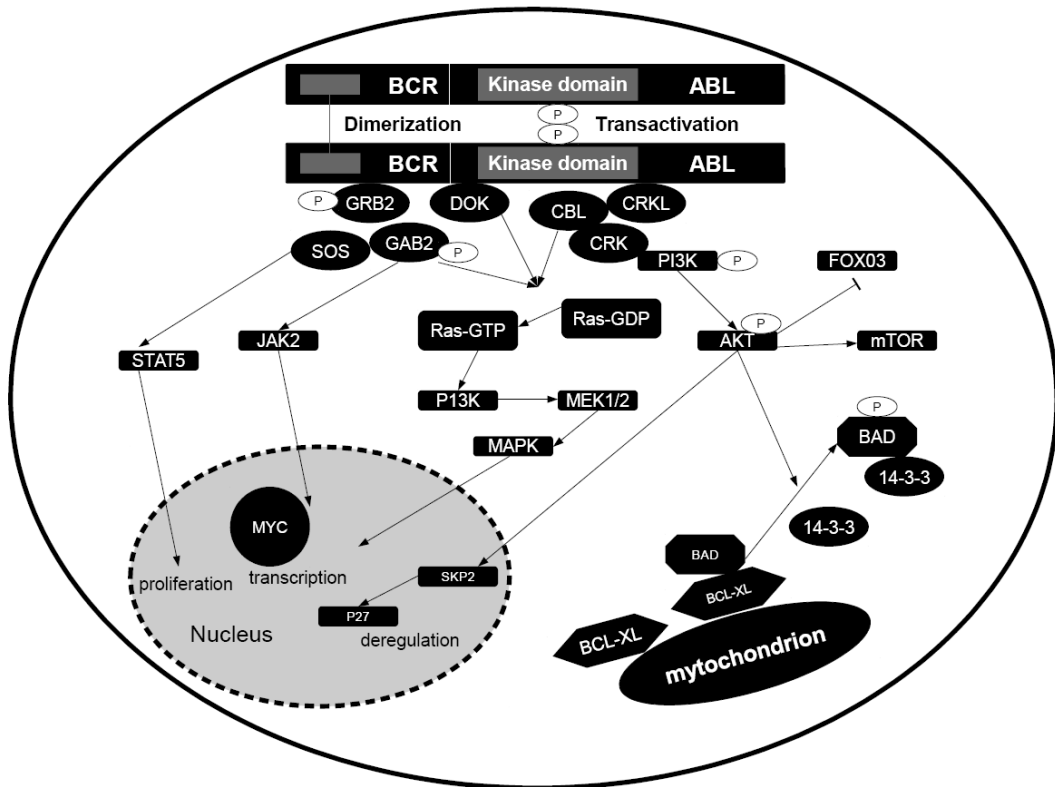
In CML, breakpoints within the *abl* gene occur over a large area at its 5' end; regardless of the exact location of *abl* the breakpoint, splicing of the primary BCR/*Abl* transcript yields an mRNA molecule in which BCR is fused to *abl* exon a2 (Figure3). BCR breakpoints can localize in three different regions; this affects the final BCR/*abl* form. In most CML patients, the break occurs within a 5.8 kb area spanning BCR exons 12-16, previously referred to as exons b1-b5, defined as the major breakpoint cluster region (M-BCR). An alternative splicing of these transcripts leads to the b2a2 or b3a2 junctions. Translation of these mRNAs results in the expression of a p210 BCR/*Abl* chimeric protein, which is present not only in most patients with CML, but also in approximately one third of patients with Ph-positive Acute Lymphoblastic Leukaemia (ALL). On the other hand, the remaining patients with ALL and rare forms of CML express the p190 BCR/*Abl* isoform. This isoform derives from breaks in the BCR region termed minor breakpoint cluster region (m-BCR) which is localized between the exon e2' and e2. The p230 BCR/*Abl* isoform originates from the third breakpoint cluster region ( $\mu$ -BCR) downstream of BCR exon 19. It is associated with the rare Ph-positive chronic neutrophilic leukaemia (Figure 3). The transforming principle of the BCR/*Abl* fusion protein is carried by the *Abl* portion, while the different phenotype of the disease is probably determined by the different portion of BCR that constitute the fusion protein. The *Abl* portion of fusion protein is in fact almost

invariable, while the BCR portion varies greatly. Moreover, the BCR gene can be replaced with different genes and still cause leukaemia; e.g., in rare cases of ALL, the TEL-abl fusion gene is expressed [16, 17].



**Figure 3. Location of the breakpoints in the ABL and BCR genes.** Structures of the mRNAs transcribed from various breaks. The arrows indicate the region where the break can occur. Minor-breakpoint cluster region: m-bcr. Major breakpoint cluster region: M-bcr.

The expression of BCR/Abl is directly responsible for leukaemogenesis. Three major mechanisms are involved in the malignant transformation: altered adhesion to stromal cells and extracellular matrix, constitutively-active mitogenic signaling and reduced apoptosis [18-20] (Figure 4).



**Figure 4. Signaling pathways activated in BCR/Abl expressing cells.**

To date, chronic-phase CML patients are treated with TKi, first of all IM, which specifically target BCR/ABL. However, TKi, despite their impressive efficacy as first-line therapy, are unable, in most cases, to prevent the relapse, inducing a state of MRD, apparently due to the persistence of LSC. The survival of LSC, in fact, is independent of BCR/ABL kinase activity [21-24].

## Haematopoietic Stem Cells

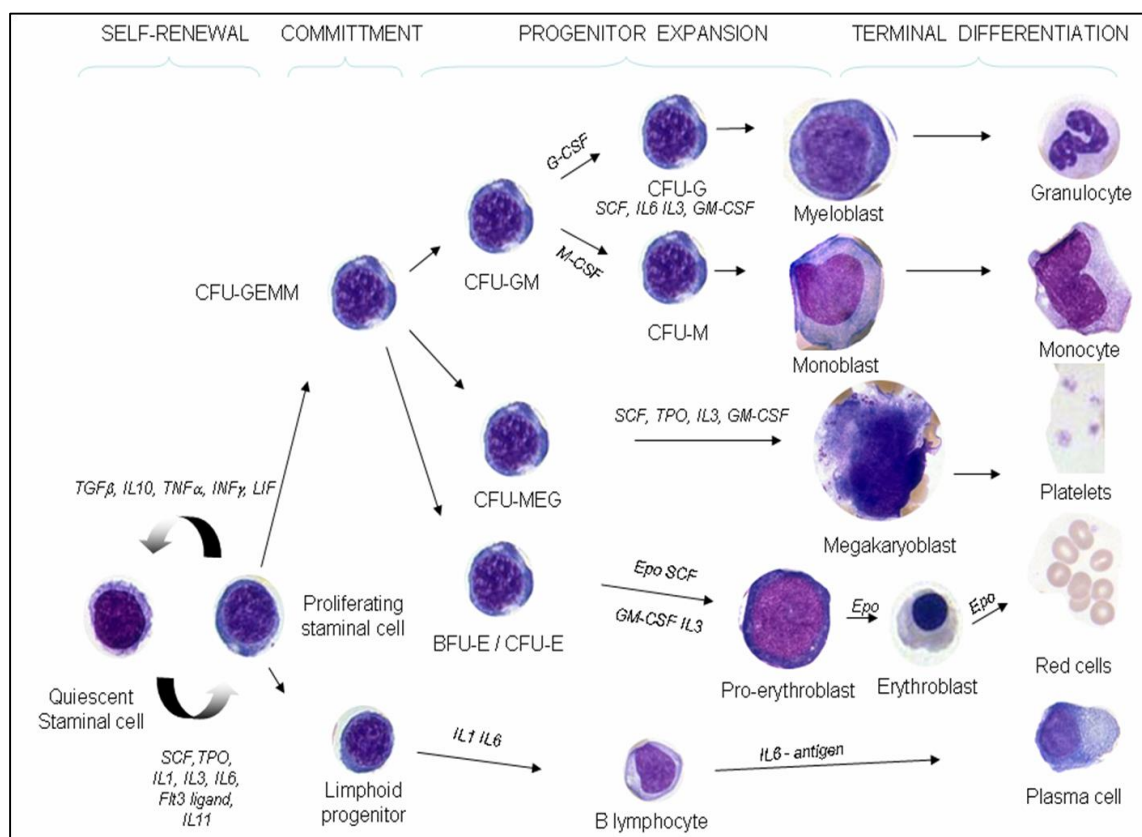
Blood cells can be divided in two main groups: lymphoid cells (T, B and Natural Killer cells) and myeloid cells (granulocytes, monocytes, erythrocytes and megakaryocytes). Haematopoiesis is the process of blood cell formation during both the embryonic and adult stage of an organism (Figure 5). In the adult animal, all blood cells derive from a small common pool of multipotent HSC [25], that represent less than 0.01% of the total number of BM cells. In the zebrafish embryo, pluripotent HSC arise directly from haemogenic endothelium, lining the ventral wall of the Dorsal Aorta (DA). In particular, HSC seem 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 haematopoietic 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 the mouse, these cells (expressing Sca1, c-Kit and CD41) emerge in the lumen of the aorta, budding directly from ventral CD31+/CD41- endothelial cells [26-28]. In mammals, the site of haematopoiesis changes during the development; during the early embryonal development, the sequential sites of haematopoiesis include the yolk sac, an area surrounding the DA termed the Aorta-Gonad Mesonephros (AGM) region, the placenta, the foetal liver, thymus and spleen. At 14 to 20 weeks of gestation, HSC migrate to the BM, where they are definitively established. The interaction with these different environments confers different properties upon HSC: for example, HSC are in cycle in foetal liver while are largely quiescent in BM. During the adult life, BM is responsible for the generation of all blood cells. In particular, human adult haematopoiesis is restricted to the proximal regions of long bones of the axial skeleton (cranium, sternum, ribs and vertebrae) in addition to the ilium [29]. However, in pathological conditions, such as leukaemia or myelofibrosis HSC can re-migrate to sites of foetal haematopoiesis, e.g. liver and spleen.

Haematopoiesis includes several stages such as commitment to a specific cell lineage, increase of proliferation, terminal differentiation of lineage-restricted progenitor cells, growth arrest and apoptosis. Such a hierarchical model of haematopoiesis has provided a paradigm for the development and regeneration of tissues in general as well as for tumorigenesis. All the steps of haematopoiesis are highly regulated via the progressive expression or repression of transcription factors and cell surface cytokine receptors, driving the interaction of different sub-populations of haematopoietic cells with the environment (stromal cells, extracellular matrix, etc.).

HSC, like all other Stem Cells (SC), possess the capacity to maintain themselves ("self-renewal") and at the same time to sustain clonal expansion generating a rapidly proliferating progeny. HSC self-renewal and concomitant clonal expansion are ensured by asymmetric cell division, in which molecular determinants of cell fate are redistributed unequally to the two daughter cells, or

via environmental asymmetry, in which 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]. In this fashion, only few SC at a time move from quiescence to a cycling state, giving rise to rapidly proliferating Haematopoietic Progenitors Cells (HPC), which directly sustain the generation of maturing blood cells. HPC derive directly from Short-Term Reconstituting (STR) HSC, capable to sustain haematopoiesis for only few weeks. STR-HSC in turn derive from Long-Term Reconstituting (LTR) HSC. The latter cell subset is the one typically involved in quiescence and asymmetric division.

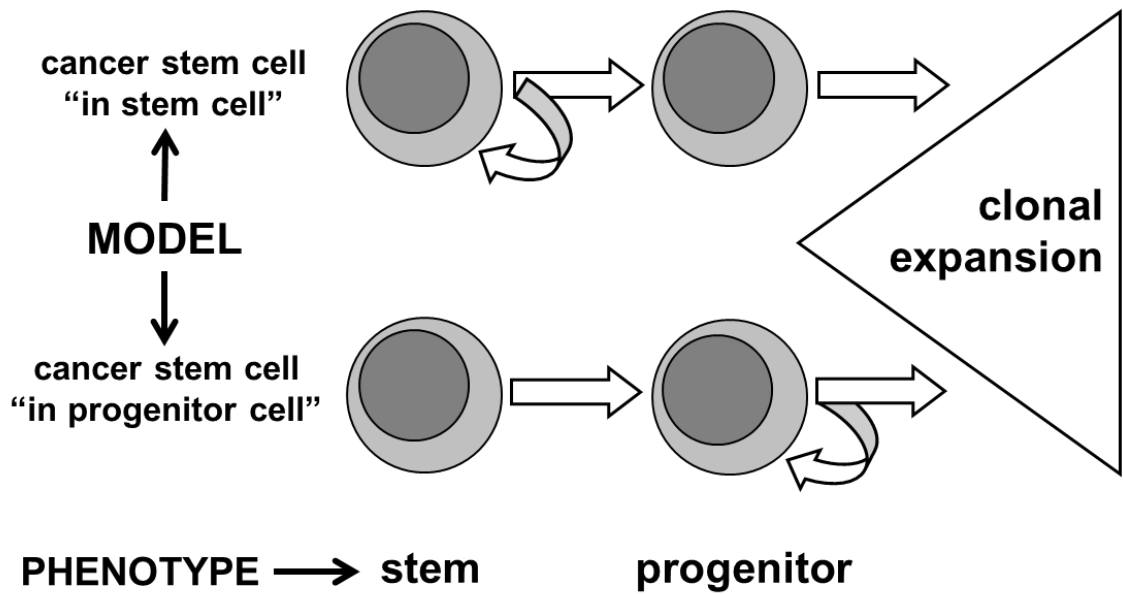


**Figure 5. Normal Haematopoiesis.**

## Leukaemia Stem Cells

The concept of the existence of a primitive population of cancer cells that escape the normal control of self-renewal, resulting in unrestrained clonal expansion, led to the formulation of the Cancer Stem Cells (CSC) hypothesis. This theory postulates that tumours are maintained from a small minority of stem-like

cancer cells, which possess sufficient self-renewal capacity to recapitulate the entire tumour population. CSC could originate from the neoplastic transformation of HSC which physiologically possess the capacity of self-renewal, or from HPC which acquire this capacity aberrantly, as a consequence of oncogenic transformation (Figure 6). In any case, CSC are responsible for the persistence of MRD after successful treatment and for resistance to therapy.



**Figure 6. The two models for cancer stem cell generation.** Oncogenic transformation of stem cells or acquisition of self-renewal by transformed progenitor cells; correspondence of cancer stem cells to normal phenotypes.

LSC were the first CSC described, in 1994, by Dick and co-workers, who dissociated LSC from the bulk of AML cells. In this work, the authors identified LSC 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 leukaemic, like normal, haematopoiesis is hierarchically organized and that LSC, like HSC, exhibit peculiar and detectable phenotypes [31-33].



## The haematopoietic stem cell niche

The decision between self-renewal and maintenance of stem cell potential or massive clonal expansion and loss of this potential largely depends on the interaction of HSC with the extracellular environment where HSC reside, the HSC “niche” [30, 34]. The concept of the haematopoietic niche was first proposed by Schofield, who designed the specific features of a microenvironment that ensures HSC maintenance by controlling the balance between self-renewal and clonal expansion. The niche environment favors HSC maintenance and quiescence, privileging self-renewal over clonal expansion, yet allowing, under controlled conditions, the SC to generate proliferating HPC. Leaving the HSC niche, HPC are enabled to proliferate and eventually differentiate [35] (Figure 7.). These Schofield’s 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 haematopoiesis [35-38].

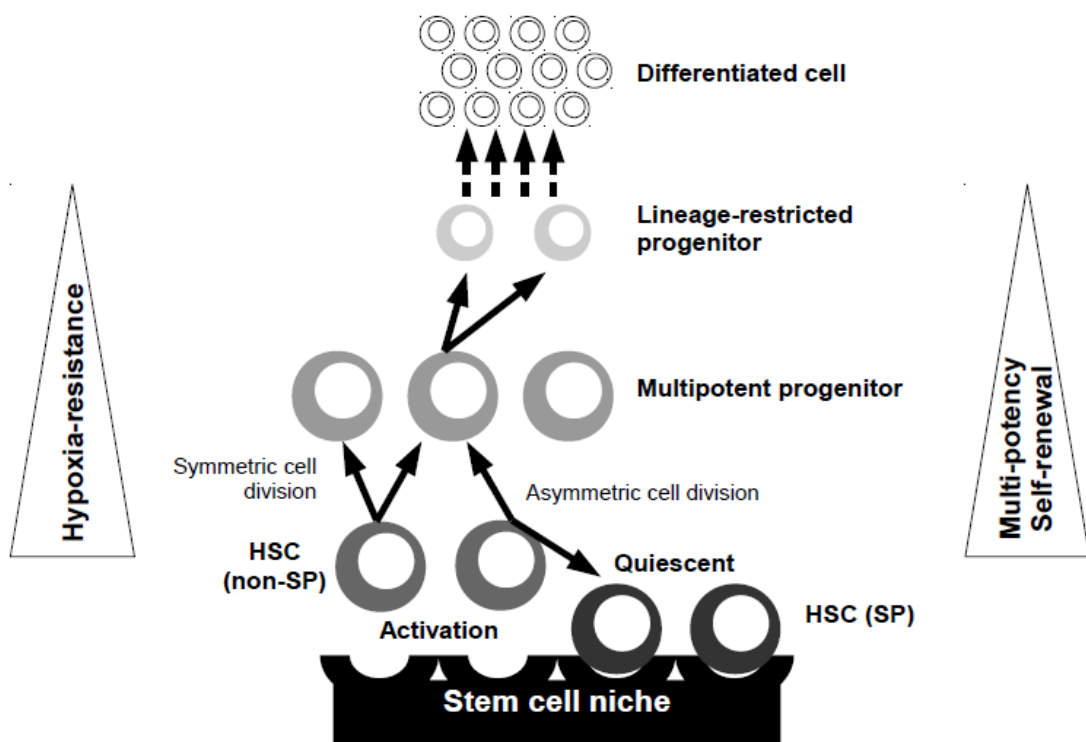


Figure 7. The haematopoietic stem cell niche.

Although the idea of the niche was proposed for HSC, the first SC niches were experimentally characterized in *Drosophila* gonads and in *Caenorhabditis elegans*. Today, SC 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 [39, 40].

HSC niches in BM consist of non-haematopoietic cells including fibroblasts, adipocytes and blood vessel cells, as well as nerves. Evidences suggest the existence of two types of BM niches: the “osteoblastic niche”, located near the endosteum, and the perivascular niche, located in the sinusoidal vasculature. To date, the functional differences between these spatially distinct niches have not been completely characterized and it is not clear if the osteoblasts and endothelial cells represent distinct or overlapping niches, and if separate niches, if the HSC occupying the niches are the same or have different properties [41-44]. The osteoblastic niche, which is a hypoxic niche, privileges the maintenance of HSC in a quiescent state (slow cycling or  $G_0$ ). From this niche, the progeny of LTR-HSC (STR-HSC) can move to progressively better oxygenated areas, reaching the vascular niche where extensive clonal expansion and differentiation are allowed following activation of aerobic metabolism in proliferating cells [45-48]. When the supply of mature cells is no longer needed and if the LT-HSC’s stem potential is not exhausted, stimuli inducing HSC activation and division are withdrawn and ST-HSC located in the osteoblastic niche can return to  $G_0$  [45]. Quiescence is critical to sustain the stem cell compartment. Loss of quiescence, as occurs with  $p21^{cip1}$  deficiency [49], results in a long-term decrease of stem cell pool and its eventual exhaustion, so that the shuttling of HSC between the two types of niches might be a key feature of well-balanced haematopoiesis. In this respect, Quesenberry proposed the so-called *Chiaroscuro* model of haematopoiesis based on a flexible and reversible transition between the stem and progenitor cell phenotypes [50].

Quiescence of HSC is, also, closely associated with the protection of the HSC pool from the various stresses induced by myelotoxic insults; quiescent HSC are, in fact, resistant to 5-fluorouracil (5-FU)-induced myelosuppression [42].

## Endosteal (osteoblastic) niche

Osteoblasts are bone cells in charge of the secretion of unmineralized bone matrix proteins, as well as of the regulation of osteoclast differentiation, and usually reside in a layer along the endosteum, at the interface between BM and bone [29, 51].

Studies of the 1970s indicate that undifferentiated haematopoietic cells localize close to the endosteal bone surface, but that differentiated cells move toward the central axis of BM.

The concept of “endosteal” or “osteoblastic” SC niche is supported by findings that osteoblasts produce factors that have the ability to regulate quiescence and maintenance of HSC, *e.g.* angiopoietin-1, thrombopoietin, osteopontin [41, 42, 52, 53]. Moreover, osteoblasts express the chemokine CXC Chemokine Ligand (CXCL)-12, which regulates chemotaxis, homing, and survival of HSC and has an important role in the retention of HSC in BM [54]. Many other *in vitro* and *in vivo* studies demonstrated that osteoblasts play an important role as part of the HSC niche. Human osteoblasts have been shown to support HSC in *ex vivo* cultures of primitive haematopoietic cells [55-58]. Using mice conditionally deleted of bone morphogenetic protein receptor 1A, Zhang and colleagues demonstrated that the ectopic formation of trabecular bone-like area and the increase of the number of N-cadherin-positive osteoblasts (SNO cells) correlates with an increased HSC number. Moreover, they found label-retaining cells thought to be HSC attached to the SNO cells, in association with N-cadherin [59]. In another study, Calvi and colleagues examined the effects of the constitutively-activated parathyroid hormone (PTH)-related peptide receptor (PPR). The PPR mice had increased HSC in conjunction with increased trabecular bone. They also observed an elevated number of trabecular osteoblasts that expressed the Notch ligand Jagged-1, required for the HSC-potentiating effects of the osteoblasts. The addition of a  $\gamma$ -secretase inhibitor (which inhibits Notch activation) to stromal cell cultures prevented this effect. These results were confirmed using wild-type stromal cultures or wild-type mice treated with PTH [41]. Moreover, PTH, regulating the size of HSC niche, has therapeutic potential for both HSC mobilization and haematopoietic recovery post-transplantation in mouse models [41, 60]. However, while the expansion of osteoblasts was demonstrated to

increase HSC, their decrease could not be consistently linked to a HSC reduction [61, 62].

## **Perivascular niche**

Endothelial cells, lining the interior surface of blood and lymphatic vessels, in BM form a barrier between the developing haematopoietic cells and the blood.

The hypothesis of a “perivascular” SC niche emerged mainly thanks to the discovery of the SLAM (Stimulated Lymphocyte-Activating Molecule) family antigens (CD150+/CD244-/CD48-/CD41-), marking HSC. Histochemical analyses of BM sections showed that the majority of HSC resides in the perivascular niche, with only 16% of HSC being in the osteoblastic region [43]. However, whether the perivascular zone represents a true niche still requires experimental definition. Indeed, it is possible that HSC accumulate around the vessels because it is an impedance point in their trafficking into and out of the vasculature. Moreover, to date, no modification in endothelial function *in vivo* has been shown to affect HSC other than what might be expected from perturbed trafficking.

However, developmental changes in haematopoiesis would suggest that perivascular sites are likely to serve as niches. With the exception of BM, all sites where HSC can be isolated from during embryonic development contain endothelial cells, which have been thus closely associated with the generation of HSC [63-65].

Additional evidence for the vascular niche was also provided using the GFP reporter in the CXCL-12 locus. This study identified cells that secrete high levels of CXCL-12, named CAR cells (CXCL-12-Abundant Reticular cells) and interact with over 90% of HSC (recognized by either SLAM markers or lin-/cKit+/Sca1+). Reticular cells around the sinusoids are an important component of the vascular niche [44]. In human BM, these cells constitute the sub-endothelial (adventitial) layer of sinusoidal walls, projecting a reticular process that is in close contact with HSC. Interestingly, reticular cells are derived from a specific subset of mesenchymal cells (CD146+) that produces either reticular or endosteal cells. Finally, other *in vitro* studies demonstrated that endothelial cells derived from various tissues support HSC in culture, suggesting their contribution to the establishment of vascular niche.

# Hypoxia and Hypoxia Inducible Factor

## Role of hypoxia in normal haematopoiesis and Leukaemia

One of most important distinguishing features of the haematopoietic stem cell niche, with respect to other districts, is the very low percentage of oxygen; in the different areas of BM, the physiological oxygen level correspond to an *in vitro* 0.1-5.0% range. This implies that an environment which is hypoxic for the overall BM cell population is instead normoxic for the HSC. Hypoxia is a consequence of both the intense crowding of proliferating/differentiating BM cells and the peculiar vascularization of BM. The steep  $pO_2$  gradient in function of cell distance from blood vessels accounts for the paradoxical situation that, even in the relatively well vascularized regions of haematopoietic tissue, hypoxic areas can be easily determined within the cell conglomerates, thus constituting the “hypoxic stem cell niches” [66] (Figure 8). Oxygen availability in leukaemic BM is possibly even lower; due to the further increase of cell density determined by the unrestrained expansion of neoplastic cell clones.

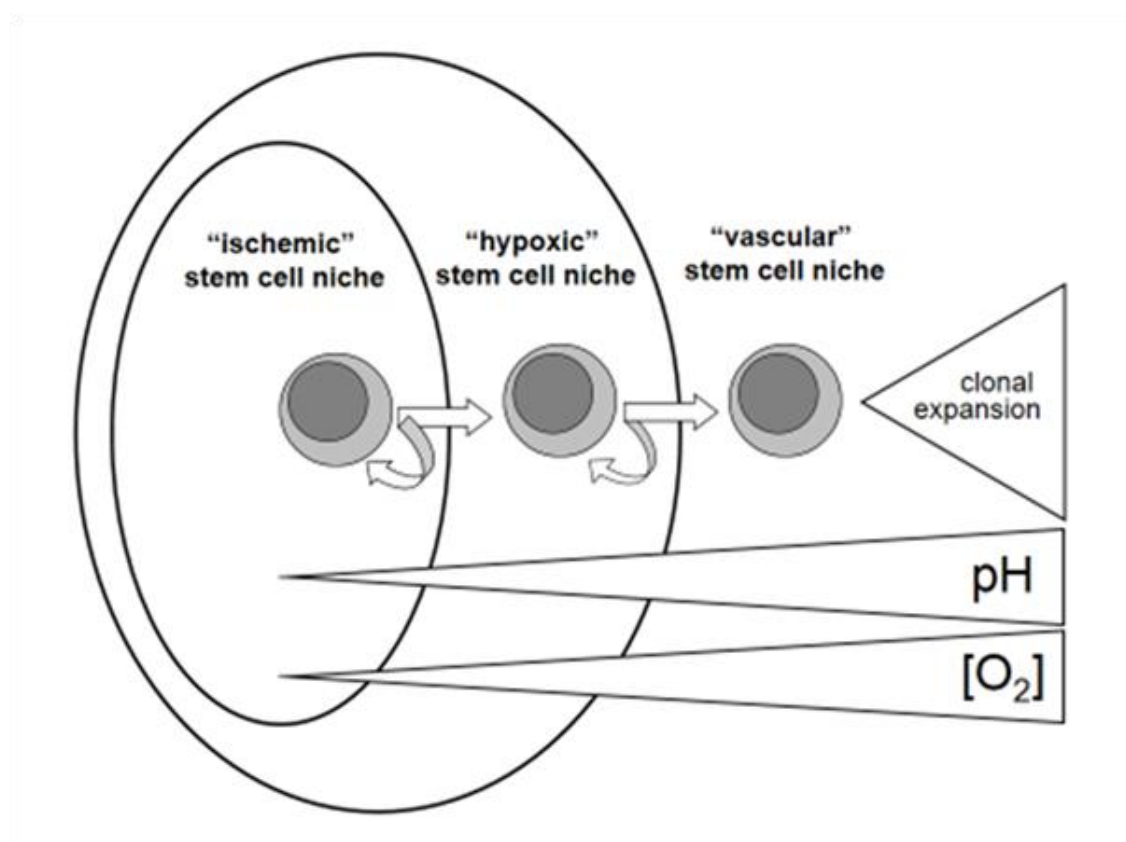


Figure 8. The ischemic core of the hypoxic haematopoietic stem cell niche.

Furthermore, the core of the hypoxic HSC niche can be defined as “ischemic”. Due to hypoxia, indeed, the glucose consumption rate and glycolysis are enhanced (the Pasteur Effect), generating areas where glucose is constantly at extremely low levels [45, 67] (Figure 8).

The hypoxic HSC niche is also an acid environment; low pH, due to the high lactate and H<sup>+</sup> concentration generated via the enhanced glycolysis, is, in fact, a common characteristic of hypoxic regions [68] (Figure 8).

The importance of the micro-environment, in particular oxygen tension, in maintaining HSC and regulating their behavior emerged first from a series of studies carried out by our group. Severe hypoxia (1% oxygen in the incubation atmosphere) enhances the maintenance of stem cell potential of murine and human haematopoietic populations. STR-HSC as well as LTR-HSC are completely hypoxia-resistant. Less immature progenitors such as Colony-Forming Cells (CFC) are, on the contrary, eliminated in the course of hypoxic incubation. Resistance to hypoxia was shown indeed to be the higher, the higher the level of progenitor in the haematopoietic hierarchy [37]. Interestingly, oxygen levels around 1% allow HSC to cycle, being thereby compatible with HSC self-renewal, also a finding of our group [69, 70]. Hypoxia modulates the effects of Reactive Oxygen Species (ROS) and cytokines such as Erythropoietin (EPo), Thrombopoietin (TPo), InterLeukin-3 (IL-3), Granulocytic Colony-Stimulating Factor (G-CSF) and InterLeukin-7 (IL-7). Under these conditions, it is possible to ensure self-renewal and thereby to maintain the stem cell compartment while the initial clonal expansion is triggered (asymmetric division). *In vivo*, this situation corresponds to the endosteal vascular niche where interaction with CAR cells and activation of pathways such as Wnt and Notch sustain the stemness and the self-renewal of HSC. In these areas, it is possible to maintain and expand stem cells, while the extensive generation of fast-growing progenitors necessary for clonal expansion is not allowed. When oxygen decreases to below 0.1%, HSC are maintained but become quiescent. Such a low oxygen tension is a typical feature of the endosteal osteoblastic niche. Here quiescence is maintained by the interaction of HSC with Extra Cellular Matrix (ECM) and by signaling mediated by N-Cadherin, Osteopontin, Transforming Growth Factor (TGF)  $\beta$  and Stem Cell Factor (SCF). Quiescent HSC are probably to be considered at the highest level of the stem cell hierarchy and have been identified as LTR-HSC. This is, as mentioned above, a

very small fraction of the CD34+/CD38- cell subset, the firm anchorage of which to the niche ensures resistance to a number of haematopoietic stresses [71].

The high percentage of quiescent HSC in the niche is due to the fact that hypoxia induces the expression of cyclin-dependent kinase inhibitor genes, p21<sup>cip1</sup>, p27<sup>Kip1</sup> and p57<sup>Kip2</sup>. It has been shown in our laboratory that in hypoxia (but not in normoxia) the potential of generating CFC *in vitro* (a feature of HSC) of CD34+ cells which have undergone one replication cycle is maintained or increased with respect to that of undivided cells or of cells which have undergone more than one replication cycle [70]. Interestingly, IL-3 increases the number of CD34+ cells that divide more than once in hypoxia and thereby decreases the stem cell potential. Thus, proliferation of HSC beyond the first cycle, under the effects of factors inducing lineage commitment, results in the loss of self-renewal ability. A possible conclusion is that the maintenance of stem cell potential requires cycling, but this cycling needs to be limited to one cycle after exit from quiescence, and that hypoxia enforces cycling limitation, resulting in the maintenance of HSC self-renewal. Other studies carried out in our laboratory showed that Vascular Endothelial Growth Factor (VEGF), a factor which is also directly active on HSC, is sufficient alone (*i.e.* in the absence of other cytokines) to maintain the stem cell potential of HSC in hypoxia and that this effect is also antagonized by IL-3. Thus, VEGF is capable to sustain survival of HSC in hypoxia directly, and then their oxygen-dependent clonal expansion indirectly, by stimulating neoangiogenesis.

A number of studies carried out in our laboratory showed that, not only HSC, but also LSC are able to adapt to severe hypoxia [22, 72-74], as they retain many characteristics of HSC. In leukaemia, hypoxia plays the same role as in normal haematopoiesis, *i.e.* the maintenance of SC features combined with the inhibition of clonal expansion.

Hypoxia-selectable LSC subsets are likely to exist within any type of leukaemia, including stabilized cells lines [22, 72-74]. In CML, either cell lines or CD34+ cells explanted from CML patients, hypoxia-resistant LSC are also resistant to IM. This resistance is due to the fact that, in a hypoxic environment, BCR/Abl, the molecular target of IM, is suppressed. However, hypoxia-selected LSC remain genetically leukemic (*BCR/abl*-positive), as they re-express BCR/Abl if cultures are shifted to growth-permissive conditions (incubation in air). This intrinsic resistance of LSC to TKi treatment is defined refractoriness [22, 72-74].

The suppression of BCR/Abl can be explained with the fact that oncogenic growth-promoting signals antagonize the maintenance of LSC self-renewal. This predisposes IM-resistant LSC to long-term homing in hypoxic stem cell niches, from where BCR/Abl-driven clonal expansion of LPC can be rescued following shift to relatively better-oxygenated areas of BM.

We addressed the effects of oxygen and glucose shortage on different subsets of LSC and LPC within CML cell populations [73]. It was possible to identify different hypoxia-resistant cell subsets, exhibiting different phenotypes and functional properties. Some of these subsets display features of LSC, others of LPC. These leukaemia cell subsets are functionally and metabolically characterized and reflect the organization of normal haematopoiesis, where the regulatory role of glycolysis and respiration within the stem/progenitor cell hierarchy is been characterized [36-38, 75, 76]. Three cell subsets were detected by modulating the duration of hypoxia and glucose availability. First: LPC metabolically similar to cell bulk, in which BCR/Abl suppression in hypoxia has not occurred yet and which are therefore IM-sensitive. Second: LPC/LSC where oxygen and glucose shortage has determined suppression of BCR/Abl. Third: LSC obtained after selection of CML cells following a long incubation in hypoxia and glucose shortage, where BCR/Abl is deeply suppressed. The latter subset is characterized by complete refractoriness to IM. It is worth noting that BCR/Abl suppression is not determined by hypoxia *per se*, but rather depends on glucose shortage in hypoxia. Glucose availability is therefore to be considered the regulator of BCR/Abl expression in hypoxia. These observations directly link LSC metabolism to resistance to therapy, suggesting that this resistance is linked to the depression of not only aerobic production of energy, but also glycolysis [73] and that the environment where LSC home *in vivo* is, like the normal HSC niche, characterized by the shortage of both oxygen and glucose. However, in these conditions, LSC are still capable to cycle, although the most recent data indicate that the longer is the incubation under oxygen/glucose shortage, the larger the proportion of quiescent LSC. On the other hand, BCR/Abl expression, which is necessary to drive the expansion of leukaemic population, occurs only under growth-permissive metabolic conditions [73]. The rescue of BCR/Abl expression likely occurs *in vivo* in relatively well oxygenated tissue areas, such as the vascular niches. Thus, the flexible and reversible stem/progenitor cell phenotype



shift described by the *Chiaroscuro* model [50] may be largely conditioned by the environmental “metabolic pressure”.

Hypoxia can also contribute to LSC and HSC maintenance through the regulation of the expression of signaling molecules, including HIF, as described in detail below.

## **HIF and their role in cancer**

As described in the previous section, hypoxia is an important hallmark of the tumor micro-environment and it is one of the most important factors favoring neoplastic progression. In normal as well as cancer cells, adaptation to hypoxia is driven by HIF, which regulate the expression of genes involved in many cellular processes, including pH regulation, glucose uptake, metabolism, angiogenesis, cell proliferation and apoptosis. Likewise, in cancer cells, HIF regulate a number of steps of tumorigenesis, including tumor formation and progression, as well as response to therapy.

HIF belong to the PAS (PER-ARNT-SIM) family of basic Helix-Loop-Helix (bHLH) transcription factors. Active HIF is a heterodimer, composed of an  $\alpha$  and a  $\beta$  subunit, which binds DNA in the specific sequence 5'-RCGTG-3' called Hypoxia Response Elements (HRE). There are three isoforms of the alpha subunit: HIF-1 $\alpha$ , HIF-2 $\alpha$  (also known as Endothelial PAS-domain protein 1, EPAS1), and HIF-3 $\alpha$ . The subunits HIF-2 $\alpha$  and HIF-3 $\alpha$ , in contrast to HIF-1 $\alpha$ , which is ubiquitously expressed, have an expression pattern restricted to particular tissues. Regarding the beta subunit, two isoforms have been identified: HIF-1 $\beta$  isoform, also known as ARNT (Aryl Hydrocarbon Receptor Nuclear Translocator) and HIF-2 $\beta$  (or ARNT2). HIF activity is regulated by oxygen tension through the stabilization of the alpha subunit. In the presence of oxygen (>7%), HIF- $\alpha$  is extremely unstable, due to its hydroxylation, in the Oxygen Dependent Degradation (ODD) domain of the protein, by specific prolyl-hydroxylases. Hydroxylation causes interaction with the von Hippel-Lindau (vHL) protein, a component of an E3 ubiquitin ligase complex, and the consequent HIF ubiquitination and degradation within the 26S proteasome. When oxygen levels are lower than 7%, HIF- $\alpha$  is stabilized and rapidly translocated to the nucleus, where it binds the HIF- $\beta$  subunit, which is

constitutively expressed (Figure 9). In addition, the HIF-3 $\alpha$  subunit seems to have a regulatory function in both HIF-1 $\alpha$  and HIF-2 $\beta$  expression.

As described previously, hypoxic and/or ischemic areas are common within tumor masses, where HIF is therefore frequently activated. Hypoxia is indeed the best-characterized mechanism of HIF activation in tumors and normal tissues. In keeping with this, many studies found that the expression of both HIF-1 $\alpha$  and HIF-2 $\alpha$  is increased in a variety of human tumors, including bladder, breast, colon, glial, hepatocellular, ovarian, pancreatic, prostate and renal tumors. Moreover, an increase of HIF $\alpha$  expression correlates with poor prognosis [77]. However, there are some significant differences between the functions played by HIF-1 $\alpha$  and HIF-2 $\alpha$ . During adaptation to hypoxia, HIF expression depends on both the oxygen level and the kinetics of its disappearance. In neuroblastoma cells, HIF-1 $\alpha$  is activated during the early phase of hypoxia (within the first hour), and remains well expressed for 48 hours. Later, HIF-1 $\alpha$  decreases while HIF-2 $\alpha$  increases. Thus, HIF-1 $\alpha$  stabilization is primarily an acute response to hypoxia, while the role of HIF-2 $\alpha$  is predominant in prolonged hypoxia [78].

Another important finding is that HIF $\alpha$  subunits are often expressed also in oxygenated areas of tumors. This is mainly due to genetic alterations of the oxygen-sensing pathway which cause HIF $\alpha$  stabilization irrespective of oxygen concentration in the environment [79, 80]. Inactivation of vHL in renal carcinomas and mutations of the Wnt/ $\beta$ -catenin signaling pathway in colon carcinoma are the most common alterations determining HIF-1 $\alpha$  stabilization. Another important hypoxia-independent mechanism of HIF $\alpha$  activation in tumor cells is mediated by the PI3-Kinase (PI3K)/Akt-signaling pathway. A number of mechanisms, including growth factor signaling, loss-of-function PTEN mutations, gain-of-function mutations of HER2<sup>neu</sup> or insulin-like growth factor-1 receptors, Ras activation, result in increased signaling via the PI3K/AKT pathway, leading to increased mTOR activity and HIF-1 $\alpha$  expression [81]. In addition, p42/p44 mitogen-activated protein kinases, which regulate cell proliferation in response to extracellular growth factors, also phosphorylate HIF-1 $\alpha$  (and HIF-2 $\alpha$ ) and activate transcription of HIF target genes [82, 83]. Collectively, these results suggest that HIF play a central role in tumorigenesis. As described previously, the importance of HIF is due to its capacity to activate many important genes involved in pH regulation, like Carbonic Anhydrase (CA) IX, in angiogenesis, like VEGF, in cell metabolism, like GLUcose

Transporter (GLUT) and Lactate De-Hydrogenase (LDH)-A, in cell proliferation, like TGF and EPo, and in the metastatic process, like CXCR4, E-Cadherin and Lysyl Oxidase (LOX).

Hypoxia induces first the so-called “glycolytic switch”. Otto Warburg discovered in the 1920s that tumors, unlike normal cells, convert glucose to lactate (via pyruvate) even in the presence of abundant oxygen, a process termed “Warburg effect”. Tumor cells, in order to make enough ATP, increase their rate of glycolysis using the HIF-1 $\alpha$  pathway to induce the expression of GLUT1, GLUT3 (enhancement of glucose up-take), LDH-A (restoration of NAD<sup>+</sup> via the increased conversion of pyruvate to lactate) and Pyruvate Dehydrogenase Kinase (PDK) 1. Interestingly, pyruvate itself inhibits HIF-1 $\alpha$  degradation. Recent work showed that HIF-1 $\alpha$  also negatively regulates mitochondrial respiration via the transcriptional activation of the c-Myc repressor MXI-1 and the regulation of c-Myc protein stability [84]. Finally, HIF-1 $\alpha$  blocks the oxidative arm and promotes the non-oxidative arm of the pentose-phosphate pathway, which converts glycolytic intermediates into ribose-5-phosphate, a substrate for nucleotide biosynthesis [85]. HIF-2 $\alpha$ , on the other hand, promotes fatty acid storage via inhibition of the genes of  $\beta$ -oxidation, thereby blocking another important pathway of oxidative metabolism. In addition, HIF-2 $\alpha$  specifically inhibits ROS production via the induction of anti-oxidant enzymes such as SuperOxide Dismutase-2 and Heme-Oxygenase 1.

The pathways described above are extremely important in the regulation of hypoxia-induced cell proliferation. Hypoxia induces indeed the expression of many growth factors capable to increase the proliferation rate of cancer cells, such as TGF- $\beta$ , TGF- $\alpha$ , Platelet-Derived Growth Factor (PDGF) and EPo. HIF-1 $\alpha$  primarily induces TGF- $\beta$  and PDGF, while HIF-2 $\alpha$  induces primarily TGF- $\alpha$ . Both TGF are involved in cancer angiogenesis and proliferation. EPo is also a preferential HIF-2 $\alpha$  target [86, 87]. However, HIF-2 $\alpha$  controls cell proliferation mainly through modulation of c-Myc activity. In this fashion, HIF-2 $\alpha$  regulates the expression of genes involved in cell cycle control including cyclin D2 (and D1) and cyclin kinase inhibitors p21<sup>cip1</sup> and p27<sup>kip1</sup> [88].

A later effect of hypoxia is the “angiogenic switch”, a multi-step process driven by a number of pro-angiogenic factors, of which VEGF-A is the most important, expressed in a large number of human tumors. VEGF-A is induced by

either HIF-1 $\alpha$  or HIF-2 $\alpha$  and binds its receptors VEGF-R1 (also called Flt-1, which is induced in turn by HIF-1 $\alpha$ ) and VEGF-R2 (also called Flk-1, induced by HIF-2 $\alpha$ ). HIF-1 $\alpha$  also promotes the stabilization of the new blood vessels, inducing the expression of angiopoietin-1 and -2 and their receptor Tie-1. Tie-2 expression seems instead primarily HIF-2 $\alpha$ -dependent. Other important factors for tumor angiogenesis are PDGF-B and the Matrix Metalloproteinases (MMP)-2 and -9, both induced by HIF-1 $\alpha$ , and the membrane type-1-MMP, mainly regulated by HIF-2 $\alpha$ . Although cancer angiogenesis is defective, it is in most cases able to support the rapid growth of tumor mass. It is evident that a crucial contribution to this outcome depends on the decreased oxygen requirement of cancer cells due to the resetting of their metabolism also induced by HIF.

The last step in the natural history of malignant neoplasias is the formation of metastasis. HIF are involved also in this mechanism, via the induction of the axis CXCL12/SDF1-CXCR4. CXCL-12, also known as Stromal cell-Derived Factor (SDF) 1, is a member of a large family of structurally-related chemo-attractive cytokines, important for recruitment and homing of several types of cells including stem cells. CXCL12/SDF1, through the interaction with its receptor CXCR4, plays an important role in the directional migration of metastatic tumor cells. CXCR4 is the most common chemokine receptor expressed in tumors and CXCL12/SDF1 is highly expressed at the sites of metastasis, including lung, BM, and liver [89]. It has been shown that HIF-1 $\alpha$  is a potent inducer of the expression of both CXCR4 and CXCL12/SDF1 in a variety of cell types, such as BM and endothelial cells, as well as non-small cell lung cancer and glioblastoma cells.

Homing of cancer cells is favored by the Epithelial-to-Mesenchymal Transition (EMT). EMT is a key feature of invasive cells and it is characterized by the loss of epithelial cell-to-cell contacts and the acquisition of mesenchymal features and motility. HIF-1 $\alpha$  influences the expression of many EMT regulators such as E-Cadherin (important for cell-to-cell contact) and LOX (an extracellular matrix remodeling enzyme). HIF-1 $\alpha$  down-regulates E-Cadherin and enhances LOX, thereby favoring cell mobilization.

## HIF in Cancer Stem Cells

Hypoxia has been shown to regulate pluripotency as well as proliferation of human Embryonic Stem (hES) cells. HIF-2 $\alpha$  maintains pluripotency and proliferation of hES cells by regulating the expression of POU5F1, SOX2 and NANOG, which are transcription factors essential to maintain the pluripotent hES cell phenotype. In these cells, interestingly, HIF-3 $\alpha$  seems to play a central role in hypoxia as well as normoxia by regulating the expression of both HIF-1 $\alpha$  and HIF-2 $\alpha$  [90]. Recently, it has been found that either HIF-1 $\alpha$  or HIF-1 $\beta$  induce Wnt/ $\beta$ -catenin in both hES cells and Neural Stem Cells (NSC), drawing key parallels between embryonic and adult stem cells. In ES cells, HIF-1 $\alpha$  directly binds the promoters of Lef1 and Tcf1 and mediates Wnt activation [91]. Several reports showed a strong correlation between hypoxia, HIF- $\alpha$  and stem cell genes, indicating in particular that HIF-1 $\alpha$  induces the expression of the Notch transcriptional targets HES1 and HEY2, while HIF-2 $\alpha$  promotes that of Oct4 [92]. Finally, other studies showed that incubation in hypoxia significantly improves the generation of colonies by induced Pluripotent Stem (iPS) cells [93].

Evidences for a strong correlation between HIF and a more immature phenotype are found also in many types of cancer. For example, HIF-1 $\alpha$  expands a sub-population of glioma cells positive for CSC marker, such as CD133 and CD44 [94], while the expression of HIF-2 $\alpha$  is characteristic of neuroblastoma and breast cancer cells with a more immature phenotype that correlates to poor prognosis [95]. In glioblastoma cells, different (protein and mRNA) expression levels of HIF-1 $\alpha$  and HIF-2 $\alpha$  have been shown between CSC and the cancer cell bulk, being HIF-2 $\alpha$  expressed in stem cells and HIF-1 $\alpha$  in both stem and non-stem cells. However, HIF-1 $\alpha$  is stabilized only under the more severe hypoxic conditions (oxygen <1%) and CSC with low levels of HIF-1 $\alpha$  are unable to form tumors and exhibit reduced survival *in vitro* [96].

On the basis of all above, we can reach three conclusions: A) HIF-1 $\alpha$  is especially important during response to acute hypoxia (within 48 hours), whereas HIF-2 $\alpha$  is more important for long permanence in hypoxia, where it drives the selection of an immature/stem phenotype; B) the latter property supports the concept of “stem cell niche” as a tissue site where one of the most characteristic micro-environmental features is hypoxia; C) the CSC phenotype may be flexibly

induced in cancer cells depending on internal and external signals such as hypoxia, so that the fraction of a cancer cell population which can be considered to represent CSC is phenotypically variable.

## **HIF in Chronic Myeloid Leukaemia**

Many studies demonstrated that HIF has important roles in the leukaemia. HIF is overexpressed in many types of blood cell cancer, such as ALL and CML, often even under normoxic conditions [97].

Recent studies demonstrated an up-regulation of CXCR4 and CXCL12/SDF1 in AML as well as niche cells, suggesting that HIF-1 $\alpha$  is important to recruit LSC in the hypoxic niche and thereby to the maintenance of MRD and progression of the disease [98, 99]. HIF-1 $\alpha$ -mediated VEGF expression is also involved in blood cell cancer, as it stimulates proliferation and the expression of anti-apoptotic proteins such BCL2.

With respect to CML, Mayerhofer *et al.*, demonstrated that BCR/ABL promote the expression of HIF-1 through a pathway involving PI3-kinase and mTOR [100].

Using a CML mouse model, Zhang and colleagues demonstrated that HIF-1 $\alpha$  plays an important role in the survival maintenance of CML LSC. HIF-1 $\alpha$  deletion impaired indeed the propagation of CML by restraining cell-cycle progression and inducing apoptosis of LCS. Moreover, they demonstrated that LSC, compared with normal HSC, are more addicted to HIF-1 pathway [101].

## **HIF-1 pharmacological inhibitors**

Several pharmacological inhibitors of HIF-1 have been identified. In this study, our attention was focused on three different inhibitors (Figure 9) with different mechanism of action:

KC7F2

Compound X

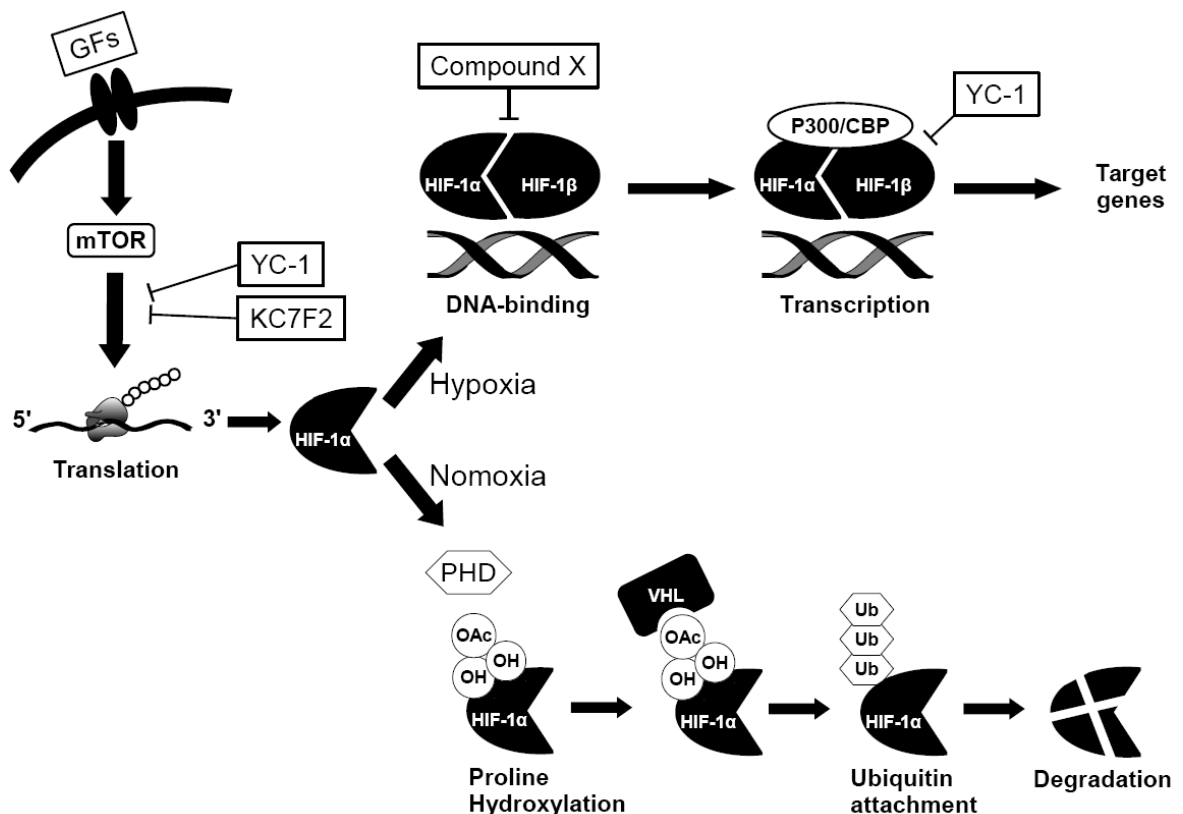
YC-1

KC7F2 is a nonspecific HIF-1 $\alpha$  inhibitor, which down-regulates HIF-1 $\alpha$  protein synthesis via the PI3K-Akt-mTOR pathway, in particular via the inhibition of the protein translation mediated by the complex mTORC1. KC7F2, in fact, inhibits the phosphorylation of 4EBP1. This results in the inactivation of the eukaryotic

translation initiation factor 4E (eIF-4E) and in the consequent inhibition of protein synthesis [102]. KC7F2 treatment *in vitro* of different neoplastic cell lines, e.g. glioma, breast and prostate cancer, inhibits HIF-mediated transcription and induces growth arrest. The cytotoxic effect of the treatment is enhanced under hypoxic incubation [102].

Compound X is another inhibitor of HIF-1 activity. Compound X is already FDA approved for a non-oncological use in humans.

YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole] inhibits HIF-1 $\alpha$  activity by both promoting its degradation [103, 104] and inhibiting its synthesis via the mTOR pathway [104]. More recently, Shan Hua Li and colleagues identified another and more specific mechanism by which YC-1 inhibits HIF-1 activity: it disrupts the binding between HIF-1 $\alpha$  and p300, a co-activator indispensable for the transcription initiation of the HIF-1 $\alpha$ -downstream genes [105]. However, the authors underlined that HIF-1 $\alpha$  inhibition resulted different in different cell lines, being some cells more sensitive than others.



**Figure 9. HIF regulation and proposed mechanism of action of HIF-1 $\alpha$  pharmacological inhibitors.**

# Materials and Methods

## Cells and culture conditions

K562, KCL22 and primary CML cells were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin and 50 mg/ml streptomycin (all from Euro-Clone, Paington, UK, E.U.) and incubated at 37°C in a water-saturated atmosphere (21% O<sub>2</sub> and 5% CO<sub>2</sub>).

K562 [106] and KCL22 [107] are immortalized BCR/Abl positive cell lines derived from CML patients in blast crisis.

K562 cells transfected with shRNA against HIF-1 $\alpha$  (shHIF-1 $\alpha$ ), HIF-2 $\alpha$  (shHIF-2) or with control shRNA (shRFP), all carrying Red Fluorescent Protein were transduced at low multiplicity infection, *i.e.* about 1 copy of the pro-virus (containing two copies of the shRNA) per cell and sorted by FACS on the basis of RFP expression. All shHIF-1 $\alpha$ , shHIF-2 $\alpha$  and shRFP-K562 cells were kindly provided by Dr. F. Mazurier, Université Bordeaux 2, Bordeaux.

Primary CML cells were 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 3x10<sup>5</sup>/ml and incubated in hypoxia (containing 0.1% O<sub>2</sub>, 94.9% N<sub>2</sub> and 5% CO<sub>2</sub>) in a Whitley DG250 Anaerobic Workstation (Don Whitley Scientific, Shipley, Bridgend, UK, E.U.) or normoxia (21% O<sub>2</sub> and 5% CO<sub>2</sub>), at 37°C in a water-saturated atmosphere.

Human Embryonic Kidney (HEK) 293T [108] and Mouse embryonic fibroblast NIH/3T3 [109] cell lines were routinely cultured in DMEM medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin and 50 mg/ml streptomycin (all from Euro-Clone, Paington, UK, E.U.) and incubated at 37°C in a water-saturated atmosphere (21% O<sub>2</sub> and 5% CO<sub>2</sub>).



## Reagents

YC-1 (Sigma-Aldrich, No.CAS 170632-47-0) powder was dissolved in dimethylsulfoxide (DMSO) and added to cultures at 25  $\mu$ M at the indicated times.

KC7F2 (Calbiochem, #400088) powder was dissolved in DMSO and added to cultures at 40  $\mu$ M at the indicated times.

IM (Santa Cruz, #202180) powder was dissolved in PBS and added to cultures at 1  $\mu$ M at the indicated times.

Compound X (artificial designation for a biochemical compound that we want to keep hidden since the patent is in processing) powder was dissolved in PBS and added to cultures at 5  $\mu$ M at the indicated times (cell lines and primary cells). Compound X was added to cultures at 1  $\mu$ M when was administered in combination with IM. In the colony assay, Compound X was added to cultures at the indicated concentrations. In the *in vivo* experiments, the mice were treated daily with Compound X (2 mg/kg) via intraperitoneal injection for 2 weeks.

5-FU (Sigma, Cat # 6627) powder was dissolved in PBS and injected into donor mice via tail vein (200 mg/kg).

For *in vivo* experiments all the drugs were freshly prepared.

## Isolation of mononuclear cells from human BM aspirates by density gradient

Primary CML cells were collected at the Division of Hematology of Azienda Ospedaliera Universitaria Careggi (AOUC). Informed consent for *in vitro* research and approval of the Ethics Committee of AOUC had been previously obtained. Human bone marrow aspirates, diluted 1:1 with Phosphate-Buffered Saline (PBS), were carefully layered on top of Ficoll (1.5 ml of Ficoll/2 ml BM aspirate diluted in PBS; Cedarlane Laboratories, Ontario, Canada) and centrifuged at 2500 rpm for 20 minutes at room temperature. The mononuclear ring at the plasma/ficoll interface was harvested and washed twice with PBS. Cell pellet, suspended in RPMI, was seeded in a 100-mm tissue culture dish and incubated for 3 hours at 37°C to allow adherent cells to attach to the plate. Cells in suspension were then collected and placed in RPMI with the addition of cytokines for subsequent experiment (see above).

## **Measurement of cell viability.**

Cell viability was measured by the trypan blue (Sigma-Aldrich, #F-7378) (0.2 gm, 99.8ml Distilled Water, 0.2% Sodium Azide) exclusion test, that consist on counting trypan-negative cells in a haemocytometer.

## **Protein separation and detection**

Cells were washed twice with ice-cold PBS containing 100  $\mu$ M orthovanadate and solubilized by incubation for 10 minutes at 95°C in Laemmli buffer. Lysates were then clarified by centrifugation (20000 g, 10 minutes, room temperature) and protein concentration in supernatants was determined by the BCA method. Aliquots containing the same amount of proteins (30-50  $\mu$ g 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 room temperature in Odyssey Blocking Buffer (LI-COR® Biosciences, Lincoln, NE, U.S.A.) diluted 1 : 1 with PBS. Membranes were then incubated over night at 4°C in Odyssey Blocking Buffer diluted 1:1 with PBS containing 0.1% Tween-20 and the primary antibody. Primary antibodies used were: rabbit polyclonal anti-phospho (Tyr245) c-Abl (#2862), rabbit monoclonal anti-CAIX (D47G3) (#5649), rabbit polyclonal anti-cleaved PARP (#9541), rabbit polyclonal anti-cleaved caspase 3 (#9661), rabbit polyclonal anti-phospho-histone H<sub>2</sub>AX (#9718), all from Cell Signaling Technology (Danvers, MA, U.S.A.); rabbit polyclonal anti-histone H4 (#07-108) from Millipore (Darmstadt, Germany); mouse monoclonal anti-vinculin (V9131), from Sigma-Aldrich (St. Louis, MO, U.S.A.); mouse monoclonal anti-HIF-1 $\alpha$  (sc-53546), goat polyclonal anti-EPAS-1 (sc-8712), mouse monoclonal anti-caspase 3 (sc-7272); goat polyclonal anti-GAPDH (sc-20357), rabbit polyclonal anti-c-Abl (sc-131), rabbit polyclonal anti-ERK1 (sc-93), from Santa Cruz Biotechnology (S. Cruz, CA, U.S.A.), rabbit polyclonal anti-VEGF (bs-0279R) from Bioss Antibodies (Boston, MA, U.S.A.) Anti-phospho c-Abl, anti-c-Abl, anti-EPAS-1, anti-VEGF antibodies were diluted 1:500 in PBS containing 0.1% Tween-20. Other antibodies were diluted 1:1000 in the same buffer mentioned before.

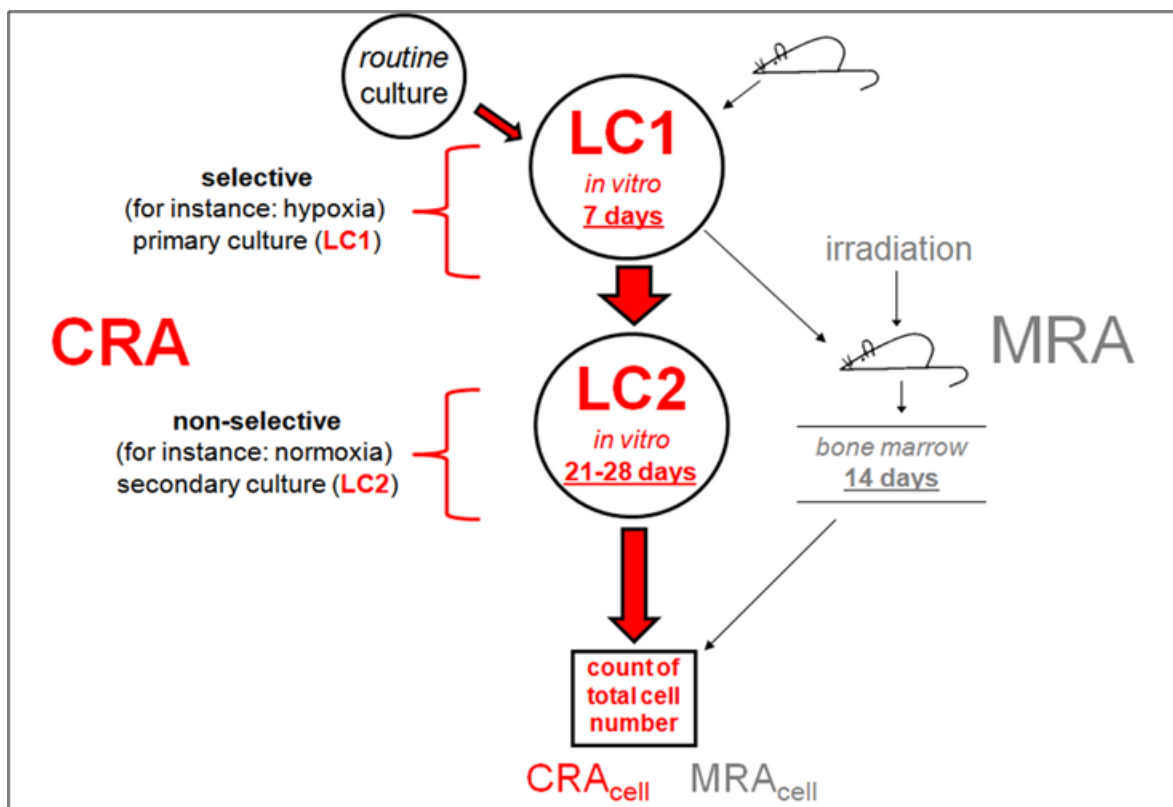
After extensive washing with 0.1% Tween-20 in PBS, membranes were incubated for 1 hour at room temperature in Odyssey Blocking Buffer diluted 1:1 with PBS containing an IRDye800CW- or IRDye680-conjugated secondary antibody (LI-COR®). Mouse and rabbit IRDye800CW- conjugated secondary antibodies were diluted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

Densitometric analysis was performed using the ImageJ program.

## Culture-Repopulation Ability assay

This assay estimates the culture repopulating power of normal [110, 111] or leukaemic [22, 72, 73] haematopoietic cells undergone a selection treatment (e.g. hypoxia, drug treatment) in a primary liquid culture (LC1) by means of their



**Figure 10. The Culture-Repopulation Ability (CRA) and the Marrow-Repopulation Ability (MRA) assays.**

transfer in fresh medium to non-selective conditions (e.g. normoxia, without

treatment) in a growth-permissive secondary culture (LC2) and the measure of its repopulation following a further incubation therein. Cell subsets rescued from LC1 at different times repopulate LC2 with different kinetics, the time necessary to reach the peak of LC2 repopulation reflecting the hierarchical level of stem/progenitor cells enriched in LC1. The CRA assay is a non-clonogenic assay capable to reveal *in vitro* stem cells endowed with marrow-repopulating ability *in vivo* [110]. The adaptation of CRA assay to leukaemia cell populations enabled to detect different subsets of leukaemia stem or progenitor cells [22, 72-74]. The standard version of CRA assay used for leukaemia studies is shown in Figure 10.

## **Mice**

C57BL/6J-CD45.1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME, U.S.A.). All mice were bred and maintained in a temperature- and humidity-controlled environment and given unrestricted access to 6% chow diet and acidified water.

## ***In vitro* methylcellulose colony formation assay**

Murine BM cells were obtained by flushing femurs and tibiae. The recovered cells were washed once in PBS and incubated 10 minutes at 4°C with red blood cell lysis buffer (150 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub> and 0.1 mM EDTA, pH 7.4) to remove red blood cells. Cells were washed with PBS to eliminate residual buffer. The cell pellet was suspended in RPMI and cells were counted by trypan blue exclusion assay.

Human mononuclear cells were harvested by centrifugation in a Ficoll-Hypaque gradient from BM aspirates (see above).

$3 \times 10^4$  (murine or human) cells were suspended in 100  $\mu$ l of IMDM with 2% FBS, 0.1% ciprofloxacin and 100U/ml Penicillin/Streptomycin. Murine or human cells were then added to 1ml of Methylcellulose medium H4435 (Stem Cell Technology, #04435) or M3234 (Stem Cell Technology, #03234) respectively. Compound X or IM were added to the mixture and the cells were plated onto 35-mm tissue culture dishes and incubated at 37°C in humidified air. Each treatment condition was performed in duplicate. Colonies were counted under microscope at day 10, 17 or 21 of incubation.

## **Transformation of competent cells**

50µl of Subcloning Efficiency DH5α Competent Cells (Life Technologies, # 18265-017) were thawed on ice. 1-10 ng of DNA (MSCV-IRES-GFP (MIG), MSCV-BCR/ABL-IRES-GFP (MIG-BCR/ABL) or MCV-Ecopac [112] plasmids) were added to the 1.5 ml microcentrifuge tube containing DH5α cells. Tube was incubated for 30 minutes on ice, then 20 seconds in a 42°C water bath and on ice for 2 additional minutes. 950 µl of autoclaved Luria Broth (LB) (10 g Tryptone, 5 g Yeast Extract, 10 g NaCl, 1 L H<sub>2</sub>O) were added to the tube containing DNA and DH5α cells and incubated for 1 hour at 37 °C at 225 rpm. 200 µl of the solution were spread on pre-warmed LB Agar plates (10 g Tryptone, 5 g Yeast Extract, 10 g NaCl, 15g Agar, 1 L H<sub>2</sub>O) containing Ampicillin (75 µg/ml). The plates were incubated overnight at 37°C.

Single colonies were then picked, transferred to flasks containing 500 ml LB, and incubated overnight at 37°C.

## **Plasmid extraction**

500 ml culture of DH5α cells in LB (see above) were centrifuged (6000g, 4°C, 10 minutes in Sorvall GSA/GS-3 and Beckman JA-10 rotors) and the pellet resuspended in 4 ml glucose/Tris/EDTA solution (20 ml 0.5M EDTA, 25 ml 1M Tris HCl pH 8, 9 g D-Glucose) with the addition of 25 mg/ml hen egg white lysozyme (American Bioanalytical, #01148-00005) in glucose/Tris/EDTA solution and incubated at room temperature for 10 minutes. The pellet was then incubated on ice for 10 minutes with 10 ml of freshly prepared NaOH/SDS solution (10% SDS, 0.5ml 10N NaOH, 22ml H<sub>2</sub>O). 7.5 ml of potassium acetate solution (240 ml 5M KAc, 46 ml Acetic Acid, 114 ml H<sub>2</sub>O) were added and the pellet was incubated for 10 additional minutes on ice. After centrifugation (10 minutes at 4°C, 20000 g) the supernatant was decanted into a clean tube and incubated 5-10 minutes at room temperature with 6% isopropanol. The solution was centrifuged at room temperature for 10 minutes at 15000 g and the pellet was washed with 70% Ethanol and dried under vacuum. The pellet was then suspended with 2 ml of TE solution (10 mM Tris HCl pH8, 1 mM EDTA pH 8). When the pellet was solubilized 2.2 g of CsCl and 200 µl Ethidium Bromide (EB) were added and the tube was

centrifuged at room temperature for 5 minutes at 4000 rpm. The supernatant was transferred into a new ultracentrifuge tube using an 18 ½ G needle and centrifuged overnight (90000 rpm, 20°C). The middle dark-pink layer containing the plasmidic DNA was collected, transferred into a new ultracentrifuge tube and centrifuged for 7 hours (90000 rpm, 20°C). The middle dark-pink layer was transferred into a clean 15ml tube and EB trough several washing with Isobutanol (until the solution turned clear). The clear solution was then transferred into a dialysis membrane and incubated for 16-18 hours at 4°C in 1 L of TE 1x. The solution was replaced with fresh TE and the membrane was incubated in the same condition for 3 additional hours. The membrane content was collected into a 15ml tube and incubated for 1 hour at 4°C with 100% Ethanol (2 times of the volume transferred into the tube) and 3 M NaOAc (10% of the final volume). The tube was then centrifuged (3000 g, 4°C, 10 minutes). The pellet was washed with 70% ethanol, dried and resuspended in 500 µl-1 ml of sterile H<sub>2</sub>O. The DNA content was measured with a NanoDrop spectrophotometer (Thermo scientific).

## **Generation of retroviral stocks**

The retroviral constructs MIG, MIG-BCR/ABL were used to generate high-titer, helper free, replication-defective ecotropic viral stock by transient transfection of 293T cells. The day before transfection, HEK293T cells were plated in 60-mm tissue culture dish (4x10<sup>6</sup> cells/dish) in RPMI. On the day of transfection, the cell medium was removed and 4ml of fresh RPMI were added to the dish. 10 µg of MIG or MIG-BCR/ABL plasmid and 5 µg of MCV-Ecopac plasmid [112] were added to a 15 ml tube with 62 µl CaCl<sub>2</sub> and sterile water (to 500 µl total volume). 2X HBS (500 µl) were added to the tube. The solution was briefly vortexed and gently dropped to the 293T cells. Cells were incubated at 37°C for 24 hours. The medium was then removed and 4 ml of fresh RPMI were added to the dish. 48 hours post-transfection of HEK293T the supernatant was collected, filtered through 0.45µm syringe and aliquoted in 15 ml tubes (4 ml of virus supernatant/tube). The tubes were stored at -80°C.

To test the viral titer the frozen supernatants were thawed and diluted 1:2 and 1:8 in RPMI medium. Polybrene (80 µg/mL) was added into the retroviral supernatant. The supernatant was then added to NIH/3T3 cells (plated the day

before in 100-mm tissue culture dishes,  $0.6 \times 10^5$  cells/dish). Cells were incubated at 37°C for 3 hours. After the incubation, the virus supernatant was removed and 10 mL of DMEM were added to the plate containing NIH3T3 cells. The medium was collected after additional 48 hours of incubation at 37°C. The percentage of GFP expressing cells was evaluated using flow cytometry analysis. Good retroviral supernatant means the GFP% can reach 90-95% at the 1:2 dilution and 75-85% at 1:8 dilution.

## **BM cells transduction and transplantation**

5-FU powder was freshly suspended in PBS and incubated 10-30 minutes at 37°C in a water bath. Donor mice were injected with 5-FU via tail vein (200mg/kg) and after 4 days they were sacrificed with CO<sub>2</sub> and femurs and tibiae were collected. BM cells were flushed out with RPMI, usually  $2-3 \times 10^7$  total BM cells were harvested from 10 mice. Cells were centrifuged (1500 rpm, 10 minutes) and the pellet was resuspended in 10 ml of the first stimulation medium: 77% DMEM, 15% FBS, WEHI-3B conditioned medium, penicillin-streptomycin, 1mg/ml ciprofloxacin, 200nM L-glutamine, 6 ng/ml recombinant murine IL-3 (Peprotech, # 213-13), 10 ng/ml recombinant murine IL-6 (Peprotech, # 250-16) and 50-100 ng/ml recombinant murine SCF (Peprotech, # 250-03). Cells were incubated at 37 °C for 24 hours. Cells were then collected, centrifuged (10 minutes, 1500 rpm, room temperature) and resuspended in 4 ml of transduction medium: 50% retroviral supernatant, 27% DMEM, 15% FBS, 5% WEHI-3B conditioned medium, penicillin-streptomycin, 1 mg/ml ciprofloxacin, 200 nM L-glutamine, 6 ng/ml recombinant murine IL-3 (Peprotech, # 213-13), 10 ng/ml recombinant murine IL-6 (Peprotech, # 216-16), 50-100 ng/ml recombinant murine SCF (Peprotech, # 250-03), 1% HEPES and 20 µg/mL polybrene. BM cells were transferred into a 6-well plate and centrifuged at 2300 rpm at room temperature for 90 minutes. Cells were then resuspended and incubated at 37°C for 3 hours. At the end of the incubation, the supernatant was removed and BM cells were incubated overnight at 37 °C with 4ml of stimulation medium (see above) per well. At the end of the incubation, 2 ml of stimulation medium were removed and 2 ml of transduction medium (2 ml of retroviral supernatant, 20 µg/ml polybrene, 1% HEPES) were added to each well. Cells were centrifuged (2300 rpm, for 90 minutes) and incubated at 37°C for 3

hours. At the end of the incubation BM cells were collected, centrifuged (1500 rpm, 10 minutes) and resuspended in PBS. The amount of PBS was adjusted to inject 300  $\mu$ l/recipient mouse ( $0.5 \times 10^6$  BM cells).

Recipient mice had been previously treated by two doses of 550-cGy gamma, separated by two hours.

### **Flow cytometry analysis of murine BM LSK cells.**

Mice BM cells harvested from femurs and tibiae were incubated 10 minutes at 4°C with red blood cell lysis buffer (see above) to eliminate red blood cells. Cells were washed once, resuspended in PBS and incubated for 30 minutes at 4°C with a biotin-labeled lineage antibody cocktail (Miltenyl Biotec, #120-003-582) containing a mixture of antibodies labeling lineage-committed cells. Cells were then washed with PBS and incubated for 15 minutes at 4°C with the fluorochrome-labeled secondary antibody (allophycocyanin-Cy7-conjugated streptavidin) for recognizing biotin, PE-conjugated anti-c-kit (eBioscience, # 12-1171-82) and allophycocyanin-conjugated anti-Sca-1 (eBioscience, # 17-5981-82). Cells were then washed and resuspended in PBS. 7AAD was added to the cells before flow cytometric analysis. Flow cytometry analysis was performed on lineage-negative/Sca-1-positive/c-kit-positive (LSK) cells. Results were analyzed using the FlowJo program.

### **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.

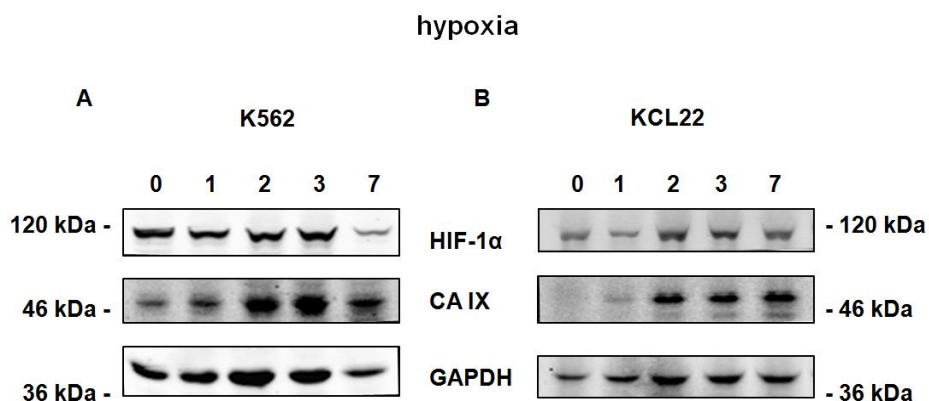


# Results and Discussion

## Genetic inhibition of HIF1- $\alpha$ or HIF2- $\alpha$ impairs the Culture-Repopulation Ability of hypoxia-selected BCR/Abl-negative Leukaemia Stem Cells.

The aim of this study was to address the effects of the pharmacological inhibition of HIF-1 $\alpha$  on the maintenance of CML stem cells potential. It has already been demonstrated, in fact, that the genetic suppression of HIF-1 $\alpha$  antagonized the propagation of CML by impairing cell-cycle progression and inducing apoptosis of LSC [101].

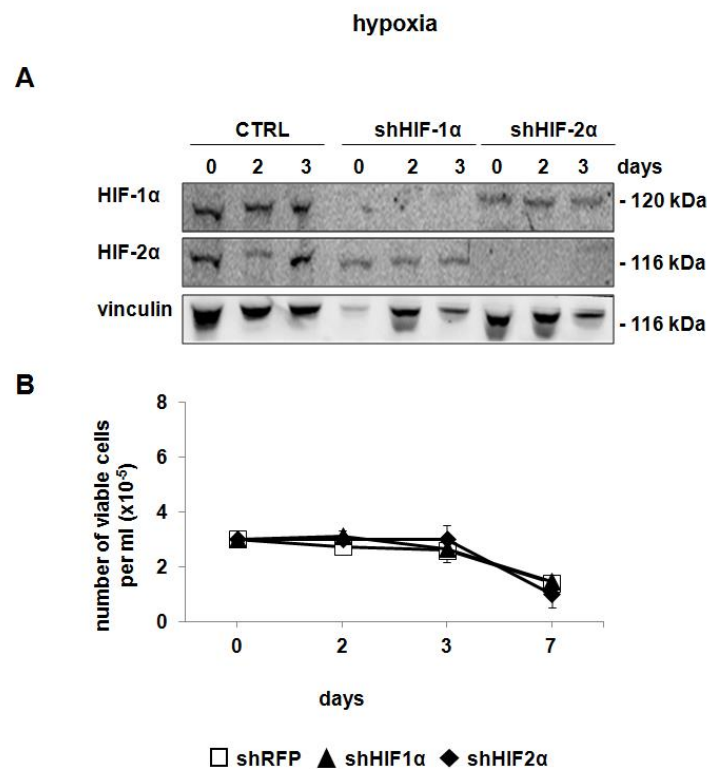
First we wanted to evaluate the effect of CML cell lines hypoxic incubation on both the expression and the activity levels of HIF-1 $\alpha$ . As shown in Figure 11, in K562 and KCL22 cells HIF-1 $\alpha$  is expressed also in normoxia (time 0 of incubation). HIF-1 $\alpha$  expression in normoxia has already been observed in other blood cancers [97]. Nevertheless, HIF- $\alpha$  transcriptional activity increased in hypoxia, as indicated by



**Figure 11. Hypoxia enhances CA-IX expression in CML cell lines.** K562 (A) and KCL22 (B) cells were incubated in deep hypoxia ( $\sim 0.1\% \text{ O}_2$ ) for the indicated times. Total cell lysates in Laemmli buffer were subjected to immuno-blotting with the indicated antibodies. Anti-GAPDH antibody was used to verify equalization of protein loading. One representative experiment out of 3 is shown.

the expression of CA-IX, one of the HIF-1 $\alpha$  target genes, in both K562 and KCL22 cells. Different mechanisms may explain this result. In aerobic conditions, nuclear shuttling of HIF-1 $\alpha$  could be diminished resulting in a predominant cytoplasmic localization of HIF-1 $\alpha$  and binding to the HRE sequence on the DNA partially (K562) or totally (KCL22) impaired. Hypoxia would then relief this block thus allowing gene target expression. Another possibility is that the promoter of the CA-IX gene could be more accessible in hypoxic conditions. This second hypothesis is supported by the fact that hypoxia does not change the expression of VEGF, another HIF-1 $\alpha$  target gene (Figure 15 B).

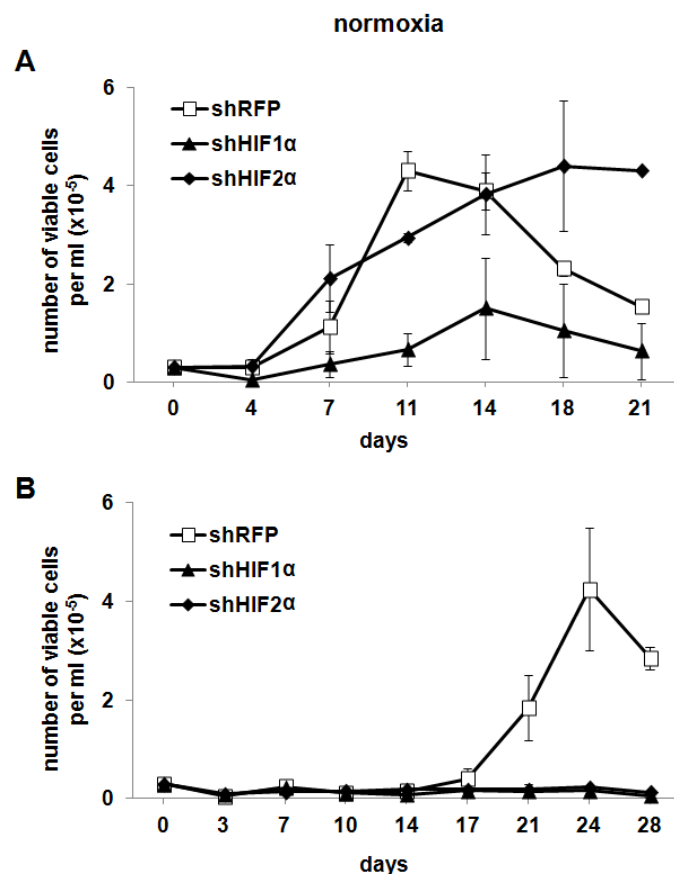
The effects of HIF-1 $\alpha$  or HIF-2 $\alpha$  suppression on the selection of LPC/LSC subsets in hypoxic cultures were then tested. We used K562 cells stably transfected with shRNA against HIF-1 $\alpha$  (shHIF-1 $\alpha$ ) or HIF-2  $\alpha$  (shHIF-2  $\alpha$ ) or control scrambled shRNA (shRFP), all containing the Red Fluorescent Protein (RFP). The genetic



**Figure 12. Genetic suppression of HIF-1 $\alpha$  or HIF-2 $\alpha$  and its effects on the kinetics of K562 cell number in hypoxia.** K562 cells stably transfected with shRNA against HIF-1 $\alpha$  (shHIF-1 $\alpha$ ), HIF2- $\alpha$  (HIF-2 $\alpha$ ) or control scrambled shRNA (shRFP) were incubated in deep hypoxia ( $\sim 0.1\%$  O<sub>2</sub>) for the indicated times. **(A)** Total cell lysates in Laemmli buffer were subjected to immuno-blotting with the indicated antibodies. Anti-vinculin antibody was used to verify equalization of protein loading. One representative experiment out of 3 is shown. **(B)** Trypan blue-negative cells were counted at the indicated times. Values represent mean  $\pm$  S.E.M. of data obtained from 3 independent experiment.

inhibition of HIF-1 $\alpha$  or HIF-2 $\alpha$  protein was successful, as revealed by immunoblotting (Figure 12A). Incubation of these cells in hypoxia showed that their number in culture decreased with a kinetics unaffected by HIF-1 $\alpha$  or HIF-2 $\alpha$  suppression. This result indicates that HIF-1 $\alpha$  and HIF-2 $\alpha$  are not necessary for the maintenance of K562 cell bulk in the course of incubation in hypoxia (Figure 12B).

The effects of the genetic inhibition of HIF1-  $\alpha$  or HIF-2 $\alpha$  on the maintenance of LPC and LSC in hypoxia were then determined by CRA assay. Cell transfected with shHIF-1 $\alpha$ , shHIF-2 $\alpha$  or shRFP were incubated in hypoxic primary cultures (LC1) for different times (3 or 7 days) and then replated into



**Figure 13. Effects of HIF-1 $\alpha$  or HIF-2 $\alpha$  genetic suppression on the maintenance of LPC or LSC potential in hypoxia.** K562 cells stably transfected with shRNA against HIF-1 $\alpha$  (shHIF-1 $\alpha$ ), HIF2- $\alpha$  (HIF-2 $\alpha$ ) or control scrambled shRNA (shRFP) were incubated in hypoxic (~0.1% O<sub>2</sub>) LC1 and transferred at day 3 (**A**) or 7 (**B**) into normoxic LC2, to determine the maintenance of LPC or LSC (respectively) in LC1. Trypan blue-negative cells were counted at the indicated times of LC2. Values represent mean  $\pm$  S.E.M. of data obtained from 3 independent experiments.

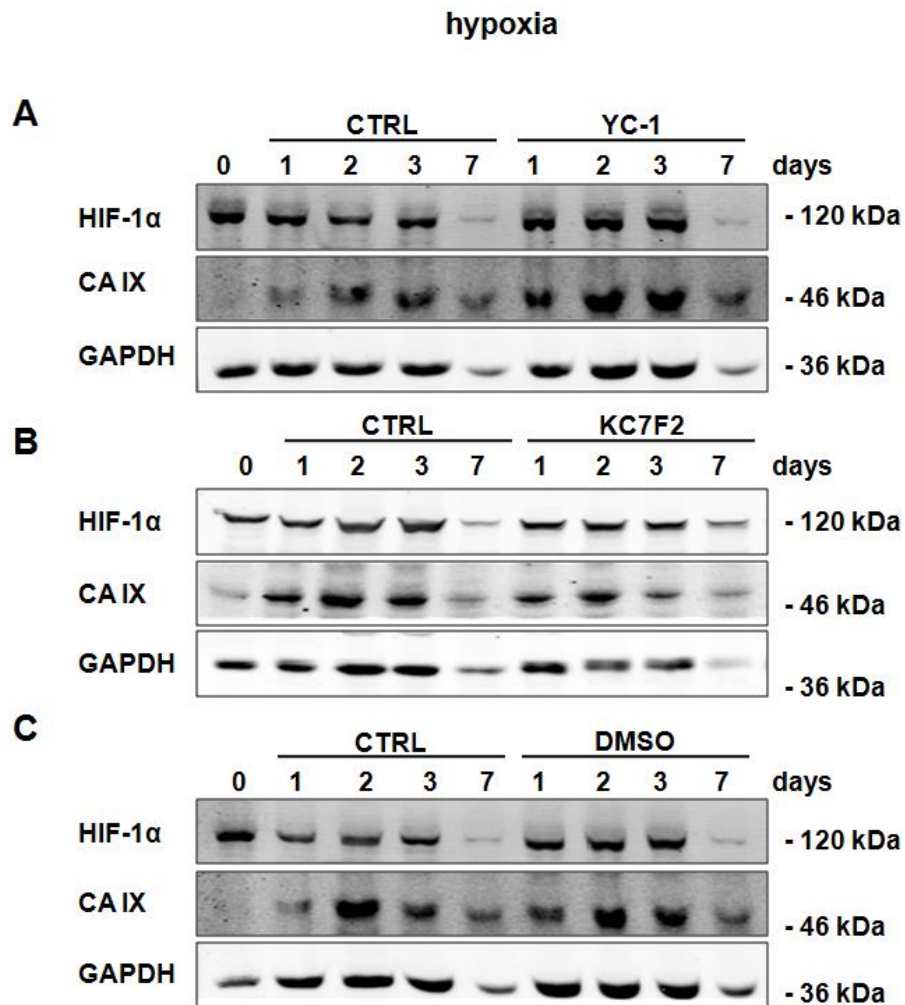
normoxic secondary cultures (LC2). The kinetics of the LC2 repopulation is shown in Figure 13. Cells transferred from day-3 hypoxic LC1 repopulated LC2 after a 4-day long lag-phase, to reach the peak at day 11 (Figure 13A). Such a CRA pattern corresponds to LPC resistant to hypoxia but not hypoxia-selected [73]. The suppression of HIF-2 $\alpha$  did not significantly affect the CRA of LPC. On the contrary, hypoxia strongly inhibited the CRA of cells where shHIF1- $\alpha$  was suppressed (Figure 13A). Thus, clonal expansion of LPC requires the expression of HIF-1 $\alpha$  but not HIF-2 $\alpha$ . The CRA of cells transferred to LC2 from day-7 hypoxic LC1, is shown in Figure 13B. At day 7 of incubation in hypoxia, the selection of BCR/Abl-negative LSC of CML is complete [73]. Control cells repopulated LC2 after a 17-day lag-phase, reaching the peak at day 24. This repopulation was suppressed by cell transfection with either shHIF-1 $\alpha$  or shHIF-2 $\alpha$ . Thus, the maintenance of LSC in hypoxia requires the activity of both HIF-1 $\alpha$  and HIF-2 $\alpha$ .

### **Pharmacological inhibition of Hypoxia-Inducible Factor-1 $\alpha$ induced apoptosis in K562 and KCL22 cell bulk.**

The effects of three HIF- $\alpha$  pharmacological inhibitors, with different mechanism of action, were then tested. KC7F2 and YC-1 inhibit HIF-1 $\alpha$  activity only [102-104], while Compound X inhibits both HIF-1 $\alpha$  and HIF-2 $\alpha$  activity. K562 and KCL22 cells were incubated in hypoxia for 1, 2, 3 or 7 days in the absence or the presence of one of the three drugs. Inhibitors were added at time zero, *i.e.* immediately before the beginning of incubation in hypoxia.

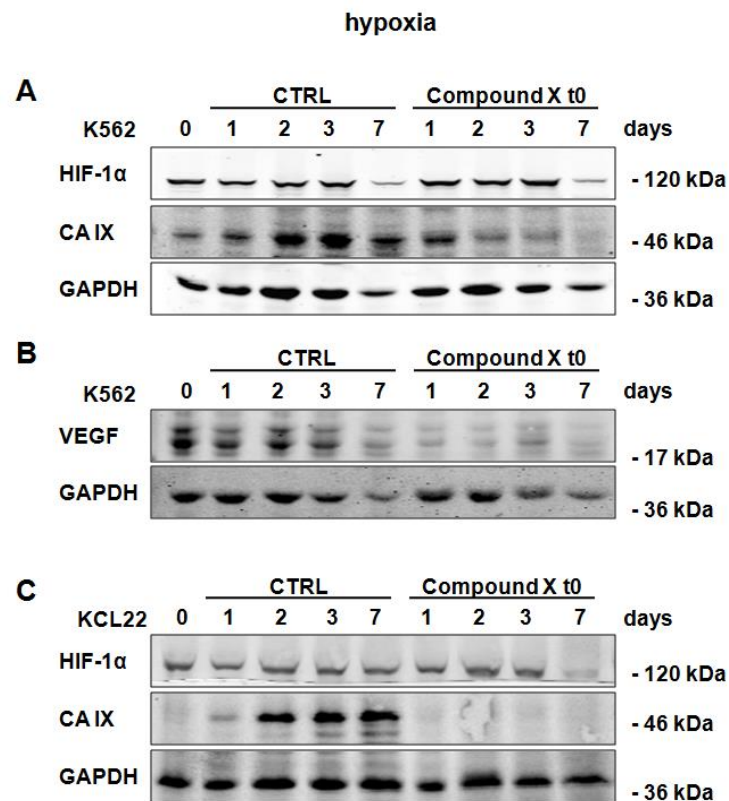
When added to hypoxia-incubated K562 cell cultures, YC-1 and KC7F2 did not inhibit HIF-1 $\alpha$  activity, as determined by testing their effects on the expression of CA-IX (Figure 14A and 14B). It is worth pointing out that the proposed mechanism of action of the two drugs should operate at the translational level, implying a decrease of HIF-1 $\alpha$  protein expression, which was not observed in our conditions. Similar results were obtained for KCL22 cells (data not shown). Since YC-1 and KC7F2 were solubilized in DMSO we tested possible effects of this compound, in the absence of the inhibitors, on HIF-1 $\alpha$  or CA-IX expression level. DMSO did not affect either HIF-1 $\alpha$  or CA-IX (Figure 14C).

Compound X was also tested, under conditions identical to those of Figure 14. Compound X, differently from YC-1 and KC7F2, inhibited HIF-1 $\alpha$  transcriptional activity. Treatment indeed, inhibited the expression of CA-IX (K562 or KCL22 cells; Figure 15A and 15C) as well as of VEGF (K562 cells), another HIF-1 $\alpha$  target protein (Figure 15B). As expected, the level of HIF-1 $\alpha$  protein expression was not



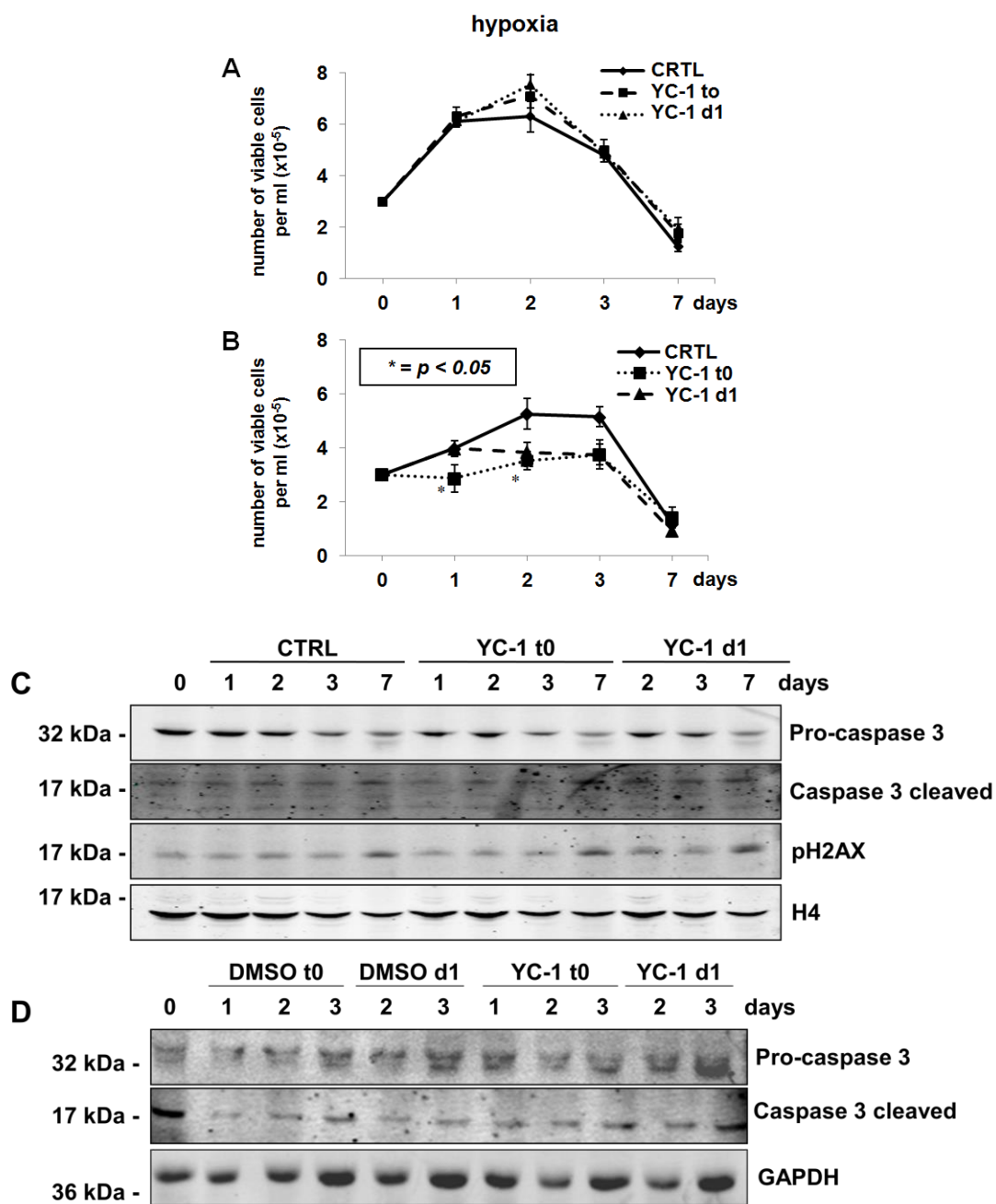
**Figure 14. Effects of YC-1, KC7F2 or DMSO on HIF-1 $\alpha$  and CA-IX expression in K562 cells.** Exponentially-growing K562 cells from routine cultures were replated in fresh medium at  $3 \times 10^5$  cells/ml and incubated in hypoxia ( $\sim 0.1\%$  O<sub>2</sub>) for the indicated times. Cultures were treated or not with 25  $\mu$ M YC-1 (**A**) or 40  $\mu$ M KC7F2 (**B**) or 0.1% DMSO (**C**) at time 0 of incubation, *i.e.* at the beginning of incubation in hypoxia. Total cell lysates in Laemmli buffer were subjected to immuno-blotting with the indicated antibodies. Anti-GAPDH was used to verify equalization of protein loading. One representative experiment out of 3 is shown.

affected by Compound X, which inhibits the binding between the HIF subunits  $\alpha$  and  $\beta$  (Figure 15A and 15C). To determine the effects of the three drugs on survival and growth of CML cell lines, exponentially-growing KCL22 or K562 cultures were incubated in hypoxia for 1, 2, 3 or 7 days without treatment or in the presence of one of the three inhibitors or DMSO. Inhibitors were added at time 0, or after 1 day of hypoxic incubation. Growth kinetics were determined by counting viable cells via trypan-blue exclusion at days 1, 2, 3 and 7 of incubation. The



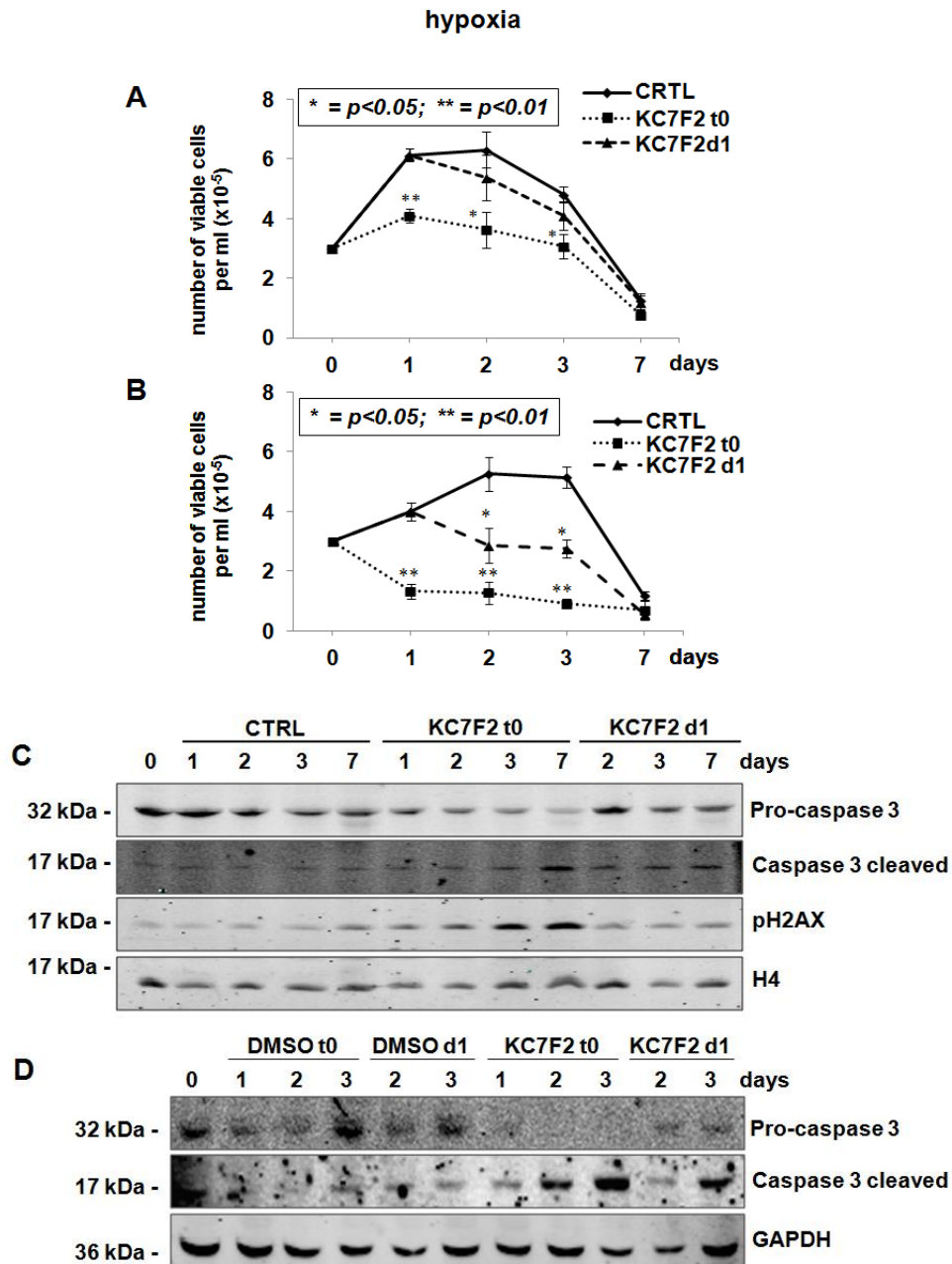
**Figure 15. Effects of Compound X on the expression of HIF-1 $\alpha$ , CA-IX and VEGF in K562 or KCL22 cells.** Exponentially-growing K562 (A and B) or KCL22 (C) cells from routine cultures were replated in fresh medium at  $3 \times 10^5$  cells/ml and incubated in hypoxia ( $\sim 0.1\% \text{ O}_2$ ) for the indicated times. Cultures were treated or not with  $5 \mu\text{M}$  Compound X at time 0 of incubation, *i.e.* at the beginning of incubation in hypoxia. Total cell lysates in Laemmli buffer were subjected to immuno-blotting with the indicated antibodies. Anti-GAPDH antibody was used to verify equalization of protein loading. One representative experiment out of 3 is shown.

number of viable cells, in control K562 or KCL22 cultures, increased about 2-fold over the first 3 days of incubation, to decrease thereafter (Figure 16A and 16B, 17A and 17B, 18A and 18B). YC-1 treatment of K562 cells did not affect growth



**Figure 16. Effects of YC-1 on cell growth, apoptosis and DNA damage in hypoxia.** Exponentially-growing K562 (**A** and **C**) or KCL22 (**B** and **D**) cells from routine cultures were replated in fresh medium at  $3 \times 10^5$  cells/ml and incubated in hypoxia ( $\sim 0.1\%$   $O_2$ ) for the indicated times. Cultures were treated or not with  $25 \mu M$  YC-1 or  $0.1\%$  DMSO (the vehicle of drug administration) at time 0, *i.e.* at the beginning of incubation in hypoxia (t0) or after a 1 day long pre-incubation in hypoxia (d1). (**A** and **B**) Trypan blue-negative cells were counted at the indicated times. Values represent mean  $\pm$  S.E.M. of data obtained from 3 independent experiments. (**C** and **D**) Total cell lysates in Laemmli buffer were subjected to immuno-blotting with the indicated antibodies. Anti-GAPDH or anti-H4 antibodies were used to verify equalization of protein loading. One representative experiment out of 3 is shown.



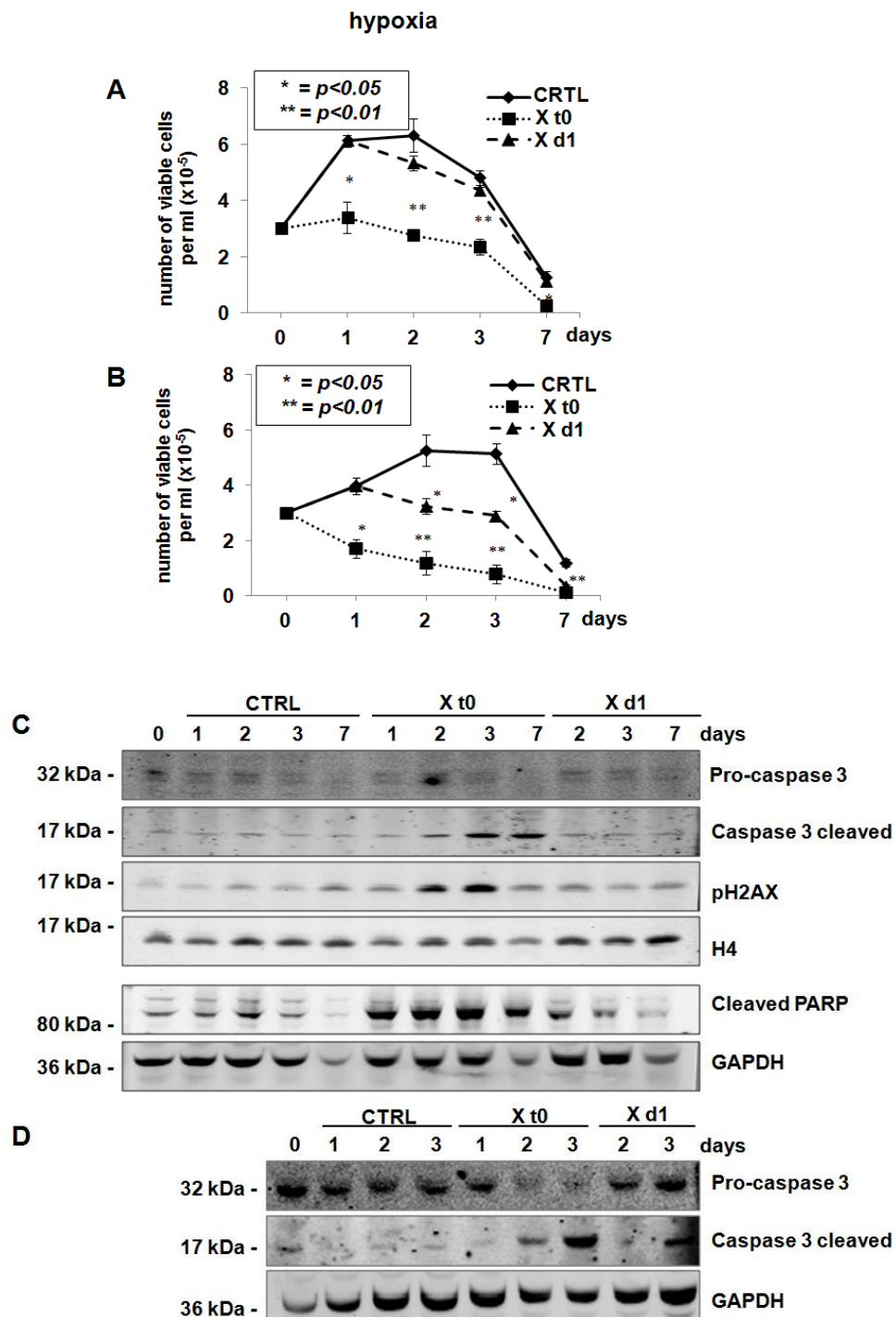


**Figure 17. Effects of KC7F2 on cell growth, apoptosis and DNA damage in hypoxia.** Exponentially-growing K562 (**A** and **C**) or KCL22 (**B** and **D**) cells from routine cultures were replated in fresh medium at  $3 \times 10^5$  cells/ml and incubated in hypoxia ( $\sim 0.1\% \text{ O}_2$ ) for the indicated times. Cultures were treated or not with  $40 \mu\text{M}$  KC7F2 or  $0.1\%$  DMSO (the vehicle of drug administration) at time 0, *i.e.* at the beginning of incubation in hypoxia (t0) or after a 1 day long pre-incubation in hypoxia (d1). (**A** and **B**) Trypan blue-negative cells were counted at the indicated times. Values represent mean  $\pm$  S.E.M. of data obtained from 3 independent experiments. (**C** and **D**) Total cell lysates in Laemmli buffer were subjected to immuno-blotting with the indicated antibodies. Anti-GAPDH or anti-H4 antibodies were used to verify equalization of protein loading. One representative experiment out of 3 is shown.



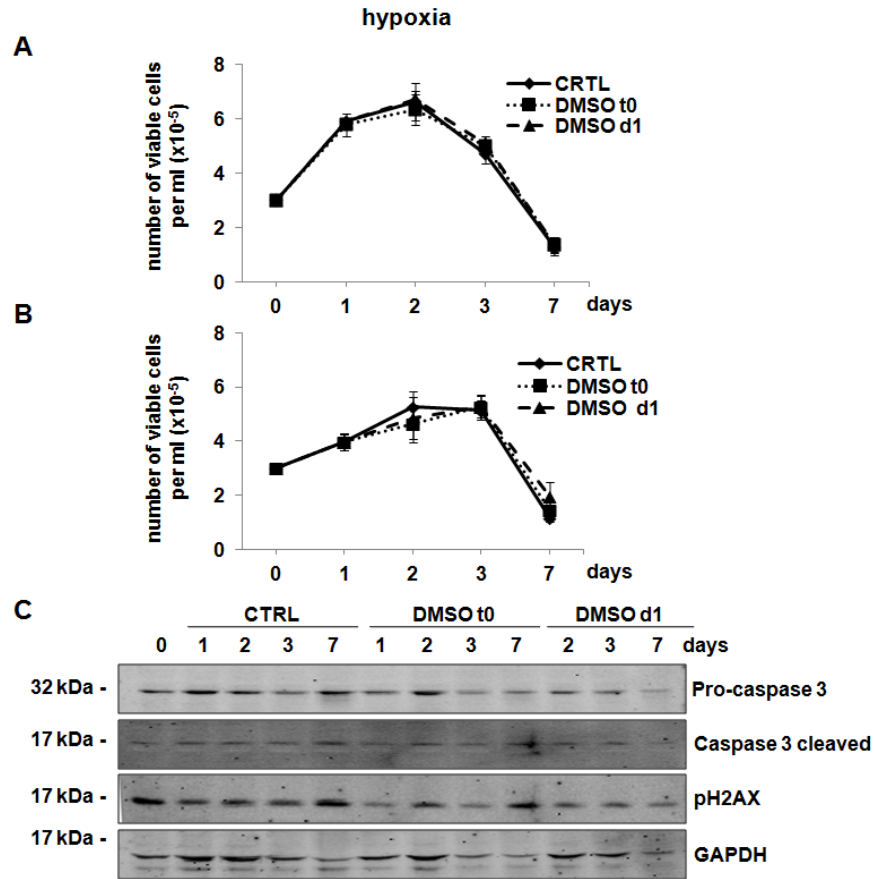
kinetics (Figure 16A), whereas that of KCL22 cells suppressed the increase of viable cell number occurring between time 0 and day 2 (Figure 16B). The immunoblotting confirmed the absence of apoptosis or DNA damage in YC-1-treated cells: the treatment, indeed, did not alter the levels of the cleaved form of caspase 3 or of histone H<sub>2</sub>AX phosphorylation (Figure 16C).

The treatment of K562 cells with KC7F2 at time 0, but not day 1, suppressed the increase of the number of viable cells occurring in control cultures from time 0 to day 2 (Figure 17A). The treatment of KCL22 cells; either at time 0 or day 1, significantly reduced the number of viable cells in culture, indicating that KCL22 cells were more sensitive than K562 cells to the drug (Figure 17B). This fact does not exclude that higher doses would elicit similar effects in K562 cells. The immunoblotting confirmed that KC7F2 administration at time 0 induced apoptosis in both cell lines (Figure 17C and 17D). In KCL22 cell cultures, treatment at day 1 also induced apoptosis (Figure 17D), in keeping with the results of figure 17 B. Interestingly, the treatment of K562 cells with KC7F2 at time 0 induced DNA damage, as indicated by the phosphorylation of H2AX histone (Figure 17C). The treatment of K562 cells with Compound X at time 0 suppressed the increase of the number of viable cells occurring in control cultures from time 0 to day 2 (Figure 18A). The treatment of KCL22 cells, either at time 0 or day 1, significantly reduced the number of viable cells in culture (Figure 18B), indicating that KCL22 cells were more sensitive than K562 cells to Compound X, like they were to KC7F2. This results are in contrast with those obtained via shRNA-mediated HIF-1 $\alpha$  knockdown, which did not decrease the number of viable cells in hypoxia (Figure 12B). This discrepancy can be explained by the fact that, in those experiments, the lack of HIF1- $\alpha$  could be compensated for by the expression of HIF2- $\alpha$ . On the contrary, Compound X, inhibiting simultaneously both HIF1- $\alpha$  and HIF2- $\alpha$ , impaired this compensation. The activation of the apoptotic pathway, by Compound X, is shown in the immunoblotting of Figure 18C and 18D: the caspase 3 cleavage occurred in both cell lines following treatment at time 0 and in KCL22 cells also at day 1. The activation of caspase pathway was confirmed by the increase of the cleaved Poly ADP-Ribose Polymerase (PARP) (Figure 18C). DNA damage as indicated by the phosphorylation of the histone H<sub>2</sub>AX, occurred in K562 cells following treatment at time 0 (Figure 18C).



**Figure 18. Effects of Compound X on cell growth, apoptosis and DNA damage in hypoxia.** Exponentially-growing K562 (A and C) or KCL22 (B and D) cells from routine cultures were replated in fresh medium at  $3 \times 10^5$  cells/ml and incubated in hypoxia ( $\sim 0.1\%$   $O_2$ ) for the indicated times. Cultures were treated or not with  $5 \mu M$  Compound X at time 0, *i.e.* at the beginning of incubation in hypoxia (t0) or after a 1 day long pre-incubation in hypoxia (d1). (A and B) Trypan blue-negative cells were counted at the indicated times. Values represent mean  $\pm$  S.E.M. of data obtained from 3 independent experiments. (C and D) Total cell lysates in Laemmli buffer were subjected to immuno-blotting with the indicated antibodies. Anti-GAPDH or anti-H4 antibodies were used to verify equalization of protein loading. One representative experiment out of 3 is shown.

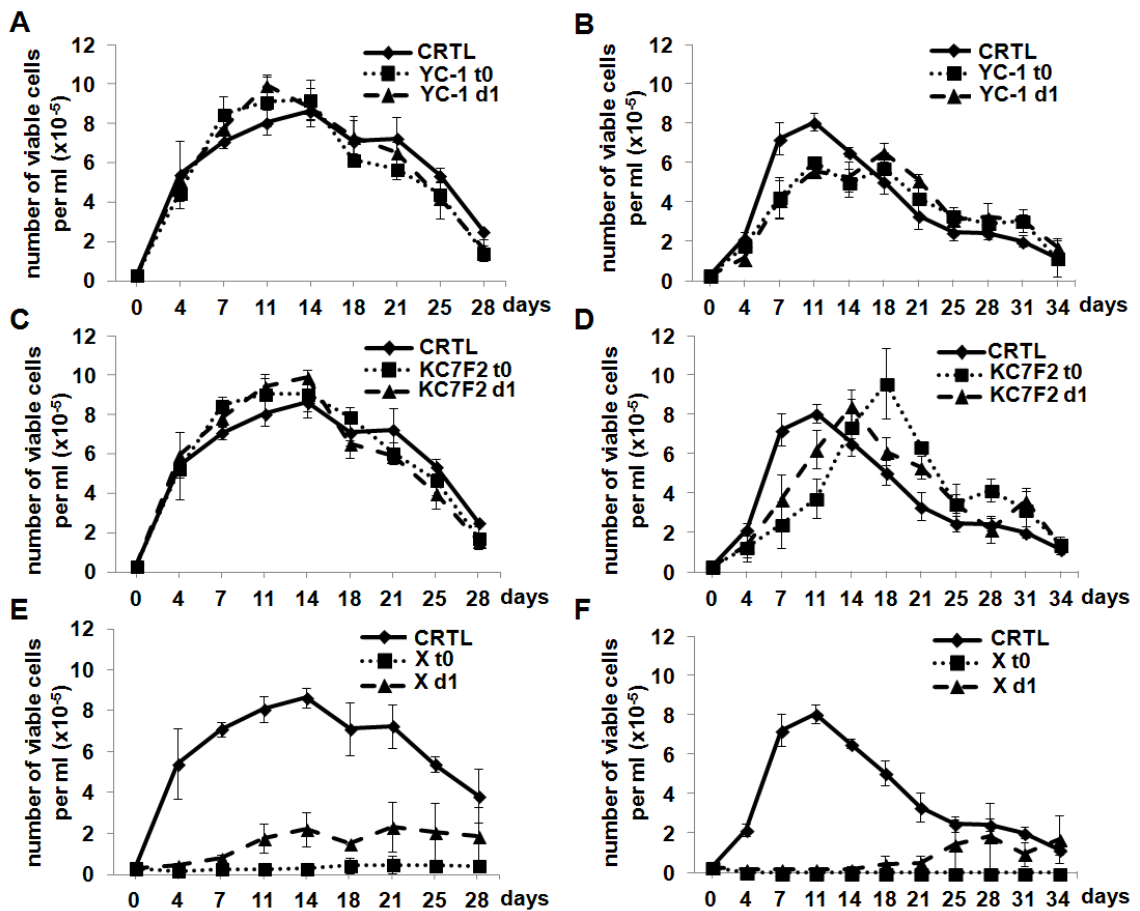
The lack of effect of cell treatment with DMSO, the vehicle of YC-1 and KC7F2 administration, alone on the number of viable cells in culture is shown in Figure 19A and B, while that on the activation of apoptosis is shown in Figure 19C. The latter results are in keeping with those of Figures 16D and 17D.



**Figure 19. Effects of DMSO alone on cell growth, apoptosis and DNA damage in hypoxia.** Exponentially-growing K562 (**A** and **C**) or KCL22 (**B**) cells from routine cultures were replated in fresh medium at  $3 \times 10^5$  cells/ml and incubated in hypoxia ( $\sim 0.1\% \text{ O}_2$ ) for the indicated times. Cultures were treated or not with 0.1 % DMSO at time 0, *i.e.* at the beginning of incubation in hypoxia (t0) or after a 1 day long pre-incubation in hypoxia (d1). (**A** and **B**) Trypan blue-negative cells were counted at the indicated times. Values represent mean  $\pm$  S.E.M. of data obtained from 3 independent experiments. (**C**) Total cell lysates in Laemmli buffer were subjected to immuno-blotting with the indicated antibodies. Anti-GAPDH antibody was used to verify equalization of protein loading. One representative experiment out of 3 is shown.

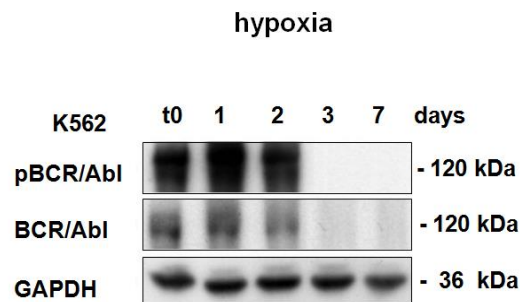
## Compound X impaired the Culture-Repopulation Ability of hypoxia-selected BCR/Abl-positive and BCR/Abl-negative Leukaemia Progenitor Cells.

The effects of the three inhibitors on the maintenance of BCR/Abl-positive and BCR/Abl-negative LPC resistant to hypoxia were tested by CRA assay. Cells were incubated, at a density of  $3 \times 10^5$  cells/ml, in hypoxic LC1 for 2 or 3 days in the absence or in the presence of DMSO or of one of the drugs. The treatment was administered at time 0 or after 1 day of pre-adaptation to hypoxia. Cells were then washed free of drug and transferred at a density of  $3 \times 10^4$  cells/ml to drug-free, growth-permissive (normoxic) LC2.



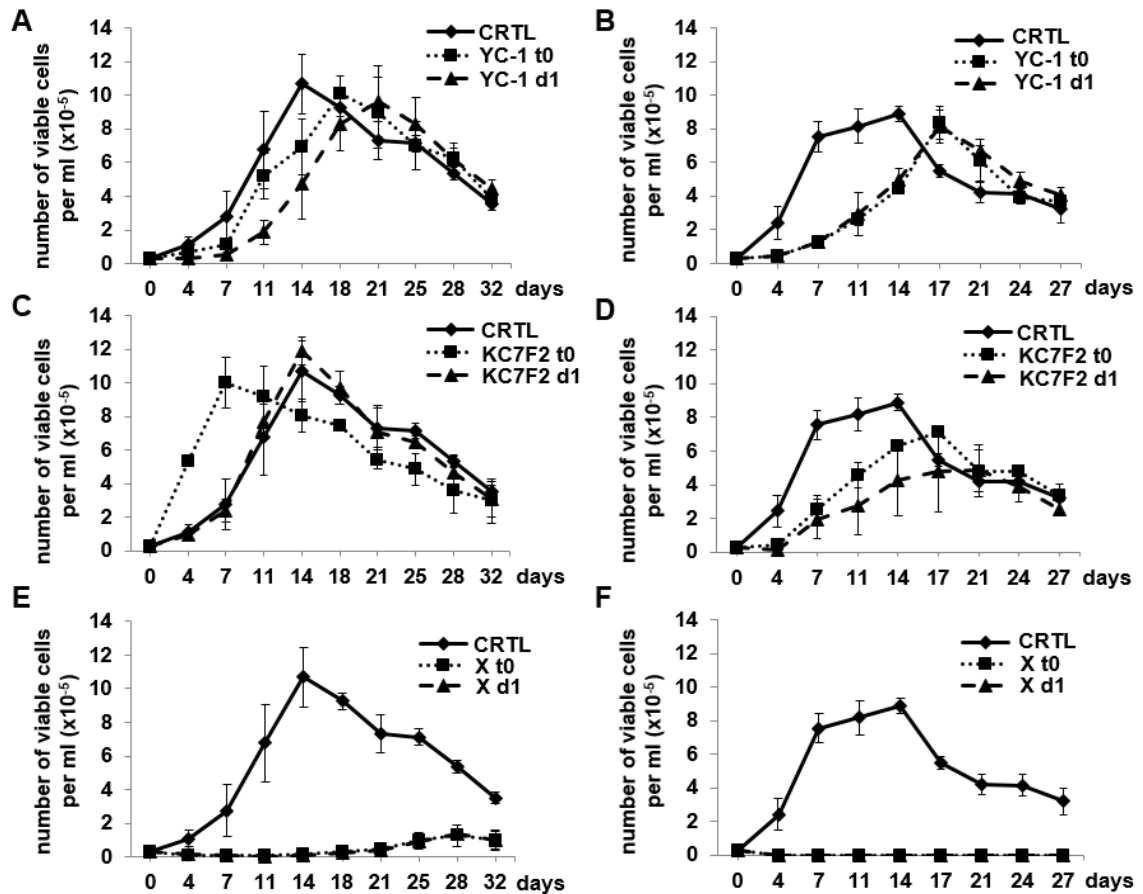
**Figure 20. Effects of YC-1, KC7F2 or Compound X on the CRA of BCR/Abl-positive LPC.** Exponentially-growing K562 (A, C and E) or KCL22 (B, D and F) cells from routine cultures were replated in fresh medium at  $3 \times 10^5$  cells/ml and incubated in hypoxic LC1 ( $\sim 0.1\%$   $O_2$ ) for 2 days. Cultures were treated with one of the drugs at time 0, *i.e.* at the beginning of incubation in hypoxia (t0) or after a 1 day long pre-incubation in hypoxia (d1). Cells were then transferred into normoxic LC2 ( $3 \times 10^4$  cells/ml) and trypan blue-negative cells were counted at the indicated times of incubation in LC2. Values represent mean  $\pm$  S.E.M. of data obtained from 4 independent experiments.

Figure 20 shows that control cells transferred from day-2 LC1 rapidly repopulated LC2, to peak at day 14 or day 11 (KCL22 or K562 cells, respectively). This kinetics is typical of cells transferred to LC2 when BCR/Abl is still fully expressed in LC1 (Figure 21). The kinetics of LC2 repopulation was unaffected by the treatment with either YC-1 or KC7F2 (Figure 20 A-D). On the contrary, treatment with Compound X of hypoxic LC1 suppressed LC2 repopulation as for either cell line.



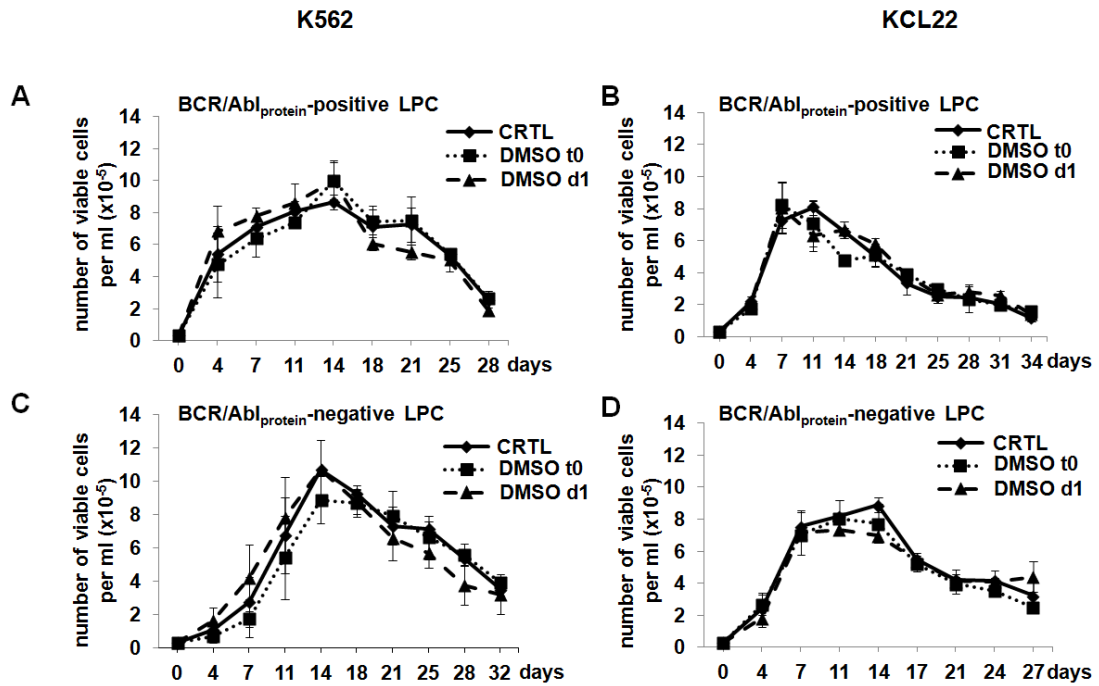
**Figure 21. Effects of hypoxia on BCR/Abl expression and phosphorylation.** Exponentially-growing K562 cells from routine cultures were replated in fresh medium at  $3 \times 10^5$  cells/ml and incubated in hypoxia ( $\sim 0.1\%$   $O_2$ ) for the indicated times. Total cell lysates in Laemmli buffer were subjected to immuno-blotting with the indicated antibodies. Anti-GAPDH antibody was used to verify equalization of protein loading. One representative experiment is shown.

The effects of cell transfer to LC2 from day-3 hypoxic LC1, where the suppression of BCR/Abl has been induced (Figure 21) are shown in Figure 22. This cell subset, mainly constituted by BCR/Abl-negative LPC, repopulated LC2 with a rather different kinetics. After an initial lag-phase of 3-4 days, cell number increased slowly to peak at day 14. This kinetics is the consequence of BCR/Abl suppression, the delay in LC2 repopulation being due to the time necessary for re-expression of BCR/Abl following transfer to normoxia. Treatment with YC-1 or KC7F2 at time 0 or day 1 of incubation in hypoxic LC1 did not significantly affect the kinetics of LC2 repopulation as for K562 (Figure 22A and 22C), or delayed this kinetics without reducing CRA substantially in the case of KCL22 cells (Figure 22B and 22D). On the contrary, treatment with Compound X of hypoxic LC1 suppressed LC2 repopulation as for either cell line. Thus Compound X suppressed CRA of hypoxia-resistant LPC of CML, irrespective whether they express BCR/Abl (Figure 20E and 20F) or not (Figure 22E and 22F).



**Figure 22. Effects of YC-1, KC7F2 or Compound X on the CRA of BCR/Abl-negative LPC.** Exponentially-growing K562 (A, C and E) or KCL22 (B, D and F) cells from routine cultures were replated in fresh medium at  $3 \times 10^5$  cells/ml and incubated in hypoxic LC1 ( $\sim 0.1\% O_2$ ) for 3 days. Cultures were treated with one of the drugs at time 0, *i.e.* at the beginning of incubation in hypoxia (t0) or after a 1 day long pre-incubation in hypoxia (d1). Cells were then transferred into normoxic LC2 ( $3 \times 10^4$  cells/ml) and trypan blue-negative cells were counted at the indicated times of incubation of LC2. Values represent mean  $\pm$  S.E.M. of data obtained from 4 independent experiments.

LC2 repopulation by either BCR/Abl-positive or -negative LPC was not affected by the addition to cultures of DMSO alone (Figure 23).



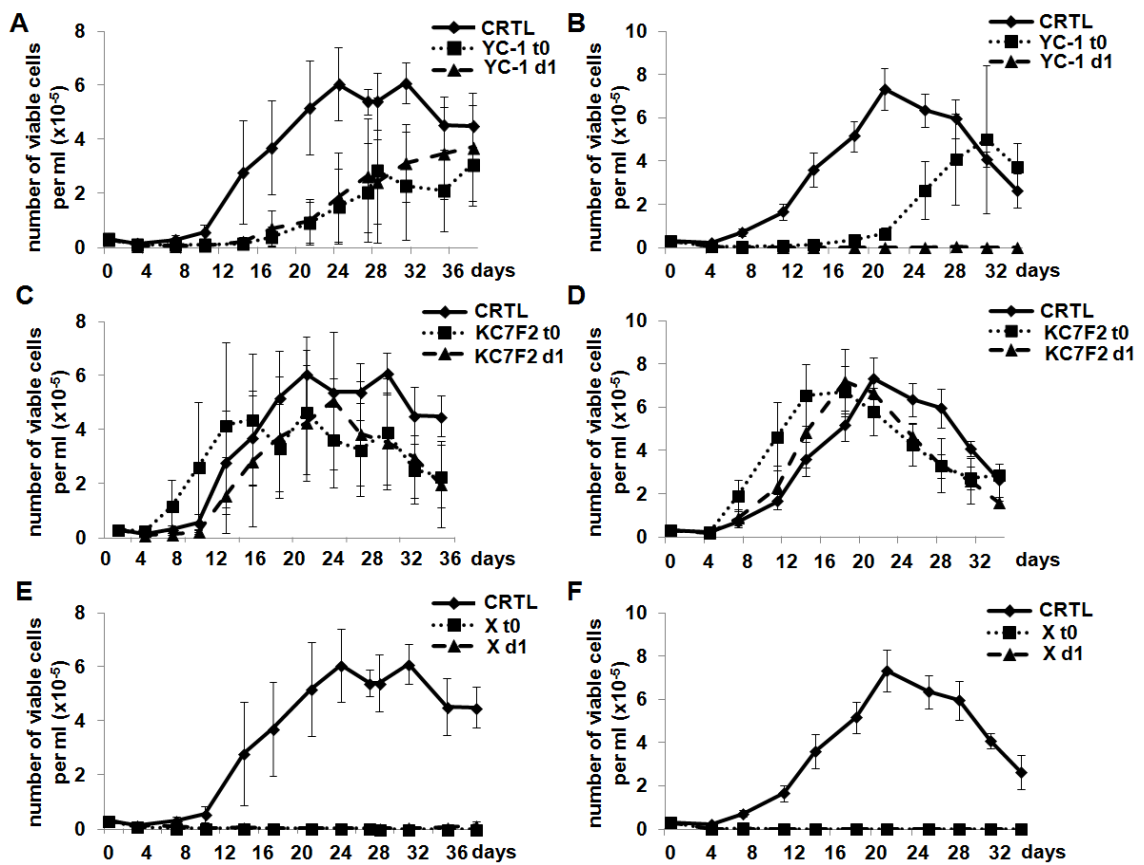
**Figure 23. Effects of DMSO on the CRA of BCR/Abl-positive or -negative LPC.** Exponentially-growing K562 (A and C) or KCL22 (B and D) cells from routine cultures were replated in fresh medium at  $3 \times 10^5$  cells/ml and incubated in hypoxic LC1 ( $\sim 0.1\% O_2$ ). 0.1 % DMSO was administered at time 0, *i.e.* at the beginning of the incubation in hypoxia (t0) or after a 1 day long pre-incubation in hypoxia (d1). After 2 (A and B) or 3 (C and D) days of incubation in LC1, cells were transferred into normoxic LC2 ( $3 \times 10^4$  cells/ml) and trypan blue-negative cells were counted at the indicated times of incubation in LC2. Values represent mean  $\pm$  S.E.M. of data obtained from 4 independent experiments.

### Compound X suppressed the Culture-Repopulation Ability of hypoxia-resistant BCR/Abl-negative Leukaemia Stem Cells.

The effects of the three inhibitors on the maintenance of LSC were tested by CRA assay. K562 or KCL22 cells were incubated, at a density of  $3 \times 10^5$  cells /ml in hypoxic LC1 in the absence or in the presence of DMSO or of one of the drugs. The treatment was administered at time 0 or after 1 day of pre-adaptation to hypoxia. Cells were rescued from day-7 LC1, *i.e.* following a one-log reduction of total cell number in LC1 and most likely an enrichment of BCR/Abl-negative cells [22, 72, 73]. Cells were then washed free of drug and transferred at a density of  $3 \times 10^4$  cells/ml to drug-free, growth-permissive (normoxic) LC2. Untreated, control K562 (Figure 24A, 24C and 24E) or KCL22 (Figure 24B, 24D and 24F) cells repopulated LC2 with an initial lag-phase of 7 or 4 days, to peak after 24 or 21

days. Such a kinetics typically reflects the content of transplanted LC1 cells with LSC [72, 73], as well as HSC [110, 111].

The treatment of hypoxic LC1 with YC-1 time 0 or day 1 determined a reduction and/or a delay of LC2 repopulation by K562 cells rescued from LC1 at day 7 (Figure 24A). A similar result was obtained with YC-1 treatment of KCL22 cells at time 0 of LC1 (Figure 24B). Unexpectedly, KCL22 cells treated with YC-1 after 1 day of hypoxic pre-incubation, did not repopulate LC2 (Figure 24B). KC7F2 treatment at time 0 or day 1 of incubation in hypoxia did not alter LC2 repopulation by K562 of KCL22 rescued after 7 days of hypoxic LC1 (Figure 24C and 24D). KC7F2 treatment at time 0 or day 1 of incubation in hypoxia did not alter LC2 repopulation by K562 of KCL22 rescued after 7 days of hypoxic LC1 (Figure 24C and 24D).



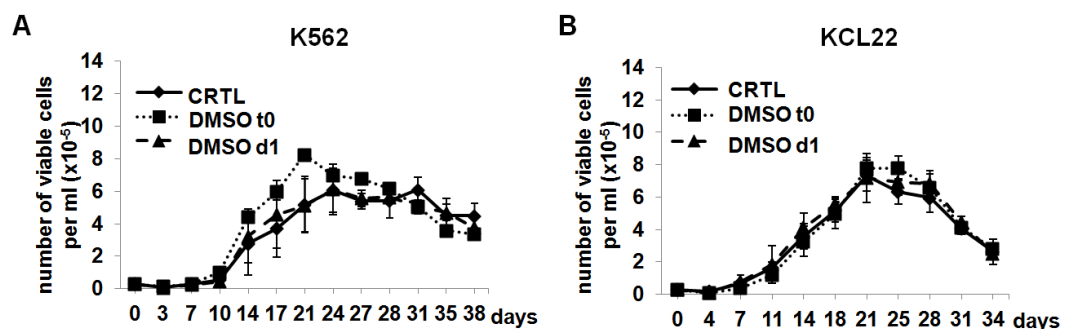
**Figure 24. Effects of YC-1, KC7F2 or Compound X on the CRA of hypoxia-resistant BCR/Abi-negative LSC.** Exponentially-growing K562 (A, C and E) or KCL22 (B, D, F) cells from routine cultures were replated in fresh medium at  $3 \times 10^5$  cells/ml and incubated in hypoxic LC1 ( $\sim 0.1\% \text{ O}_2$ ) for 7 days. Cultures were treated with one of the drugs at time 0, *i.e.* at the beginning of incubation in hypoxia (t0) or after a 1 day-long pre-incubation in hypoxia (d1). Cells were then transferred into normoxic LC2 ( $3 \times 10^4$  cells/ml) and trypan blue-negative cells were counted at the indicated times of incubation in LC2. Values represent mean  $\pm$  S.E.M. of data obtained from 4 independent experiments.



Although potentially interesting, this results does not seem due to HIF-1 $\alpha$  inhibition, as YC-1 and KC7F2 did not inhibit HIF-1 $\alpha$  in our conditions and did not alter CA-IX expression (Figure 14A and 14B). It should be noted that the latter results were obtained in different experimental condition, *i.e.* administering the drugs at time 0. On the other hand, the fact that KC7F2 was not effective on K562 cells further support the evidence that KCL22 cells are more sensitive than K562 cells to this drugs.

Compound X administration to LC1, at either time 0 or day 1 of incubation in hypoxia, suppressed LC2 repopulation by cells rescued from LC1 at day 7 (Figure 24E and 24F). The effects of Compound X are in keeping with those of genetic suppression of HIF-1 $\alpha$  (Figure 13B).

No difference was observed between control cultures and cultures where DMSO was administered (Figure 25).

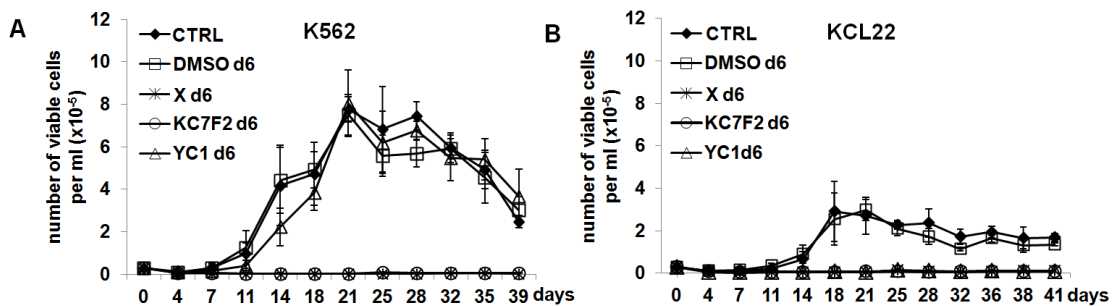


**Figure 25. Effects of DMSO on the CRA of hypoxia-resistant BCR/Abl-negative LSC.** Exponentially-growing K562 (A) or KCL22 (B) cells from routine cultures were replated in fresh medium at  $3 \times 10^5$  cells/ml and incubated in hypoxic LC1 ( $\sim 0.1\%$  O $_2$ ). 0.1% DMSO was administered at time 0, *i.e.* at the beginning of the incubation in hypoxia (t0) or after a 1 day long pre-incubation in hypoxia (d1). After 7 days of incubation in LC1, cells were transferred into normoxic LC2 ( $3 \times 10^4$  cells/ml) and trypan blue-negative cells were counted at the indicated times of incubation in LC2. Values represent mean  $\pm$  S.E.M. of data obtained from 4 independent experiments.

To better mimic the scenario most likely occurring *in vivo*, we treated K562 or KCL22 cell cultures at day 6 of incubation in hypoxia. It is indeed straightforward to think that, *in vivo*, LSC are already established within the SC niches, *i.e.* adapted to a hypoxic environment, before the beginning of treatment. Accordingly, it has been demonstrated that hypoxia can protect CSC from several treatments [74,

113-115]. Cells treated in hypoxic LC1 at day 6 were transferred to normoxic LC2 at day 9 (Figure 26). Control K562 (Figure 26A) or KCL22 (Figure 26B) cells repopulated LC2 with an initial 7-11 day-long lag-phase, respectively, to reach the peak around day 21. YC-1 did not affect LC2 repopulation by K562 cells, but suppressed that by KCL22 cells. KC7F2 suppressed LC2 repopulation (CRA) by either K562 or KCL22 cells. However, as mentioned above, this was not a consequence of HIF-1 $\alpha$  inhibition.

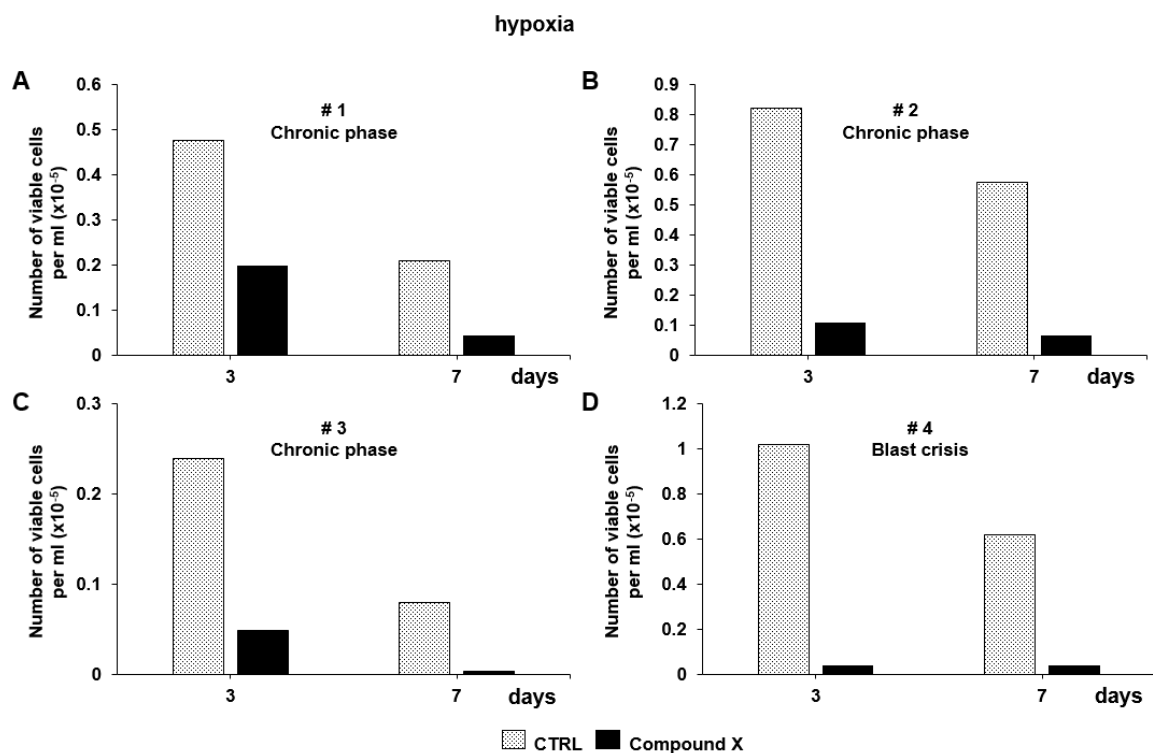
Compound X also suppressed the CRA of K562 and KCL22 cells. Thus, prolonged adaptation to hypoxia did not confer resistance to Compound X treatment, indicating that HIF-1 $\alpha$  pharmacological inhibition could be a possible therapeutic strategy to target hypoxia-adapted LTR-LSC. DMSO did not alter the CRA of K562 or KCL22 rescued after 9 days of hypoxic LC1.



**Figure 26. Effects of YC-1, KC7F2 or Compound X on the CRA of long hypoxia-adapted LSC.** Exponentially-growing K562 (A) or KCL22 (B) cells from routine cultures were replated in fresh medium at  $3 \times 10^5$  cells/ml and incubated in hypoxic LC1 ( $\sim 0.1\%$   $O_2$ ). Cultures were treated with DMSO or one of the drugs at day 6 of incubation and transferred into normoxic LC2 at day 9 ( $3 \times 10^4$  cells/ml). Trypan blue-negative cells were counted at the indicated times of incubation in LC2. Values represent mean  $\pm$  S.E.M. of data obtained from 4 independent experiments.

## Compound X impaired the Culture-Repopulation Ability of hypoxia-selected primary Chronic Myeloid Leukaemia Stem Cells.

First, we tested the effect of Compound X on the cell bulk of primary cells from CML patients. (Figure 27). Human light-density BM mononuclear cells, obtained from three CML patients in chronic phase (Figure 27A, 27B and 27C) and one CML patient in blast crisis (Figure 27D), were incubated in hypoxia for 7 days and treated with Compound X at time 0. In agreement with what observed for cell lines, Compound X reduced the number of viable cells in culture.

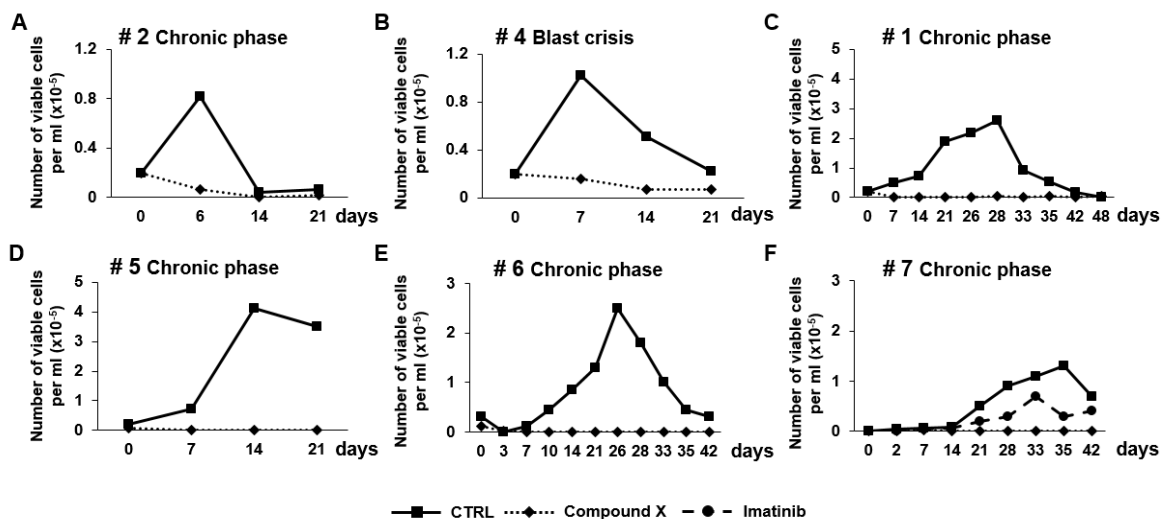


**Figure 27. Effects of Compound X on total cell number in primary CML cell cultures incubated in hypoxia.** Cultures of human light-density BM mononuclear cells obtained from CML patients were established with  $3 \times 10^5$  cells/ml, treated with Compound X at time 0 and incubated in hypoxia ( $\sim 0.1\%$   $O_2$ ). Trypan blue-negative cells were counted at the indicated times of incubation.

The effectiveness of Compound X demonstrated on hypoxia-selected LSC of stabilized CML lines was also tested on primary CML cells. Human light-density BM mononuclear cells explanted from five chronic-phase and one blast-crisis CML patients were treated with Compound X at time 0 and incubated in hypoxia for 7 days. Cells were then washed free of drug and transferred at a density of  $3 \times 10^4$  cells/ml to drug-free, normoxic LC2 (Figure 28).

Cells derived from two CML patients (# 2 and # 4) repopulated LC2 rapidly (Figure 28A and 28B) with a kinetics similar to that obtained with BCR/Abl-positive LPC of stabilized CML cell lines rescued from LC1 (Figure 20A-F). On the other hand, cells derived from patients # 6 and # 7 repopulated LC2 after an initial lag-phase of 7-10 days (Figure 28E and 28F), with a kinetics similar to that obtained replating hypoxia selected LSC of stabilized CML cell lines (Figure 24A-F). Finally, cells derived from patients # 1 and # 5 repopulated LC2 with a kinetics that was intermediate between the other two groups of patients (Figure 28C and 28D) and comparable to that of hypoxia-selected BCR/Abl-negative LPC (Figure 22A-F). In keeping with what observed for CML cell lines, Compound X impaired LC2 repopulation driven by different hypoxia-selected human primary CML cell subsets (Figure 28A-F).

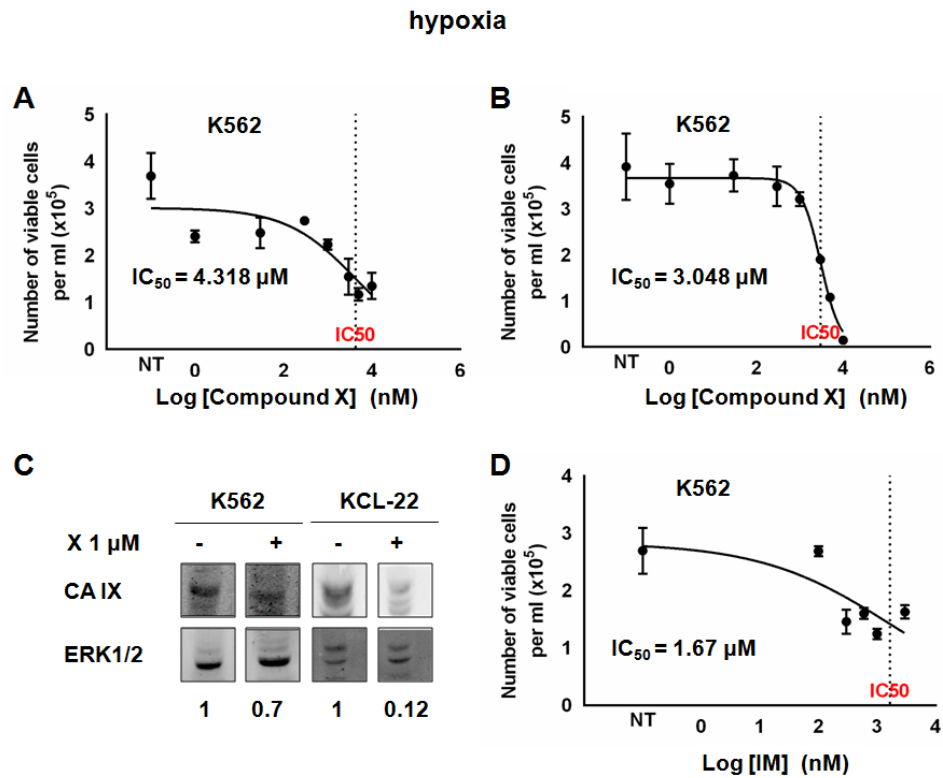
The effects of IM, the standard CML treatment, on the CRA of primary CML cells from patient # 7 were tested. Cells were treated with IM at time 0 and incubated in hypoxia for 7 days. Contrarily to Compound X, IM did not suppress LC2 repopulation. Thus, hypoxia-selected human primary CML cells were insensitive to TKi treatment (Figure 28F). This is in agreement with the notion of *refractoriness* of LSC to IM [73]; in this respect, it is worth pointing out that patient # 7 was a newly-diagnosed patient, *i.e.* never treated before, and no BCR/Abl mutations have occurred yet.



**Figure 28. Effects of Compound X on the CRA of hypoxia-selected primary LSC of CML.** Human light-density BM mononuclear cells, obtained from CML patients, were treated with Compound X (A-F) or IM (F) and incubated in hypoxic LC1 (~0.1% O<sub>2</sub>) (established at 3x10<sup>5</sup> cells/ml) for 7 days. Cells were then transferred into normoxic LC2 (established at 3x10<sup>4</sup> cells/ml) and trypan blue-negative cells were counted at the indicated times of incubation in LC2.

## The combined treatment with Compound X and IM impaired the CRA of hypoxia-selected LSC.

The effects of the combined treatment of Compound X and IM on CML cells were then tested. The estimate of half-maximal Inhibitory Concentration ( $IC_{50}$ ) of the two drugs on the number of viable cells in K562 as well as KCL22 cultures incubated in hypoxia is reported in Figure 29 (29A, 29B and 29D). Compound X, at a concentration (1  $\mu$ M) markedly lower than  $IC_{50}$ , still reduced CA-IX expression (Figures 29C).

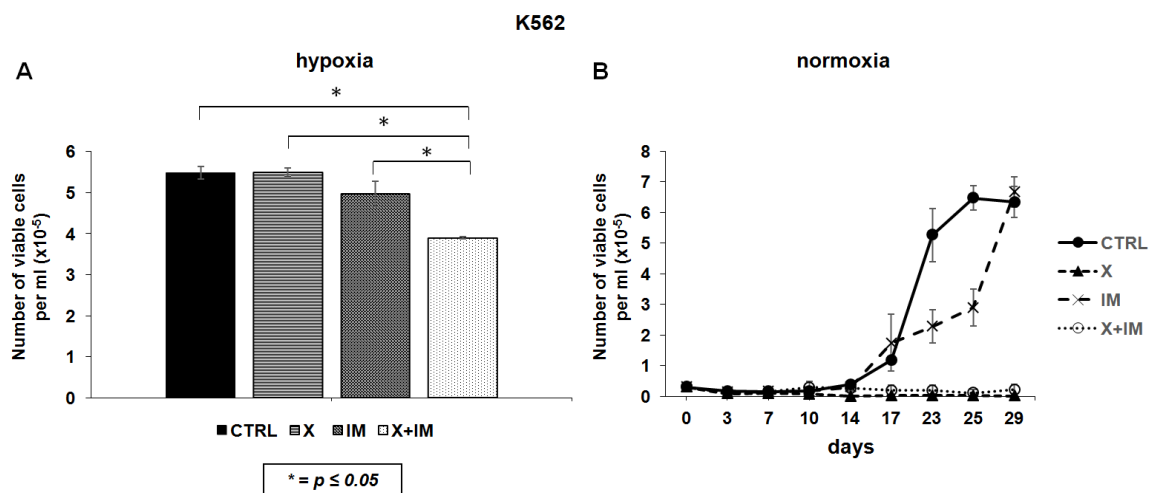


**Figure 29.  $IC_{50}$  of Compound X and IM on the number of viable cells in CML cell cultures in hypoxia.** Exponentially-growing K562 or KCL22 cells from routine cultures were replated in fresh medium at  $3 \times 10^5$  cells/ml and incubated in hypoxia. Cultures were treated with the indicated concentration of Compound X or IM. Trypan blue-negative cells were counted on day 3 of incubation. Values represent mean  $\pm$  S.E.M. of data obtained from 3 experiments (A, B and D). Total lysates in Laemmli buffer of cells incubated in hypoxia for 7 days were subjected to immuno-blotting with the indicated antibodies. Values represent ratios between Compound X-treated and -untreated samples with respect to CA-IX band densitometry, following normalization as for the relative ERK1/2 band (used to verify equalization of protein loading) (C).

The effects of the combined treatment of Compound X and IM on total number of viable K562 cells in LC1 incubated in hypoxia and on the maintenance

of LSC therein are reported in Figure 30. To prevent the possible toxicity of the combination of the two drugs at full doses, as well as to better appreciate the effects of their combination, we used both Compound X and IM at 1 $\mu$ M. Cells were incubated in hypoxia in the absence or the presence of Compound X or IM, alone or in combination, added at time 0. Compound X or IM alone did not significantly decrease the number of viable cells in LC1 after 2 days of incubation in hypoxia, while the combined treatment determined a significant reduction (Figure 30A). Thus, Compound X and IM had an at least additive effect in reducing the number of viable cells in culture.

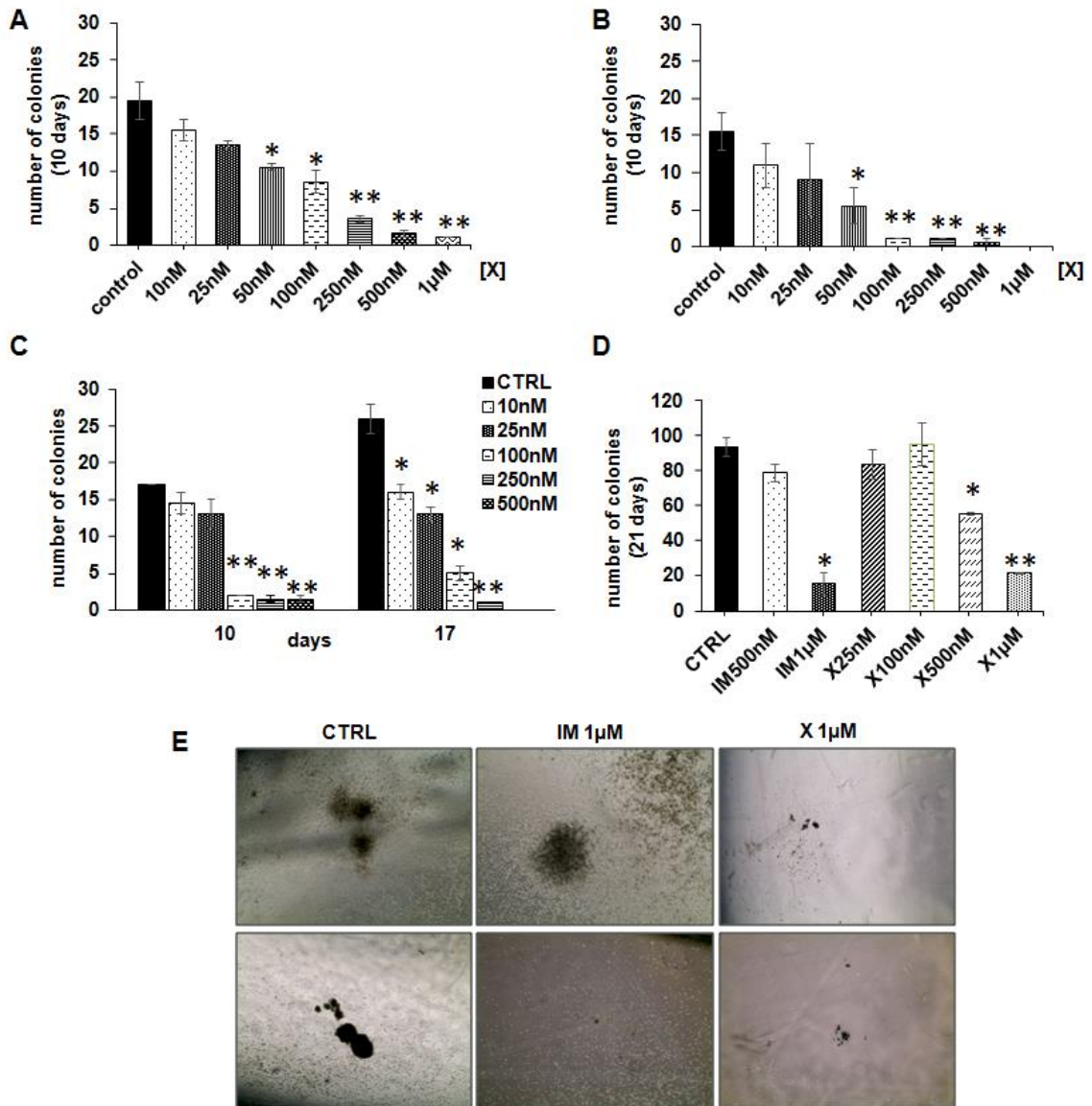
To establish the effects of drug combination on the CRA of hypoxia-selected cells K562 cells were incubated in hypoxic LC1 in the absence or in the presence of the drugs alone or in combination for 7 days and then transferred into normoxic LC2 (Figure 30B). Control cells repopulated the culture with a kinetics typical of hypoxia-selected LSC. In keeping with the results obtained with primary CML cells (Figure 28F), IM did not suppress LC2 repopulation. On the contrary, Compound X completely suppressed this repopulation. Interestingly, IM did not interfere with the effect of Compound X.



**Figure 30. Effects of Compound X and IM combined treatment on K562 cell line in hypoxia.** Exponentially-growing K562 cells from routine cultures were replated in fresh medium at  $3 \times 10^5$  cells/ml. Cultures were incubated in hypoxic LC1 in the absence (CTRL) or in the presence of 1 $\mu$ M Compound X or 1 $\mu$ M IM alone (X and IM, respectively) or in combination (X+IM). (A) Trypan blue-negative cells were counted at 2 days of LC1. Values represent mean  $\pm$  S.E.M. of data obtained from 3 experiments. (B) Cells were transferred, after 7 days of LC1, into normoxic LC2 (established at  $3 \times 10^4$  cells/ml) and trypan blue-negative cells were counted at the indicated times of incubation in LC2. Values represent mean  $\pm$  S.E.M. of data obtained from 3 experiments.

## Compound X reduced the colony-formation ability of murine or human primary CML cells.

The effects of Compound X on the colony-formation ability in normoxia of murine and human primary CML cells were tested. Murine BM cells, obtained from



**Figure 31. Effects of Compound X treatment on the colony-formation ability of murine and human primary CML cells.** (A, B and C) Murine BM cells were obtained from femurs and tibiae of three CML mice and plated in methylcellulose medium in the absence (Control) or in the presence of the indicated concentration of Compound X (X). The number of colonies was scored at the indicated times. (D and E) Human light-density BM mononuclear cells explanted from one CML patient were plated in methylcellulose medium in the absence (Control) or in the presence of the indicated concentrations of Imatinib (IM) or Compound X (X). Number of colonies (D) and representative pictures (E) after 21 days.

femurs and tibiae of three CML mice, were incubated in methylcellulose medium in the absence or the presence of different concentrations of Compound X. At day 10 of incubation, Compound X concentration-dependently inhibited colony formation, 50 nM Compound X being the lowest significantly effective concentration (Figure 31A and 31B). At day 17, interestingly, Compound X was effective at a lower concentration, 10 nM (Figure 31C). Thus, more immature colony-forming cells, generating later-appearing colonies, appeared more sensitive to Compound X than less mature colony-forming cells.

Human light density BM mononuclear cells, obtained from one CML patient, were plated in methylcellulose medium in the absence or the presence of different concentrations of Compound X or IM and incubated in normoxia. The number of colonies was scored after 21 days (Figure 31D). Compound X 0.5 $\mu$ M or 1 $\mu$ M, as well as IM 1 $\mu$ M, significantly reduced the colony-formation ability of human CML cells. Although there was no difference in the number of colonies between cultures treated with 1 $\mu$ M Compound X or IM, colony morphology was quite different; indeed only small colonies were present in Compound X-treated cultures, while several large colonies were observed following IM treatment (Figure 31E).

### **Compound X reduced the percentage and the number of LSK cells in the BM of CML mice.**

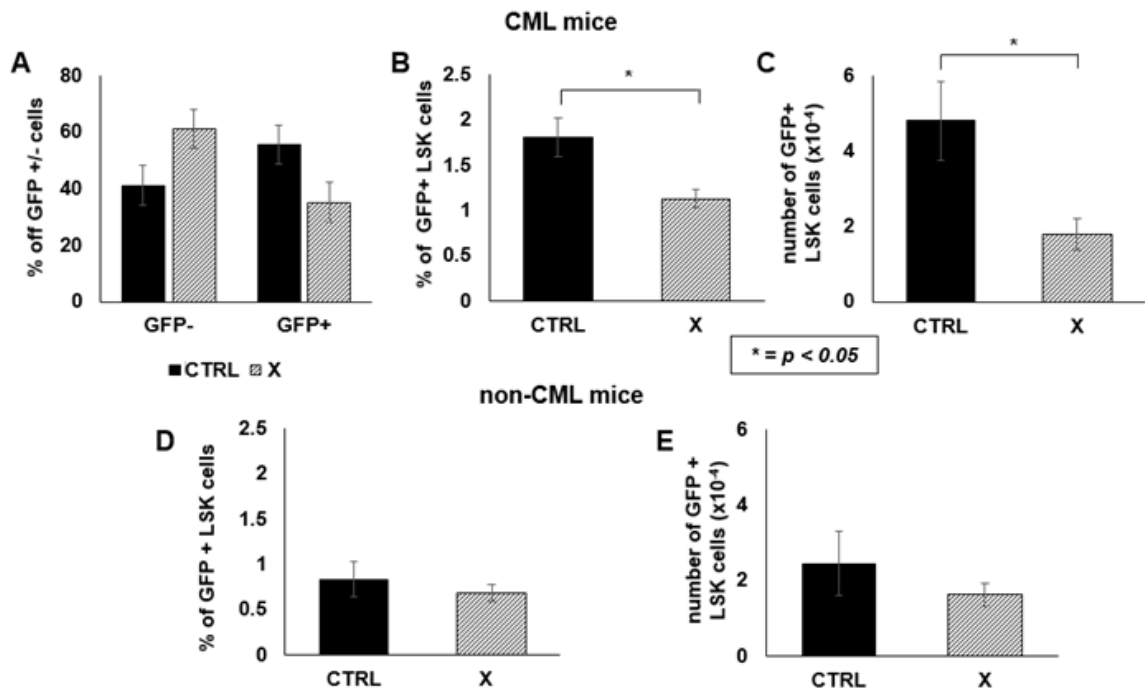
It has already been demonstrated that the *in vivo* genetic suppression of HIF-1 $\alpha$  causes an impairment of LSC function in CML mice [101]. Therefore, we examined whether inhibition of HIF-1 $\alpha$  by Compound X also suppressed LSC *in vivo* using a CML mouse model. BM cells from 5-FU-treated mice were retrovirally transduced with BCR/ABL-GFP and transplanted into lethally irradiated recipient mice. One week after the Bone Marrow Transplantation (BMT), mice were treated daily with Compound X (2mg/kg) or placebo (control group). After two weeks of treatment, mice were sacrificed and BM was analyzed by FACS.

Compound X did not affect the percentage of leukaemia cells (GFP+) in BM. Interestingly, no toxic effects of Compound X was observed on the percentage of non-leukaemia cells (GFP-) (Figure 32A).

It has been demonstrated that the Lineage-Sca-1+c-kit+ (LSK) subset functions as LSC in CML, *i.e.* capable to induce CML in recipient mice [24]. The



effects of Compound X on LSK cells are shown in Figure 32B and 32C. Compound X significantly reduced the number and the percentage of LSK cells.



**Figure 32. Effects of Compound X treatment on the percentage and the number of LSK in BM of CML mice.** BM cells from 5-FU-treated mice were retrovirally transduced with BCR/Abl-GFP (A, B and C) or GFP alone (D and E) and transplanted into lethally irradiated recipient mice. One week after BMT, mice were treated daily with 2mg/kg Compound X (X) or placebo (CTRL). After two weeks of treatment, mice were sacrificed and BM was analyzed by FACS. (A) Percentage of non-leukaemia (GFP-) or leukaemia (GFP+) cells in BM of CML mice. Percentage (B) and number (C) of GFP+LSK in BM of CML mice. Percentage (D) and number (E) of GFP+LSK in BM of non-CML mice.

To exclude toxic effects of Compound X on non-leukaemia LSK cells, BM cells from 5-FU-treated mice were retrovirally-transduced with GFP alone and transplanted into lethally-irradiated recipient mice, which were treated as indicated above. Compound X did not affect the total number or the percentage of non-leukaemia GFP+ LSK cells (Figure 32D and 32E).

# Conclusions

CML is a stem cell-driven disorder which is treated with impressive efficacy with TKi. Although TKi are unable in most cases to prevent the relapse, inducing a state of MDR, apparently due to the persistence of LSC. LSC survival *in vivo* most likely occurs, independently of BCR/Abl kinase activity, within the hypoxic environment of stem cell niches. HIF-1 $\alpha$  is a key regulator of cell adaptation to hypoxia and its role in the maintenance of HSC and LSC has already been demonstrated [101].

In this study, we wanted to evaluate the effects of HIF-1 $\alpha$  pharmacological inhibition on the maintenance of LPC and LSC of CML. We compared three different putative HIF-1 $\alpha$  inhibitors, identifying Compound X as a very effective inhibitor of CML cell survival and growth in hypoxia. Compound X also inhibited the colony-formation ability of primary human and murine CML cells. It was interesting to compare this effect with that of IM. There was no difference between Compound X and IM as for their effect on colony number. However, in Compound X-treated cultures, only small colonies were present, while some large colonies were detected in the presence of IM.

Compound X, but not IM, suppressed LSC maintenance in hypoxia. These results were obtained with either CML cell lines or primary cells explanted from CML patients. According to these results, Compound X appeared suitable to be used alone to suppress MRD as well as CML cell bulk. However, TKi represent a consolidate standard for CML therapy, as they are extremely effective in inducing remission. Thus, we had to take into consideration also the possibility of a simultaneous or sequential use of Compound X with TKi, where Compound X is directed to target LSC specifically. In this scenario, we decided to test the effects of the simultaneous combination of Compound X with TKi, thinking that such a protocol better approaches a potential clinical setting. Therefore, it appeared necessary to exclude antagonist effects of IM with respect to Compound X. We found that, in fact, the two drugs had an additive effect as far as the total number

of viable cells in hypoxic cultures is concerned. On the other hand, IM did not interfere with the detrimental effect of Compound X on LSC maintenance. These results pointed to the feasibility of a simultaneous combination of Compound X with TKi.

Using a murine model of BCR/Abl-induced CML, we analyzed *in vivo* the effects of Compound X on the maintenance of the LSC-containing LSK cell subset. Compound X significantly reduced the number and the percentage of leukaemia LSK in the BM of mice compared to the placebo treatment. Interestingly, Compound X did not affect HSC maintenance, pointing to a good therapeutic index of the drug in discriminating leukaemic from normal haematopoietic cells.

Taken together, these results indicated that TKi-resistant LSC are instead sensitive to HIF-1 $\alpha$  inhibition by Compound X. On this basis, we propose the Compound X/TKi combination as a novel therapeutic approach targeting CML cell bulk to induce remission and, at the same time, LSC to prevent the onset of MRD.

A paper containing the main results of this thesis is in preparation. Its submission will follow the acceptance of patent relative to Compound X.

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