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Targeting the p53–MDM2 interaction by the small-molecule MDM2 antagonist Nutlin-3a: a new challenged target therapy in adult Philadelphia positive acute lymphoblastic leukemia patients

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ABSTRACT

The human p53 tumor suppressor, known as the "guardian of the genome", is one of the most important molecules in human cancers. One mechanism for suppressing p53 uses its negative regulator, MDM2, which modulates p53 by binding directly to and decreasing p53 stability. In testing novel therapeutic approaches activating p53, we investigated the preclinical activity of the MDM2 antagonist, Nutlin-3a, in Philadelphia positive (Ph^+) and negative (Ph^-) leukemic cell line models, and primary B-Acute lymphoblastic leukemia (ALL) patient samples. In this study we demonstrated that treatment with Nutlin-3a induced grow arrest and apoptosis mediated by p53 pathway in ALL cells with wild-type p53, in time and dose-dependent manner. Consequently, MDM2 inhibitor caused an increase of pro-apoptotic proteins and key regulators of cell cycle arrest. The dose-dependent reduction in cell viability was confirmed in primary blast cells from Ph^+ ALL patients with the T315I Bcr-Abl kinase domain mutation. In order to better elucidate the implications of p53 activation and to identify biomarkers of clinical activity, gene expression profiling analysis in sensitive cell lines was performed. A total of 621 genes were differentially expressed ($p < 0.05$). We found a strong down-regulation of GAS41 (growth-arrest specific 1 gene) and BMI1 (a polycomb ring-finger oncogene) (fold-change -1.35 and -1.11, respectively; p-value 0.02 and 0.03, respectively) after in vitro treatment as compared to control cells. Both genes are repressors of INK4/ARF and p21. Given the importance of BMI in the control of apoptosis, we investigated its pattern in treated and untreated cells, confirming a marked decrease after exposure to MDM2 inhibitor in ALL cells. Noteworthy, the BMI-1 levels remained constant in resistant cells. Therefore, BMI-1 may be used as a biomarker of response. Our findings provide a strong rational for further clinical investigation of Nutlin-3a in Ph^+ and Ph^-ALL.

1. INTRODUCTION

1.1 Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is a malignant disorder that originates from one single hematopoietic precursor committed to the B- or T-cell lineage[1]. ALL represents a biologically and clinically heterogeneous group of B/T-precursor-stage lymphoid cell malignancies arising from genetic insults that block lymphoid differentiation and drive aberrant cell proliferation and survival[2].

As a consequence there is accumulation of an immature B- or T cell clone in the bone marrow resulting in the suppression of normal hematopoiesis and in various extra-medullary sites. 80-85% of ALL are B-cell lineage (B-cell precursor ALL), and 15-20% are T-cell lineage (T-ALL). Normal lymphocyte ontogeny takes place in the central lymphoid organ: in the bone marrow for B-cells and in the thymus for T-cells. During the maturation process, specific cell surface receptors are triggered to initiate a program of a sequential gene expression that commits developing cells to a B- or T-cells fate, drives the proliferation of progenitors and initiates the rearrangement of antigen receptor genes. The acquisition and expression of a mature and unique antigen receptor is a central feature of both B- and T-cell maturation and very similar molecular and cellular events characterize the early development of B- and T-cells[1].

Incidence and cure rates differ among children and adults. In children, ALL is the commonest malignancy accounting for approximately 25% of childhood cancer; in adults, this disease is less common and generally carries a worse prognosis. Although there is a remarkable progress made in the treatment of ALL in children and, with less efficacy in adults, several ALL subtypes continue to have a poor prognosis.

Consequently there is the need to improve the molecular dissection of subtypes, identifying the genetic alterations that predict the risk of treatment failure, and developing novel and targeted therapies[2].

1.2 Philadelphia chromosome

In adult patients with B-ALL, the most frequent chromosomal translocation is the t(9;22). The Philadelphia chromosome (Ph^+), arising from a reciprocal translocation between chromosome 9 and 22 (Fig.1) was the first defined cytogenetic abnormality recognized as linked both chronic myeloid leukemia (CML) and Ph^+ ALL. This translocation fuses the ABL1 oncogene on chromosome 9 to a breakpoint cluster region (BCR). It generates the constitutively activated BCR-ABL tyrosine kinase, which is responsible for both acute and chronic disease.

In CML, a p210 Bcr-Abl isoform is initially expressed in hematopoietic stem cells capable of giving rise to both differentiated myeloid and lymphoid progeny; whereas, in *de novo* Ph^+ ALL, the expression of p185 and p190 isoforms is restricted to the B-cell lineage.

CML typically presents as an indolent myeloproliferative disease, CML chronic phase (CP), which, if untreated, invariably evolves to blast crisis (BC), in which poorly differentiated malignant myeloid or lymphoid blast cells become resistant to any therapeutic approach.

Bcr-Abl expression increases during disease progression and promotes the acquisition of additional genetic changes, which are essential for the expansion of clones with greater malignant potential. From a clinical perspective, de novo Ph^+ ALL resembles CML lymphoid BC, but without an initial CP. Treatment of CML and Ph^+ ALL is represented by tyrosine kinase inhibitors (TKIs). CML-CP and Ph^+ ALL differ in their aggressiveness and response to therapy; Ph^+ ALL is associated with a rapid response to treatment but with frequent relapse and with poor outcome, whereas CML-CP is characterized by high and sustained rate of complete hematologic and cytogenetic responses[3].

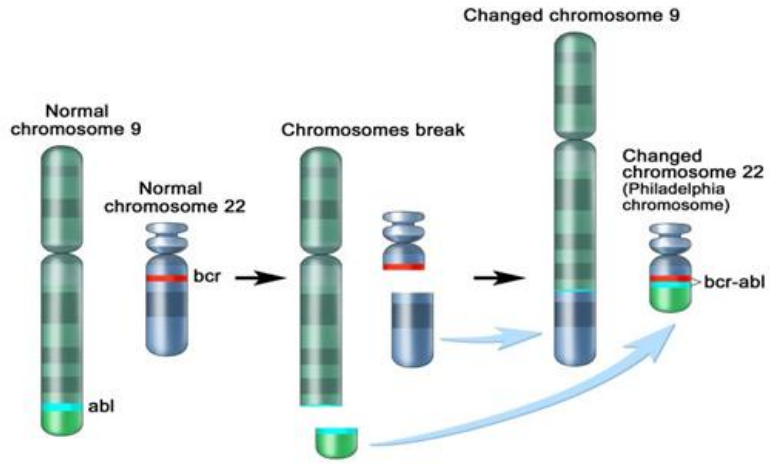

Figure 1: The t(9;22) translocation and its products. The *BCR-ABL* oncogene on the Ph chromosome and the reciprocal *ABL-BCR* on the derivative 9q+ chromosome.

1.2.1 Structure and functions of the ABL and BCR proteins

The *ABL1* gene, spanning a 230 kb region at band 9q34, encodes a 145 kDa non-receptor tyrosine kinase. The ABL1 protein (Fig.2) has three SRC homology (SH) domains called SH1, SH2, and SH3; the SH1 domain has a tyrosine kinase function while the SH2 and SH3 domains are involved in protein–protein interactions; they also regulate the tyrosine kinase activity and are necessary for signal transduction function. The ABL1 protein has also three nuclear localization signal (NLSs) domains and one nuclear export sequence (NES), three DNA-binding regions and an F-actin-binding domain[4].

The c-ABL has been implicated to function in a range of cellular processes. This protein can shuttle between the nuclear and the cytoplasmic compartment because of its NLSs and NES. Nuclear c-ABL plays a role in transcription regulation and can induce apoptosis in response to DNA damage. Cytoplasmic c-ABL is involved in actin dynamics and cell migration.

c-ABL function is tightly controlled in order to prevent oncogenic activity; it's regulated by intramolecular interactions that keep the kinase domain in a closed and inactive conformation. The c-ABL closed conformation involves the interaction between the SH3 domain and the proline-rich sequence present in SH2–kinase linker. In addition, c-ABL regulation implicates unique interactions that involve the SH2 domain, the myristoylation site and the first 80 N-terminal amino acids of the protein, which has been named the "Cap"[5].

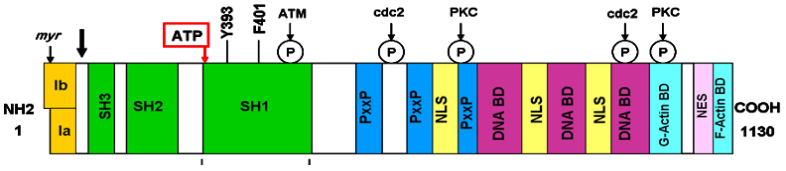

Figure 2: Schematic structure of the ABL protein. Type Ia isoform is slightly shorter than type Ib, which contains a myristoylation (myr) site for attachment to the plasma membrane. The SH domains are situated toward the NH2 terminus. Y393 is the major site of autophosphorylation within the kinase domain, and phenylalanine 401 (F401) is highly conserved in PTKs containing SH3 domains. The middle of protein is dominated by proline-rich regions (PxxP) capable of binding to SH3 domains, and it harbors 1 of 3 NLS. The C-terminus contains DNA as well as G- and F-actin–binding domains. The arrowhead indicates the position of the breakpoint in the BCR-ABL fusion protein[7].

Breakpoint cluster region (BCR) is a 160 kDa citoplasmatic protein. The BCR serine/threonine kinase contains multiple functional domains (Fig.3). Two domains in the C-terminal half of the protein are homologous to regulators of small, Ras-related GTP-binding proteins: guanine nucleotide exchange factors and GTPase activating proteins for the p2l subfamily of GTPases[6]. The center of the molecule contains a region with dbl-like and pleckstrin-homology (PH) domains that stimulate the exchange of guanidine triphosphate (GTP) for guanidine diphosphate (GDP) on Rho guanidine exchange factors (Rho-GEF), which in turn may activate transcription factors. In addition, BCR can be phosphorylated on several tyrosine residues, especially tyrosine 177 which binds Grb-2, an important adapter molecule involved in the activation of the Ras pathway[7].

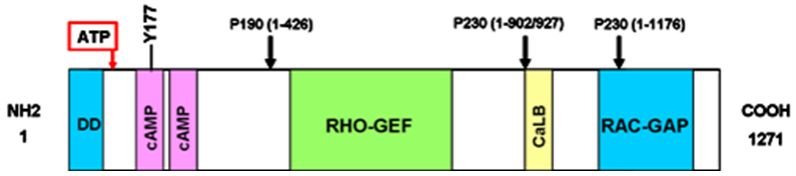

Figure 3: Structure of the BCR protein. The dimerization domain (DD) and the 2 cyclic adenosine monophosphate (cAMP) kinase homologous domains are situated in the N-terminus. Y177 is the autophosphorylation site crucial for binding to Grb-2. The center of the molecule contains a region homologous to Rho-GEF as well as dbl-like and PH domains. Toward the C-terminus a putative site for calcium-dependent lipid binding (CaLB) and a domain with activating function for Rac-GTPase (Rac-GAP) are found. Arrowheads indicate the position of the breakpoints in the BCR-ABL fusion proteins[7].

1.2.2 *BCR-ABL* fusion gene

Depending on the precise breakpoints in the translocation and RNA splicing, different forms of BCR–ABL protein with different molecular weights (p185 BCR-ABL, p210 BCR–ABL and p230 BCR–ABL) can be generated in patients[8] (Fig.4).

The breakpoints within the ABL gene at 9q34 can occur anywhere over a large (greater than 300 kb) area at its 5’ end, either upstream of the first alternative exon Ib, downstream of the second alternative exon Ia, or, more frequently, between the two[9].

In most patients with CML, the break in BCR gene occurs within a 5.8-kb area spanning BCR exons 12-16 (originally referred to as exons b1-b5), defined as the major breakpoint cluster

region (M-*bcr*). Because of alternative splicing, fusion transcripts with either b2a2 or b3a2 junctions can be formed. A 210-kDa chimeric protein ($P210^{BCR-ABL}$) is derived from this mRNA. In patients with ALL and rarely in patients with CML, the breakpoints are further upstream in the 54.4-kb region between the alternative BCR exons e2' and e2, termed the minor breakpoint cluster region (m-*bcr*). The resultant e1a2 mRNA is translated into a 190-kDa protein ($P190^{BCR-ABL}$). A third breakpoint cluster region (μ-*bcr*) was identified downstream of exon 19, giving rise to a 230-kDa fusion protein ($P230^{BCR-ABL}$) associated with the rare Ph^+ chronic neutrophilic leukemia[10].

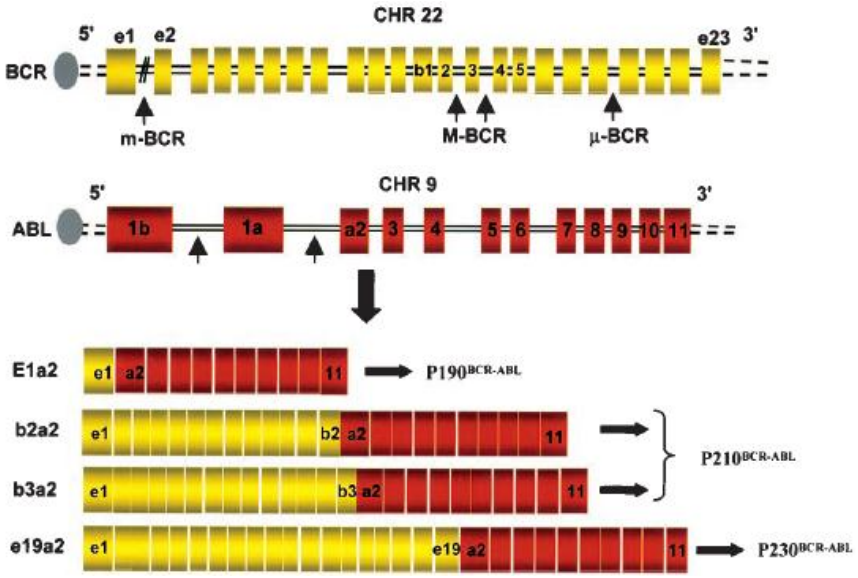

Figure 4: Locations of the breakpoints in the *ABL* and *BCR* genes and structure of the chimeric BCR/ABL mRNA transcripts derived from the various breaks.

1.2.3 Roles of BCR-ABL in leukemogenesis

In contrast to ABL, BCR-ABL exhibits deregulated, constitutively active tyrosine kinase activity and is found exclusively in the cytoplasm of the cell, complexed with a number of cytoskeletal proteins[7].

BCR-ABL expression regulates a variety of signaling molecules ranging from mitogens, antiapoptotic proteins, hematopoietic factors, and cytoskeletal components, to negative

regulators such as phosphatases (Fig.5). The key pathways implicated so far are those involving RAS, mitogen-activated protein (MAP) kinases, signal transducers and activators of transcription (STAT), phosphatidylinositol 3-kinase (PI3K), and MYC[11].

BCR-ABL interacts with the cytoplasmic protein GRB2 and the BCR-ABL/GRB2 complex recruits Son of Sevenless (SOS), which is constitutively associated with the GRB2 SH3 domain. In turn, the BCR-ABL/GRB2/SOS complex stimulates conversion of the inactive GDP-bound form of Ras to its active GTP-bound state and the activation of the scaffold adapter GRB2-associated binding protein 2 (GAB2). As a consequence, the GRB2/GAB2/SOS complex causes constitutive activation of the RAS downstream pathway, thereby activating MAP extracellular signal-regulated kinase (ERK)1/2 (MEK) and MAP kinase proteins and resulting in abnormal cell proliferation[8].

In addition, this complex activates the PI3K/AKT pathway, which promotes survival by suppressing the activity of the forkhead O (FOXO) transcription factor, and enhances cell proliferation by inducing p27 proteosomal degradation and by mTOR activation. BCR-ABL may activate PI3K by more than one pathway. Once activated, PI3K activates AKT kinase, which serves as a key downstream effector by exerting many cellular effects through the phosphorylation of downstream substrates that regulate the apoptotic machinery, resulting in prolonged survival and expansion of the abnormal clone[12].

Among the key transcription factors involved in BCR-ABL signaling, STAT1 and STAT5 are constantly active in Ph^+ CML patients. In normal cells, nuclear translocation of STATs occurs exclusively after cytokine binding to receptors and is mediated by activation of the receptor-associated JAK kinases[13]. By contrast, in CML, STATs seem to be activated in a JAK-independent manner through a direct association of STAT SH2 domains with phosphorylated tyrosines on BCR-ABL. Activation of STAT5 is at least partially responsible for protection from programmed cell death through the upregulation of the antiapoptotic molecule BCL-xL together with the inactivation of the proapoptotic molecule BAD by AKT[14].

Another target of the transforming activity of the BCR-ABL protein is the proto-oncogene MYC, transcription factor that regulates apoptosis and cell cycle, which is expressed at a high level in CML cells. MYC activation seems to be independent of the RAS pathway but directly upregulated by the ABL SH2 region[15].

BCR-ABL is involved in the inhibition of apoptosis because it may block the release of cytochrome C from the mitochondria and thus the activation of caspases. This effect upstream of caspase activation might be mediated by the Bcl-2 family of proteins. BCR-ABL has been

shown to up-regulate Bcl-2 in a Ras- or a PI3 kinase-dependent manner. Moreover, BclxL is transcriptionally activated by STAT5 in BCR-ABL–positive cells. The survival signal provided by BCR-ABL is at least partially mediated by Bad and requires targeting of to the mitochondria, where Raf-1 phosphorylates Bad on serine residues. BCR-ABL, also, inhibits apoptosis by down-regulating interferon consensus sequence binding protein (ICSBP)[7].

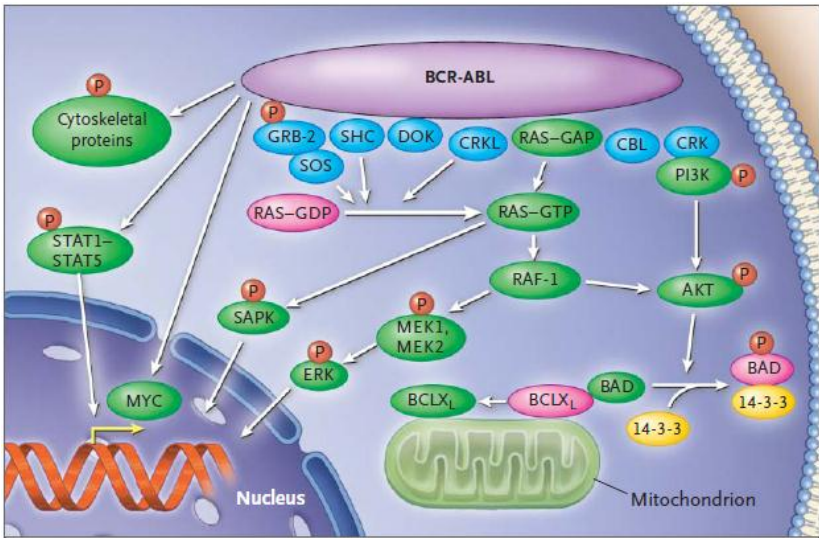

Figure 5: Signal-transduction pathways affected by BCR-ABL. The cellular effects of BCR-ABL are exerted through interactions with various proteins that transduce the oncogenic signals responsible for the activation or repression of gene transcription, of mitochondrial processing of apoptotic responses, of cytoskeletal organization, and of the degradation of inhibitory proteins[11].

1.3 Treatment of Ph$^+$ ALL

Ph$^+$ ALL is associated with poor prognosis. The outcome of ALL patients with the standard chemotherapy was improved with the incorporation of the TKI Imatinib Mesylate into frontline therapy. Unfortunately, in most cases these responses are rapidly lost despite persistent treatment[3]. Several highly potent second-generation BCR-ABL inhibitors have been developed to overcome Imatinib resistance and improve the prognosis of patients with CML and ALL. These include novel and more potent multi-TKIs such as Dasatinib, and potent selective BCR-ABL inhibitors such as Nilotinib[13], that typically induces a high rate of hematological and cytogenetic responses[3]. However, resistance or intolerance to these TKIs still leaves some patients without many treatment options. One point mutation in particular, the T315I mutation, has been shown to be resistant to first and second generation TKIs.

The third generation TKI, Ponatinib, may provide an option for these patients[16].

Most BCR-ABL inhibitors can be classified as either a type 1 or type 2 inhibitor compound, depending on their binding interactions with BCR-ABL. Type 1 inhibitors target the ATP-binding site of the enzyme in the active form, which is characterized by an open conformation of the activation loop, referred to as DFG "in" based on the position of the conserved triad aspartate-phenylalanine-glycine (DFG) at the beginning of the activation loop.

The common feature of the type I compounds is their ability to bind to the ATP site mimicking the adenine ring in its interactions with the "hinge" residues of the protein.

In contrast, type 2 inhibitors, or DFG-out compounds, are the inactive forms subsequently developed to bind to an additional hydrophobic site in addition to the same area as type 1 compounds. The ability to bind to an additional hydrophobic site provides type 2 inhibitors with the ability to be more selective and more potent than type 1 inhibitors. Examples of DFG-in inhibitors include Dasatinib, while DFG-out inhibitors include Imatinib, Nilotinib, and Ponatinib[17].

1.3.1 Tyrosine Kinase Inhibitors:

- Imatinib. Imatinib, a small-molecule TKI, was the first drug to be developed that was able to directly target BCR-ABL tyrosine kinase activity and to be tested in CML[13]. Imatinib mesylate (Glivec, previously known as STI571), a potent inhibitor of the tyrosine kinases ABL, ARG, platelet-derived growth factor receptor (PDGFR) and KIT, has been shown to selectively induce apoptosis of BCR-ABL cells and is remarkably successful in treating patients with CML and ALL Ph^{+}[8]. Imatinib resistance has been attributed to BCR-ABL dependent and independent mechanisms. BCR-ABL dependent mechanisms include amplification of the BCR-ABL gene and mutations within ABL that reactivate BCR-ABL and disrupt binding to the drug target. BCR-ABL point mutations are most common in the ATP-binding pocket (P-loop), the contact site (T315 and F317), the SH2 binding site (M351), and the A-loop. A common mutation that occurs frequently after Imatinib therapy in Ph^{+} ALL patients is the glutamic acid to lysine mutation at codon 255 (E255K).

Gatekeeper mutations (T315I and F317L) impede contact between Imatinib and BCR-ABL and, thus, contribute to Imatinib resistance and resistance to other second-generation TKIs. BCR-ABL-independent mechanisms include chromosomal abnormalities in addition to the Ph

chromosome abnormality, such as disruptions in drug uptake and efflux, and activation of alternative signaling pathways that cause proliferation or promote cell survival[18].

- Dasatinib. Dasatinib blocks BCR-ABL at low concentrations but is less selective than Imatinib. Similarly to Imatinib, it inhibits BCR-ABL, Kit, and PDGFR, but in contrast, it also blocks Src, Tec and Ephrin (Eph) kinases, as well as many other kinases[13].

- Nilotinib. Nilotinib inhibits kinase targets including BCR-ABL, PDGFR, and c-Kit, but not the Src family kinases[16]. Nilotinib blocks BCR-ABL at lower concentrations than does Imatinib, but, like Imatinib, it appears to be more selective than Dasatinib in targeting tyrosine kinases[13]. This highly specific BCR-ABL inhibitor is approximately 30-fold more potent than Imatinib and is active in vitro against 32 of 33 BCR-ABL mutations. BCR-ABL P-loop mutations (Y253F/H or E255K/V) are resistant to Nilotinib[18].

- Bosutinib. Bosutinib (SKI-606) is a small molecule, dual Src/ABL TKI, with potent preclinical BCR-ABL inhibitory activity in Imatinib-resistant CML cell lines. Unlike other second-generation TKIs, Bosutinib exhibits minimal inhibitory activity against c-KIT or PDGRF[19]. Bosutinib demonstrated preclinical activity against most Imatinib-resistant mutants of BCR-ABL, including Y253F, E255K and D276G, with the exception of T315I and V299L. It is able to bind to both inactive and intermediate conformations of BCR-ABL[3].

- Ponatinib. Ponatinib is a synthetic, multi-targeting TKI structurally designed as a pan-BCR-ABL inhibitor. This is reflected by the inhibition of in vitro tyrosine kinase activity of ABL and T315I mutant ABL with a half maximal inhibitory concentration (IC_{50}) of 0.4 nM and 2.0 nM, respectively. Ponatinib also inhibits the in vitro activity of additional oncogenic kinases, including members of the vascular endothelial growth factor receptor (VEGFR), PDGFR, fibroblast growth factor receptor (FGFR), Eph receptor, Src family kinases, c-Kit, RET, TIE2, and FLT3. The Ponatinib molecule is designed to interact with T315I in a different manner than the existing kinase inhibitors. Ponatinib was designed to avoid binding with the side chain of T315I in native BCR-ABL, and instead forms beneficial van der Waals interactions with the isoleucine side chain of the T315I mutant. This, coupled with its ability to accommodate the steric hindrance from mutations in the isoleucine variant, allows it to bind to and inhibit the ABL kinase activity of T315I point mutations[16].

1.4 Novel alterations in ALL

The ALL cells carry numerous genetic alterations. Translocation resulting in the creation of fusion genes are frequently found, like t(12;21) encoding the *ETV6-RUNX1* or fusions of MLL with various partner genes in childhood B-ALL; genomic deletions classically lead to the loss of tumor-suppressor gene like cyclin-dependent kinase inhibitor 2A gene, *CDKN2A*, encoding a cell cycle regulator. In B-cell precursor ALL, genome wide analyses have identified deletions and/or inactivating mutations of genes encoding regulators of B-cell development like *PAX5*, *EBF1* and *IKZF1*[1].

1.4.1 Ikaros

The *Ikaros* (IKZF1) gene encodes a family of zinc finger transcription factors that are important regulators of hematopoietic lymphoid development and differentiation (Fig.6). Ikaros is transcribed as a number of isoforms due to alternative splicing that essentially alter the expression of exons 3–5 coding for the N-terminal DNA binding domain. Long isoforms (Ik1 to Ik3) with at least three zinc fingers can bind efficiently to DNA, while shorter versions (Ik4 to Ik8) behave as dominant negative isoforms upon heterodimerization. These short isoforms are normally expressed at low levels compatible with their potential regulatory role on the activity of the predominant Ik1 and Ik2 isoforms. Due to the important consequences stemming from a diminution of Ikaros activity, it is conceivable that abnormalities in the splicing regulation of Ikaros transcripts could lead to significant pathological manifestations.

The role of Ikaros in lymphoid development is illustrated by the complete lack of lymphoid cells following gene inactivation by targeted deletion of exons 3–5, which generates a dominant negative Ikaros isoform.

While Ikaros is expressed throughout the ontogeny of lymphoid cells, a diminution of Ikaros activity in T lymphocytes, by means of over-expression of a dominant-negative isoform, causes hyperproliferation, loss of heterozygosity and malignant T cell development[20].

Genetic alteration of *IKZF1* is associated with a very poor outcome in B-cell–progenitor ALL. Deletions or mutations of *IKZF1* were identified as predictors of poor outcome which are associated with a high risk of relapse in ALL[21]. *IKZF1* deletion is the most frequent somatic copy number alteration in Ph^+ ALL. It is likely that Ikaros loss combines with BCR-ABL1 to induce lymphoblastic leukemia, arresting B-lymphoid maturation[22].

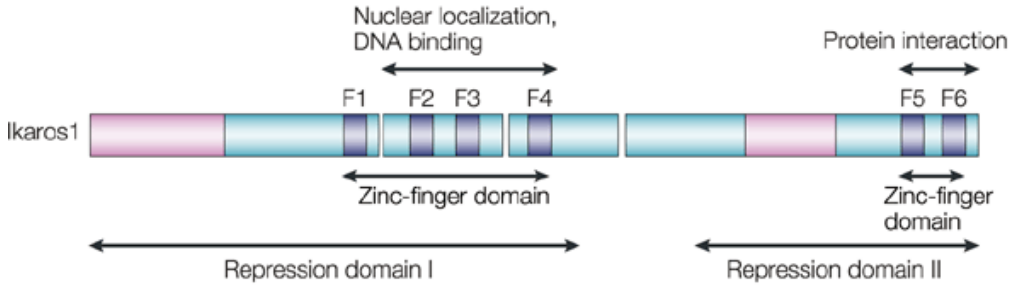

Figure 6: Schematic structure of Ikaros functional domains. The N-terminal zinc fingers are responsible for DNA binding. The C-terminal zinc fingers are essential for homo- and hetero-dimerization.

1.4.2 Pax5

PAX5 (paired-box domain 5) is the guardian of the B-cell identity. This transcription factor belongs to the family of paired-box domain transcription factors (Fig.7).
Its expression is initiated during early stages of B-cell differentiation beginning at the pro-B stage, and is turned off to allow terminal B-cell differentiation. PAX5 is thus involved both in the maintenance of B-cell identity and in the control of terminal B-cell differentiation[23].

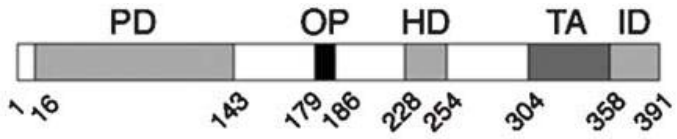

Figure 7: Schematic structure of PAX5 functional domains. The PAX5 structure consists of a DNA binding paired domain (PD), conserved octapeptide (OP), partial homeodomain (HD), transactivation domain (TA) and inhibitory domain (ID).

PAX5 is frequently mutated in adult B-cell progenitor ALL[24]. *PAX5* genomic deletions, ranging from a complete loss of chromosome 9 to the loss of a subset of exons, were identified in *BCR-ABL1* ALL patients[25]. B-ALL is also characterized by the participation of *PAX5* in specific chromosomal translocations that generate novel transcription factors by fusing the N-terminal DNA-binding domain of PAX5 with C-terminal regulatory sequences of a second transcription factor. Surprisingly, these *PAX5* translocations involving several different partner genes, as *PAX5-ETV6, PAX5-FOXP1, PAX5-EVI3* and *PAX5-ELN*, have so far been identified in B-ALL[26].

1.4.3 9p21 Locus

The chromosomal region of 9p is a frequent site of loss or deletion in several human cancers, such as glioma (60%), head and neck cancers (50%) and bladder cancers (45%)[27].
High frequencies of 9p21.3 deletions, ranging from 18% to 45%, have been documented also in ALL [28,29].

1.5 *CDKN2A* and *CDKN2B* genes

Two important tumor suppressor genes, *CDKN2A* and *CDKN2B* situated on 9p21 locus, encode three critical factors, $p14^{ARF}$, $p16^{INK4a}$ and $p15^{INK4b}$ (Fig.8), involved in cell cycle regulation and apoptosis[30]. The *INK4a/ARF* locus span approximately 30 kb of genomic DNA and comprise four exons. The Alternative Reading Frame, also called p14ARF, is derived from a distinct first exon (exon 1β), originating approximately 19 kb centromeric to the first exon of $p16^{INK4a}$ (exon 1α) and 23 kb centromeric to exon 2. Exon 1β, under the control of its own promoter, is spliced to the second and third exons that are separated by 3 kb of intronic sequence and are shared with $p16^{INK4a}$. The open reading frame of the 1.1 kb p14ARF transcript is terminated within exon 2, with exon 3 comprising an untranslated 3' exon[31]. *CDKN2b* has its own open reading frame and it encodes $p15^{INK4b}$.

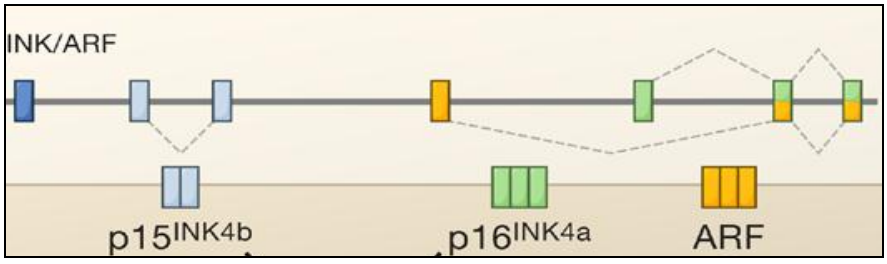

Figure 8: p16 and p14 proteins are encoded in alternative reading frames, are not isoforms and have no amino acid homology. Instead p16 and p15 are homologous inhibitors of the cyclin-dependent kinases, and are 85% similar at the amino acid level[32].

1.5.1 $p15^{INK4a}$ and $p16^{INK4b}$ proteins

The INK4 cell-cycle inhibitors, $p15^{INK4a}$, $p16^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ are homologous inhibitors of the cyclin-dependent kinases, CDK4 and CDK6, which promote proliferation[33]. The INK4 proteins bind to CDK4 and CDK6 and inhibit their kinase activity for Retinoblastoma (Rb) protein family, and perhaps other CDK targets (Fig.9).

As opposed to the CIP/KIP inhibitors of cyclin dependent kinases, INK4 binding induces an allosteric change that abrogates the binding of the cyclin dependent kinases with the D-type cyclins, which are required for catalytic activity, inhibiting CDK4/6-mediated phosphorylation of Rb family members. The expression of $p16^{INK4a}$ or other INK4 members, therefore, produces decreased CDK4/6 kinase activity and Rb hypophosphorylation, which in turn leads to E2F repression and growth arrest[32].

The Rb protein is one of the key regulators of various stages of cell development. It regulates transmission from extracellular signals and controls the response of a cell to these signals. In cycling cells, Rb regulates progression through and exit from the cell cycle, and also regulates permanent withdrawal from the cell cycle preceding differentiation[34].

Thus, expression of $p15^{INK4b}$ or $p16^{INK4a}$ maintains Rb-family proteins in a hypophosphorylated state, which promotes binding E2F to effect a G1 cell-cycle arrest.

The transition from the G1 to the S phases of the cell cycle marks an irreversible commitment to DNA synthesis and proliferation and is strictly regulated by positive and negative growth-regulatory signals. The G1-S transition is controlled by the Rb-E2F pathway, which links growth-regulatory pathways to a transcription program required for DNA synthesis, cell cycle progression, and cell division. This transcription program is activated by the E2F transcription factors and repressed by E2F-Rb complexes. In quiescent cells or cells in early G1, the Rb protein binds to the E2F transcription factors and blocks their transactivation domain. Mitogenic growth factors lead to the sequential activation of the CDK-Cyclin CDK4/6-CyclinD and CDK2-CyclinE, which hyperphosphorylate Rb and thereby cause the release of active E2F. The Rb pathway thus ensures that S phase entry strictly depends on growth-factor signals. The importance of Rb in the control of cell proliferation is underscored by the deregulation of this pathway in a majority of cancer cases, which occurs either by mutation or deletion of Rb, or by alterations in the upstream CDK, Cyclin, and CDK-inhibitory proteins[35].

1.5.2 ARF protein

ARF protein comprises 132 amino acids with a molecular weight of 13,902 Da[31].

It suppresses aberrant cell growth in response to oncogene activation by inducing the p53-pathway (Fig.9). ARF induction of p53 is mediated through two ubiquitin ligases, murine double minute 2 (MDM2), a RING finger oncoprotein, and ARF-binding protein 1/Mcl-1 ubiquitin ligase E3 (ARF-BP1/Mule), a HECT (homology to E6-AP C-terminus) containing protein. Both MDM2 and ARF-BP1 act as specific E3 ubiquitin ligases for p53, are highly expressed in various types of tumors, and have the potential to abrogate the tumor-suppressor functions of p53. ARF associates with MDM2 to inhibit the ubiquitination, nuclear export and subsequent degradation of p53. ARF physically sequesters MDM2 in nucleoli, thus relieving nucleoplasmic p53 from MDM2-mediated degradation[32].

Nucleolar relocalization of MDM2 is not required for p53 activation and that the redistribution of ARF into the nucleoplasm enhances its interaction with MDM2 and its p53-dependent growth suppressive activity[31].

In addition to MDM2, ARF-BP1 is a key regulator of the p53 cell cycle regulatory pathway; ARF-BP1 directly binds and ubiquitinates p53 in an MDM2-independent manner. Unexpectedly, ARF-BP1 also ubiquitinates and promotes the degradation of the anti-apoptotic bcl-2 family member, Mcl-1, and down-regulation of ARF-BP1 expression can also render cells more resistant to killing by genotoxic agents. Thus, ARF-BP1 has been assigned both anti-apoptotic (via p53 degradation) and pro-apoptotic (via Mcl-1 degradation) functions. Following aberrant oncogene activation, ARF expression is induced and inhibits ARF-BP1 activity toward p53 in the nucleus, thereby leading to p53-dependent cell cycle arrest or apoptosis. In the cytoplasm, where ARF is not abundant, oncogene activation may lead to ARF-BP1 mediated Mcl-1 degradation further promoting apoptosis[36].

ARF also enhances p53 function by promoting the phosphorylation and inhibiting the transcriptional activity of the RelA NF-kB subunit. The NF-kB family of transcription factors display anti-apoptotic activity and antagonize the p53 pathway through induction of MDM2 and repression of p53. Thus, by counteracting the functions of Rel A, ARF increases the effectiveness of the p53 pathway[37].

Several p53-independent functions of ARF have also been reported. ARF has been reported to interact with multiple proteins other than MDM2, including E2F-1, MDMX, HIF-1α, topoisomerase I, MYC, and nucleophosmin (NPM).

The best characterized interaction is that between ARF and NPM. NPM, also known as B23, is a protein that shuttles between the nucleolus and cytoplasm and is involved in several cellular processes including ribosome processing and centrosome duplication. The interaction of ARF with B23 is important to regulate ribosome biogenesis by retarding the processing of early 47S/45S and 32S rRNA precursors[32].

The ARF-NPM interaction modulates ARF protein stability. Additionally, it appears that this interaction sequesters ARF in the nucleolus thus preventing it from binding MDM2. Specific point mutations of the C-terminus of NPM are noted in 30% of cases of primary adult acute myelogenous leukemia (AML)[38].

These mutant forms of NPM appear constitutively localized to the cytoplasm but retain the ability to bind ARF. Therefore, these mutations are believed to compromise the ARF-p53 pathway both by decreasing ARF's protein stability and by mislocalizing ARF to the cytoplasm where it is unable to inactivate MDM2[39,33].

ARF can promote the conjugation of the small ubiquitin-like protein SUMO-1 to its binding partners, including MDM2 and B23[32].

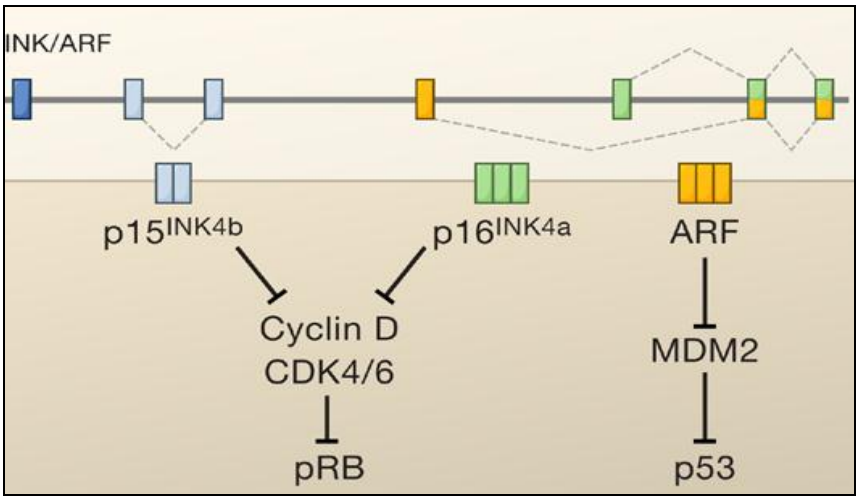

Figure 9: p15 ^{INK4b} and p16^{INK4a} inhibit CDK4/6 activity producing RB phosphorylation, which induces cell cycle arrest. p14^{ARF} inhibits MDM2-mediated degradation of p53.

1.5.3 *CDKN2A* and *CDKN2B* alteration in ALL

Inactivation of the tumor suppressor genes, *CDKN2A* and *CDKN2B*, can occur by deletion, methylation or mutation[28]. In the hematopoietic system, the INK4-ARF locus functions as a master regulator of Rb and p53 function to prevent inappropriate progenitor cell self-renewal and to eliminate incipient cancer cells driven by sustained oncogenic signaling[40].

Williams et al. induced BCR-ABL ALL in mouse models in which they demonstrated that *ARF* gene loss enhances oncogenicity, high aggressive lympholeukemia and limits imatinib response. In mouse bone marrow cells, transduced with retroviral vectors encoding p210 BCR-ABL and p185 BCR-ABL isoforms, occurred B cell lympholeukemias when transplanted into lethally irradiated mice. If the activity of the ARF tumor suppressor is compromised, these donor cells initiate a much more highly aggressive and rapidly fatal disease. When mouse bone marrow cells expressing BCR-ABL are placed in short-term cultures selectively designed to support the outgrowth of pre-B cells, only those lacking one or two *ARF* alleles can initiate lympholeukemias when inoculated into immunocompetent, syngeneic recipient mice. Although the ABL kinase inhibitor Imatinib mesylate provides highly effective treatment for BCR-ABL-positive CML, it has proven far less efficacious in the treatment of BCR-ABL-positive ALLs, many of which sustain deletions of the *INK4A-ARF*, *CDKN2A*, tumor suppressor locus. Mice receiving $ARF^{-/-}$ or $ARF^{+/-}$ p210 BCR-ABL-positive pre-B cells do not achieve remission when maintained on high doses of oral imatinib therapy and rapidly succumb to lympholeukemia[41].

In mice, the combination of BCR-ABL expression and ARF loss are sufficient to induce aggressive B-cell ALL. Constitutive BCR-ABL kinase activity triggers ARF expression and induces p53-dependent apoptosis, thereby countering BCR-ABL's proliferative effects. However, ARF inactivation cancels BCR-ABL-induced apoptosis and endows the cells with increased self-renewal capacity. Hence, ARF inactivation enables the BCR-ABL kinase to enforce unfettered B-cell proliferation, differentiation to the pre-B-cell stage, and guarantees acquisition of full leukemic potential[42].

Genomic deletion of *CDKN2A* is more prevalent alteration in childhood ALL than either hypermetilation or mutation[43].

The *CDKN2A* locus was deleted in ALL patient set of adolescents and young adults; in particular, homozygous deletions were found in 76% of the analyzed cases[29].

It's been reported that *CDKN2A/ARF* loss was detected in 29% of BCR-ABL1-positive ALL patients at diagnosis while *CDKN2B* loss was detected in 25% of cases; genomic deletions on 9p21 locus were predominantly monoallelic and in 57% of leukemia cases the minimal overlapping region of the lost area was considerably large, eliminating a large number of genes. At relapse, a strong trend in the detection rate of *CDKN2A/ARF* loss (47%) compared with diagnosis was found, suggesting that loss of this genomic region may be involved in disease progression. In this study, *CDKN2A*, *CDKN2B* and *ARF* mutations were found at very low levels. Moreover, frequent nucleotide variations, were identified in exon 2 and 3 of *CDKN2A:* rs3731249 G/A, rs11515 C/G and rs3088440 C/T. These SNPs have been phenotypically associated with other tumors. Deletions of *CDKN2A/B* were significantly associated by univariate analysis with higher white blood cell (P=0.0291) and with poor outcome in terms of overall survival (P=0.0206), disease free survival (DFS) (P=0.0010), and cumulative incidence of relapse (P=0.0014). The negative prognostic impact of CDKN2A/B deletion on DFS was thereafter also confirmed by a multivariate analysis (P=0.0051)[28].

Because the loss of *CDKN2A/B* eliminates the critical tumor surveillance mechanism and allows proliferation, cell growth and tumor formation by the action of MDM2 and CDK4/6, attractive drugs could be represented by the inhibitors of MDM2 and CDK4/CDK6.

1.6 *Tp53* gene

The *Tp53* gene encompasses 16-20 kb of DNA on the short arm of human chromosome 17. This gene is composed of eleven exons, the first of which is noncoding and localized 8-10 kb away from the exons 2-11. The *Tp53* gene has been conserved during evolution. In cross-species comparison, the p53 proteins show five highly (>90%) conserved regions among the amino acid residues 13-19, 117-142, 171-181, 234-258, and 270-286[44].

Mammalian genomes contain three members of the p53 family, p53, p63, and p73 genes located on chromosomes 17p13.1, 3q27-29, and 1p36.2-3, respectively (Fig.10). These genes encode proteins with similar domain structures and significant amino acid sequence homology in the transactivation, DNA-binding and oligomerization domains. However, only one member has been identified in invertebrates, suggesting that the mammalian *Tp53* gene family is derived from the triplication of one ancestral gene[45].

The human *Tp53* gene contains an internal promoter in intron-4, confirming that the *Tp53* gene has a dual gene structure like its homologous genes *Tp63* and *Tp73*. Moreover, intron-9

of the *Tp53* mRNA can be spliced in three different ways, leading to the expression of p53 proteins with different C-terminal domains. The intron-2 of human *Tp53* mRNA can also be alternatively spliced, leading to the expression of p53 proteins deleted of the first 40 amino acids. Altogether, the human *Tp53* gene can express nine different forms of the p53 protein containing different domains of the protein because of alternative splicing, alternative promoter usage, and alternative initiation of translation[46].

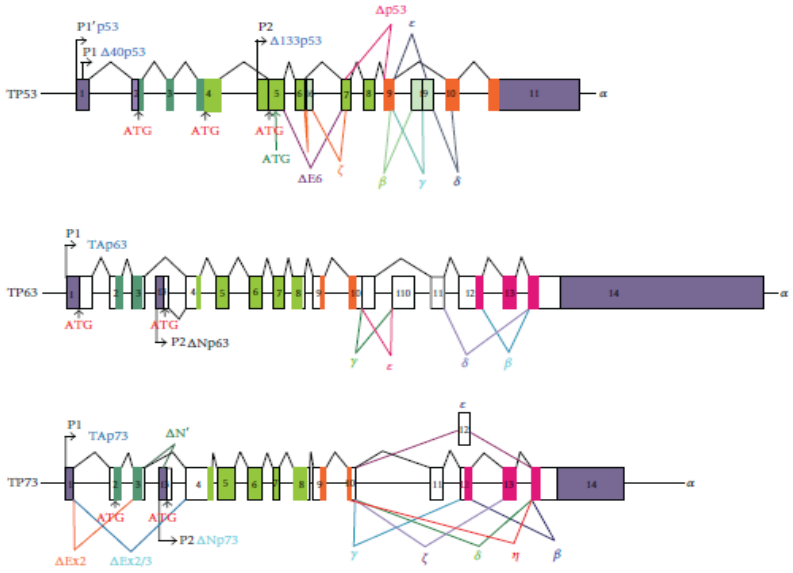

Figure 10: Schema of the *Tp53*, *Tp63* and *Tp73* genes of Homo sapiens. *TP53*, *TP63*, and *TP73* genes have two promoters (P1 and P2). The P1 promoters produce transactivation-competent full-length proteins (TA) while the P2 promoters produce TAD-deficient proteins (ΔN) with dominant-negative functions. p53 gene transcription is initiated from two distinct sites (P1 and P1').

1.6.1 p53 protein structure

The product of *Tp53* gene is a 393-amino acid nuclear phosphoprotein (about 53 kDa in molecular weight). p53 was first described in 1979 as a protein that binds to the simian virus (SV40) large T antigen[44,47]. The p53 protein is divided into regions highly conserved during evolution. The protein is composed of: an N-terminal transactivation domain (amino acid 1-42), a region rich in proline residues (amino acid 63-97) involved in the induction of apoptosis, a core sequence-specific DNA-binding domain (amino acid 102-292), containing most of the inactivating mutations found in different types of human cancers[46,48], a

tetramerization domain (amino acid 323-356), and a C-terminal region (amino acid 363-393) (Fig.11). This C-terminal region of p53 binds to the N-terminal domain of MDM2. In addition, there are also NES for exporting to the cytoplasm at the N- and C-terminal ends, as well as NLS at the C-terminal end, enabling the regulation of subcellular localization of p53[48,49].

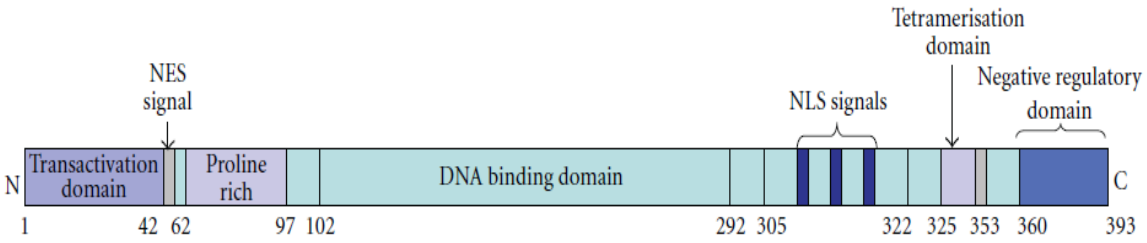

Figure 11: The p53 domain structure. The N-terminal region contains the transactivation domain (1–62) and a proline-rich region (63–97). The core domain (102–292) contains specific DNA binding sites. The C-terminal region includes the tetramerization domain (325–360) and a negative autoregulatory domain. NES signals exist on both N- and C-terminal, whereas NLS signals are located on C-terminal region.

1.6.2 p53 expression

The p53 protein was found in very low quantities in normal cells, due to its short half-life. In contrast, larger quantities of p53 (5-100-fold) could be detected in transformed cells in culture and in human tumors, due to increased half-life and an altered p53 conformational structure[50].

1.6.3 p53 protein regulation

p53 activity is controlled by two broad mechanisms. First, is a complex array of post-translational modifications, such as phosphorylation, sumoylation, or acetylation, which affect its ability to engage with relevant co-activators or negative regulators. This in turn determines p53 stability and abundance, and its ability to productively engage chromatin to regulate downstream target genes and micro-RNAs. Second, are mechanisms that control the stability and/or activity of its negative regulators, most significant of which are the related RING domain proteins, MDM2 and MDMX (also known as MDM4). The protein p53 can be also regulated by cytoplasmic or nuclear localization[51].

1.6.4 p53 function

p53 is a transcription factor that binds directly and specifically as a tetramer to target sequences of DNA through p53-responsive elements (p53REs) to transactivate several genes involved in p53 tumor suppressor activities such as p21 (cell-cycle arrest), Puma and Scotin (apoptosis)[44].

In response to a myriad of stress signals, the p53 protein is activated and thereafter depending on the tissue type and the extent of the cellular damage, triggers adequate cellular response including cell-cycle arrest, in G1 phase, and programmed cell death thus preventing the multiplication of damaged cells that could lead to cancer formation. Hence, p53 has been dubbed "the guardian of the genome"[52,53].

Multiple stimuli such as ionizing radiations, DNA damage, nitric oxide, hypoxia, chemotherapeutic agents, or oncogenic stimuli can activate p53. In response to various stimuli, p53 undergoes different changes and this activation could induce different effects. p53 is a transcription factor involved in the control of G1/S and G2/M phase transition, in DNA repair, and in induction of senescence, apoptosis, survival, autophagy, mitotic catastrophe[54] (Fig.12):

Cell Cycle Regulation. p53 regulates the control of the G1 checkpoint and can induce an arrest of the cell cycle, repair or apoptosis if DNA lesions are extensive[55]. Wild-type p53 protein can transcriptionally transactivate p21Cip1, a potent inhibitor of most cyclin-dependent kinases, involved in the cell cycle arrest[56].

p53 also stimulates the expression of the 14-3-3σ protein that sequesters the cyclin B1/CDK1 complex to block the transition G2/M. But p53 also induces the expression of many others genes such as GADD45, which interacts with PCNA to inhibit the passage to S phase, or Reprimo to block the cell cycle in G2 phase[54].

Cell Senescence. The p53 tumor suppressor seems to play a critical role in the induction and maintenance of cellular senescence. p53 activation is an essential step in the induction of senescence following DNA damage or other forms of stress. In the context of senescence, p53 is controlled by ATM/ATR and Chk1/Chk2 proteins which cause the posttranslational stabilization of p53 through its phosphorylation[54].

Apoptosis. p53 can transactivate the cell death receptors CD95 or TNF which induce the formation of the DISC complex and finally activate caspase 8. p53 also activates proapoptotic members of the Bcl2 family: Bax, Noxa, and Puma-involved in the permeabilization of the outer mitochondrial membrane. Moreover, p53 has a direct role in cell death initiation by localizing to mitochondria and regulating mitochondrial outer membrane permeabilisation directly. Thus, p53 protein can directly induce permeabilisation of the outer mitochondrial membrane by forming complexes with the protective BclXL and Bcl2 proteins, resulting in cytochrome C release[57].

Autophagy. p53 can activate but also inhibit autophagy. Under stress, p53 can activate its target gene in the nucleus, such as AMP-activated protein kinase (AMPK) β1 and β2, deathassociated protein kinase1 (DAPK-1), and damage-regulated autophagy modulator (DRAM) favoring lysosomal degradation of intracellular components and adaptation to stress and cell survival. Cytoplasmic, but not nuclear, p53 is able to repress autophagy[58].

Mitotic Catastrophe. In response to DNA damage, checkpoints are activated to delay cell cycle progression and to coordinate repair. The absence of p53 might increase mitotic catastrophe[58]. p53-deficient cells in an unchecked tetraploid G1 state reduplicate their DNA, leading to polyploidy and subsequent chromosomal instability. In the presence of wild-type p53, the polyploidy causes the activation of p21CIP1 and an irreversible arrest in cell cycle, or in cell death, thus preventing the propagation of aneuploidy[56].

Angiogenesis. The p53 protein has been shown to limit angiogenesis by few mechanisms: interfering with central regulators of hypoxia that mediate angiogenesis, inhibiting the production of proangiogenic factors, and directly increasing the production of endogenous angiogenesis inhibitors. The combination of these effects allows p53 to efficiently shut down the angiogenic potential of cancer cells. Wild-type p53 plays a role in limiting tumor vascularization. Mutant p53 plays a central role in promoting angiogenesis in colon cancer progression, and tumors carrying p53 mutations are more highly vascularised than tumors harboring wild-type p53[59]. The loss of *TP53* appears to amplify the Hypoxia Inducible Factor (HIF) pathway. HIF-1α has been shown to be physically associated with p53 in immuno-precipitation experiments. p53 promotes MDM2-mediated ubiquitination and degradation of HIF-1α, while loss of p53 leads to amplification of the HIF response[60].

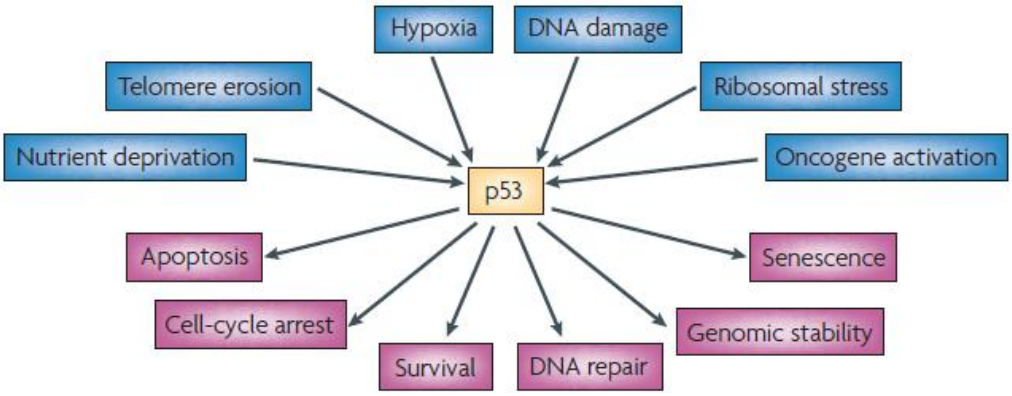

Figure 12: Activation and functions of p53. p53 has a key role in integrating the cellular responses (pink boxes) to different types of stress (blue boxes). Activation of p53 can result in a number of cellular responses, and different responses are induced by different stress signals. p53 can play a part in determining which response is induced through differential activation of target-gene expression. The role of p53 in tumor suppression, development and ageing is likely to depend on which cellular response is activated and on the context in which the activation occurs[52].

1.6.5 p53 inactivation in tumors

Disruption of the p53 response pathway strongly correlates with tumorigenesis. Because of its prominent role as a tumor suppressor, p53 is functionally impaired by mutation or deletion in nearly 50% of human cancers. Restoring p53 function to cancer cells with mutant p53 has been shown to induce tumor cell death. Mutational inactivation is the most common mechanism and occurs in a large spectrum of sporadic and familial cancers of the breast, gastrointestinal tract, lung, brain, and soft tissues[55].

The evolutionarily conserved regions are the most frequent sites of mutations occurring in many human tumors. More than 90% of the substitution mutations reported so far in malignant tumors are clustered between exon 5 and 8 and are localized in four evolutionarily conserved regions. Among these conserved regions, at least four mutation "hot spots", located at the amino-acid residues 175, 248, 273 and 282, have been identified in a variety of human neoplasms. Mutations in these "hot spot" codons account for approximately 30% of all p53 mutations. In most tumors, both *p53* alleles are inactivated, one through a point mutation, the

other through a deletion. In addition, most of these p53 mutations in human cancers are missense mutations, giving rise to an altered protein[50].

p53 mutations are much less frequent in leukemia than in other solid tumors. Although p53 mutations occur in only 10%–15% of AML at diagnosis, they are associated with the most aggressive disease courses and drug resistance. Patients with p53 mutations are generally resistant to chemotherapy and have relatively short survival[61,62].

Alterations of the *TP53* gene are of particular importance in the relapse stage of childhood ALL. *TP53* alterations might be related to an increased proliferative capacity of leukemic blasts reflected by the observed shifted distribution of cell cycle phases to an increased percentage of cells in S and G2-M phase compared with that in patients with wild-type *TP53*. This might be caused by loss of p53 wild-type function abrogating cell cycle arrest in G0/G1 phase and permitting passage through S phase. Therefore, *TP53* alteration is a promising novel molecular marker for high risk of treatment resistance and failure with particular importance in the relapse stage of childhood ALL[63].

1.7 MDM2

MDM2 was discovered on double minute chromosomes in a derivative cell line of NIH-3T3 cells[64]. MDM2 belongs to the family of E3 ubiquitin ligases that contain a RING (really interesting new gene) domain and serves as the major E3 ubiqitin ligase for p53 degradation[65].

1.7.1. *MDM2* gene

Human *MDM2* gene consists of 12 exons that can generate many different proteins. Transcription of the *MDM2* gene is controlled by two distinct promoters, referred to as P1 and P2 (Fig.13A). The P1 promoter controls basal expression of *MDM2*, and is situated upstream of the first exon of the *MDM2* gene. The P2 promoter is highly regulated, is responsible for inducible expression of *MDM2*, and is found in the first intron. As the start site for translation is contained in exon 2, the transcripts expressed from both the P1 and P2 promoters encode identical full-length MDM2 proteins (Fig.13B). The p53 response elements are located upstream of the P2 promoter, and hence the p53-inducible expression of MDM2 can be monitored by expression levels of this specific transcript. A number of other signaling

pathways lead to the interaction of specific transcription factors, including RXR, AP-1, the Ets family, Smad2 and Smad3, with sequences contained within the first intron of the *MDM2* gene, activating transcription via the P2 promoter in addition to p53. A cluster of GC boxes lie further upstream, and have been implicated in the differential expression of MDM2 due to an interesting single nucleotide polymorphism, SNP309. When this polymorphism is a guanine, it results in the ability of Sp1 to regulate MDM2 expression. In contrast, a thymine in this position abrogates this, with a concomitant reduction in MDM2 levels[66].

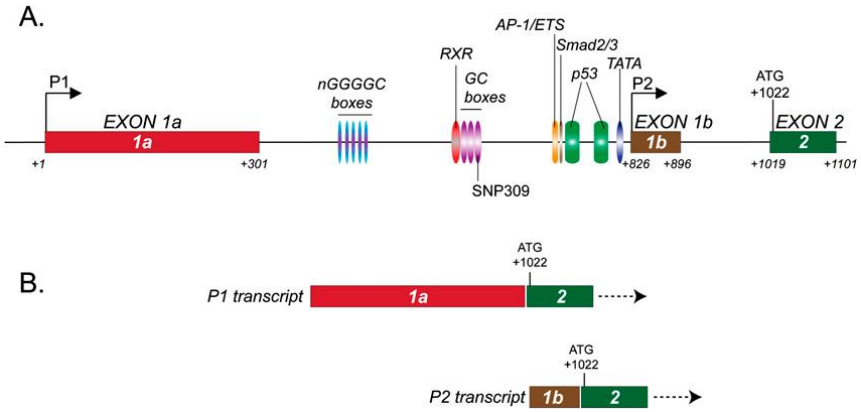

Figure 13: MDM2 is transcriptionally regulated by multiple pathways. **(A)** P1 and P2 promoters regulate MDM2 expression. P1 controls basal expression of Mdm2 and is upstream of exon 1a. P2 is further downstream and uses a start site at an alternate first exon, 1b. P2 is inducible, and is regulated by response elements for a variety of transcription factors. A cluster of nGGGGC boxes act downstream from the Erk pathway. Three GC boxes control expression of MDM2, with one of these containing the site of a SNP309 that influences the binding of the Sp-1. **(B)** The two transcripts from the P1 and P2 promoters have distinct 5'UTR and different lengths[66].

1.7.2 MDM2 protein

Human MDM2 is a 491 amino acid-long phosphoprotein (Fig.14) that interacts through its N-terminal domain with an α-helix present in the N-terminal transactivation domain of p53. MDM2 is a ubiquitously expressed protein and plays an important role in tissue development. The most evolutionarily conserved domain of MDM2 is the C-terminal RING-finger domain, which possesses ubiquitin ligase activity towards p53. The MDM2 protein contains NLS and NES signals within its structure, and as a result MDM2 constantly shuttles the nucleus and the cytoplasm. Importantly, MDM2 itself is the product of a p53-inducible gene.

Thus, the two molecules are linked to each other through an autoregulatory negative feedback loop aimed at maintaining low cellular p53 levels in the absence of stress[67].

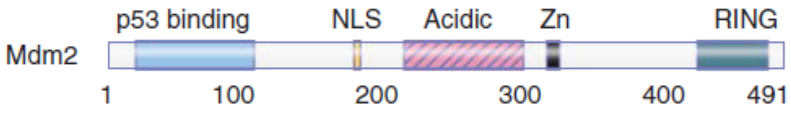

Figure 14: Domain structure of human MDM2. The N-terminal of MDM2 protein bind p53. MDM2 has a NLS. The central acidic region of MDM2 binds ribosomal proteins. The RING finger domain is required for Ubiquitin ligase MDM2 function[68].

1.7.3 MDM2 in tumors

Human MDM2 is amplified and overexpressed in several human tumors. MDM2 amplification has been identified in 19 tumor types with varying frequency, as well as in approximately one-third of human sarcomas, including those of soft tissue and bone.

In addition, MDM2 expression can be upregulated independently of gene amplification and oncogenic splice variants have been identified. Alternative splicing of MDM2 and the generation of short proteins also occurs in many human tumors[69]. Overexpression of MDM2 provides cells with a growth advantage, promotes tumorigenesis, and correlates with worse clinical prognosis and poor response to cancer therapy[70].

It has been shown that SNP309 correlates with the incidence of tumorigenesis in certain patient populations, thereby emphasizing the role of MDM2 as an oncogenic modifier in human cancer[71].

1.8 p53-MDM2 axis

The MDM2-p53 interaction maps to the 106 amino acid-long N-terminal domain of MDM2 and the N-terminus of the transactivation domain of p53 (residues 15–29). The direct interaction between p53 and MDM2 involves four key hydrophobic residues (Phe19, Leu22, Trp23 and Leu26) in a short amphipathic helix at the N-terminus of p53 and a small but deep hydrophobic pocket at the N-terminus of MDM2 (residues 25–109)[72].

Upon activation, p53 binds to the P2 promoter of the *MDM2* gene and transcriptionally induces MDM2 protein expression. In turn, MDM2 protein binds to p53 protein and inhibits it

through multiple mechanisms. One of the consequences of MDM2 binding is to inhibit p53-mediated transcriptional activity by blocking the p53-transcriptional coactivator interactions. This effect may be further enhanced by MDM2-mediated inhibition of the acetylation of p53 by factors such as p300 and an ability of MDM2 to function directly as a transcriptional repressor. Another function of MDM2 that efficiently abolishes all p53 activity is the ability of MDM2 to target p53 for degradation through the ubiquitin-dependent proteasome pathway and this is likely to play a key role in maintaining p53 at low levels in normal cells. Inhibition of MDM2 in response to stress leads to the rapid stabilization of the p53 protein and activation of the p53 response[49].

In the absence of stress signals, p53 is bound to its negative regulator MDM2 that ubiquitinates p53, targeting it for degradation by the proteasome (Fig.15A). MDM2 harbors a self- and p53-specific E3 ubiquitin ligase activity within its evolutionarily conserved C-terminal RING finger domain (Zinc-binding), and its RING finger is critical for its E3 ligase activity. MDM2 transfers monoubiquitin tags onto lysine residues mainly in the C-terminus of p53. Monoubiquitination is involved in receptor endocytosis, virus budding, transcription, DNA repair, and caspase recruitment in apoptosis, while polyubiquitination generally causes protein degradation. Because a chain of at least four ubiquitin molecules is believed to be necessary for efficient proteosomal degradation, other proteins must aid in polyubiquitination of p53; the p300/CREB binding protein (CBP) transcriptional coactivator proteins serving as scaffolding cooperates with MDM2 in polyubiquitination and degradation of p53[72].

Because MDM2 inhibits p53 activity, this forms a negative feedback loop that tightly regulates p53 function. In turn, decreased p53 activity results in decreased MDM2 to constitutive levels. MDM2 can also ubiquitinate itself and induce its own degradation.

Upon DNA damage, p53 is post-translationally modified to inhibit interactions with MDM2. Several kinases also phosphorylate MDM2 and modulate interactions with p53. This ability of p53 to regulate MDM2 provides a feedback loop with an important role in regulating cell cycle progression and apoptosis[73] (Fig.15B).

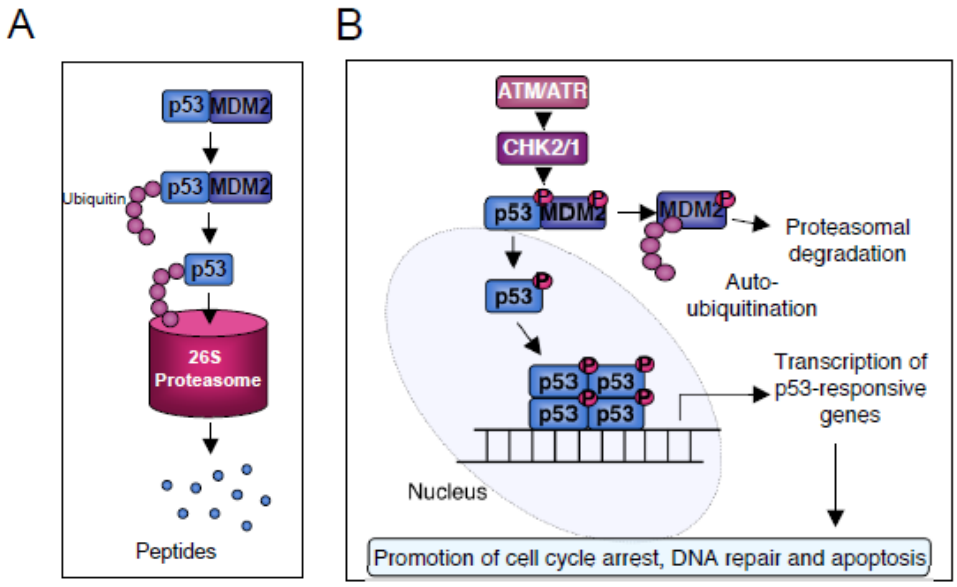

Figure 15: Schematic representation of the interactions between p53 and MDM2. **(A)** In the absence of stress signals, p53 is bound to its negative regulator MDM2. MDM2 ubiquitinates p53, targeting it for degradation by the 26 S proteasome. **(B)** Cellular stress signals, such as DNA-damage, lead to activation of ATM/ATR that mediates the phosphorylation of MDM2 and p53. Phosphorylated MDM2 undergoes auto-ubiquitination and degradation by the 26 S proteasome. Phosphorylated p53 undergoes nuclear localization, tetramerization, and binds to p53-responsive promoters to induce transcription of genes involved in the DNA-damage response[74].

1.9 Disruption of p53-MDM2 axis

Pharmacological inhibitors of MDM2 have been developed that may enhance the anti-cancer activities of p53 (Fig.16). The potential effectiveness of MDM2 inhibitors is supported by the preclinical findings that genetic restoration of p53 activity results in rapid and extensive tumor regression in experimental mouse cancer models[75,76].

One potential advantage of the MDM2 inhibitors is that unlike many current forms of chemotherapy, they activate p53 without first damaging DNA.

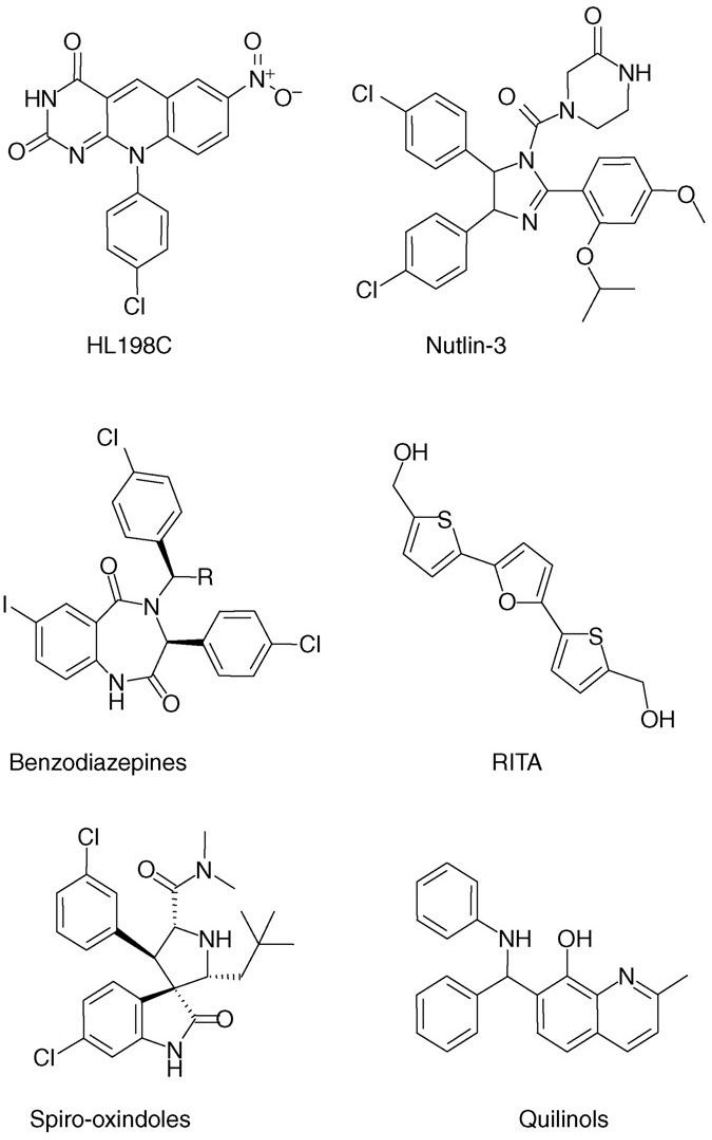

Figure 16: Chemical structure of small-molecule MDM2 inhibitors.

The first potent and selective small-molecule MDM2 antagonists, the Nutlins, were identified from a class of cis-imidazoline compounds. Nutlin-1 and Nutlin-2 are racemic mixtures and Nutlin-3a is an active enantiomer isolated from racemic Nutlin-3. These inhibitors could displace p53 from MDM2 in vitro with nanomolar potency (IC_{50}=90 nM for Nutlin-3a, the active enantiomer of Nutlin-3). Crystal-structure studies demonstrated that Nutlins bind to the p53 pocket of MDM2 in a way that remarkably mimics the molecular interactions of the crucial amino acid residues from p53 (Fig.17). Nutlins have been shown to enter multiple types of cultured cells and inhibit the p53–MDM2 interaction in the cellular context with a

high degree of specificity, leading to stabilization of p53 and activation of the p53 pathway. Proliferating cancer cells were effectively blocked in G1 and G2 phases, and underwent apoptosis when exposed to low micromolar concentrations of Nutlins[77].

The Nutlins activate the p53 pathway and suppress tumor growth in vitro and in vivo. They represent valuable new tools for studying the p53 pathway and its defects in cancer.

Nutlins induce p53-dependent apoptosis in human cancer cells but appear cytostatic to proliferating normal cells. MDM2 antagonists may have clinical utility in the treatment of tumors with wild-type p53[78].

Several studies have reported the use of MDM2 inhibitors to reactivate p53 pathway in cancer[79,80,81,82]. Thus it's interesting to evaluate the Nutlin-3 effects on ALL tumor.

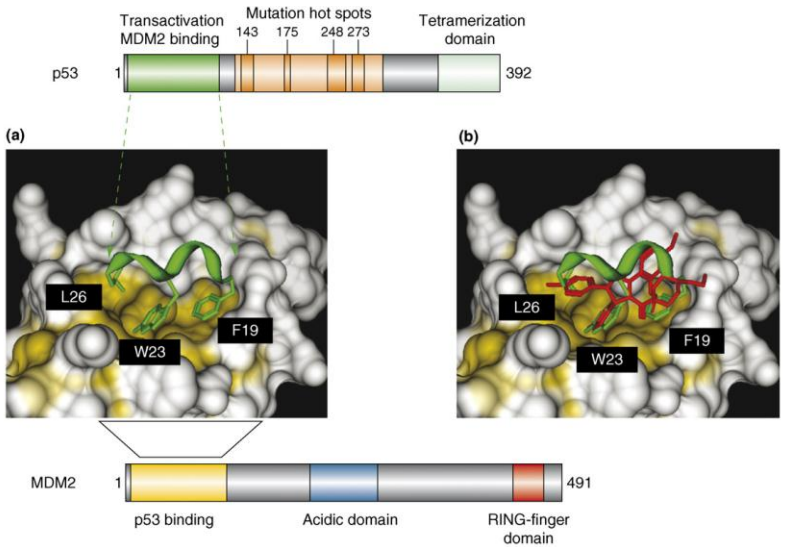

Figure 17: Structural aspects of the p53–MDM2 interaction and Nutlin binding. **(a)** MDM2 and p53 interact with each other with their N-terminal domains through a well defined p53 binding pocket. The crystal structure of the binding revealed that three AA residues of the p53 peptide (green) are essential for the binding with MDM2, and they are inserted into a fairly deep cavity on the MDM2 surface (yellow). The figure depicts the most important functional domains of p53 and MDM2 proteins. **(b)** Nutlin (red) binds to the p53 pocket of MDM2 by mimicking the interaction of the three crucial AA residues from the p53 peptide (green)[79].

2. AIMS

The BCR-ABL TKIs are highly effective for treatment of Ph^+ ALL, the most frequent and prognostically unfavorable subtype of ALL in adults. However, relapses with emerging TKI-resistance mutations in the BCR-ABL kinase domain pose a significant problem.

Here, we plan to investigate the efficacy and in vitro activity of a small-molecule antagonist of MDM2, Nutlin-3, that binds the p53-binding pocket of MDM2, thus inhibiting MDM2-mediated degradation of p53.

The rational of this study is based on the fact that theoretically hematological malignancies are attractive candidates for the MDM2 inhibitor-based therapy, because *TP53* gene is relatively infrequently mutated.

We performed a mutational screening of *TP53* gene in B-ALL adult patients by Sanger Sequencing.

Furthermore, previous studies using cell lines and primary samples from patients with various hematological malignancies (AML, ALL, chronic lymphocytic leukemia, multiple myeloma, and malignant lymphomas) have already shown that apoptosis is effectively induced by Nutlin-3. However, the effects of Nutlin-3 or other MDM2 inhibitors on BCR-ABL1-positive leukemic cells have not been reported so far.

The main aim of this study has been to investigate the biological activity of Nutlin-3 in Ph^+ and Ph^- ALL. To this purpose, we assessed:

- mutational screening of *TP53* gene in ALL cell lines and patients;
- the *in vitro* activity of Nutlin-3 on Ph^+ and Ph^- cells;
- the *in vitro* activity of Nutlin-3 on primary patient cells, resistant to previous TKIs treatment, with wild-type or mutated BCR-ABL;
- the *in vitro* activity of the combination of Nutlin-3 and TKIs (Imatinib and Nilotinib) on Ph^+ cells.
- the determinants of Nutlin-3 efficacy and the signal transduction pathways which are affected by exposure to Nutlin-3.

2. MATERIALS AND METHODS

PATIENTS

Primary blast cells from 6 ALL patients were obtained, upon written informed consent, from bone marrow and peripheral blood samples by density gradient centrifugation over Lymphoprep (Nycomed UK, Birmingham).

CELL LINES

Human Ph^+ (BV-173 and SUP-B15) and Ph^- (NALM-6, NALM-9 and REH) ALL cell lines, and Ph^+ CML cell line (K562) were used.

BV-173 cells derived from the peripheral blood of a 45-year-old man with CML in blast crisis in 1980, contain the t(9;22) leading to BCR-ABL1 e13-a2 (b2-a2) fusion gene. SUP-B15 derived from the bone marrow of a 9-year-old boy with ALL (B cell precursor ALL) in second relapse in 1984, described to carry the ALL-variant (m-bcr) of the BCR-ABL1 fusion gene (e1-a2). K562 cells were isolated by bone marrow of a 53-year-old female with CML in terminal blast crises. NALM-6 cells were established from the peripheral blood of a 19-year-old man with ALL in relapse in 1976. NALM-9 cells derived from the peripheral blood of a 26-year-old man with acute undifferentiated leukemia at diagnosis in 1988. REH were established from the peripheral blood of a 15-year-old North African girl with ALL at first relapse in 1973; carries t(12;21) leading to ETV6-RUNX1 (TEL-AML1) fusion gene.

Ph^+ cell lines, BV-173, SUP-B15 and K562, were maintained in RPMI 1640 medium supplemented with 20% fetal bovine serum, 1% penicillin streptomycin and 2 mM glutamine. Ph^- ALL cell lines, NALM-6, NALM-9 and REH, were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin streptomycin and 2 mM glutamine. Cells were maintained at 37°C in 5% CO_2.

METHODS:

- RNA extraction

Total RNA was extracted using the RNA Blood Mini Kit (Qiagen) from mononuclear cells isolated from peripheral blood or bone marrow aspirate samples. RNA was quantified using the Nanodrop Spectrophotometer and quality was assessed using the Nanodrop and by agarose gel electrophoresis.

- Sequencing

For the *TP53* mutation screening, one µg of total RNA was used to synthesize the cDNA using High-Capacity cDNA Archive Kit (Applied Biosystems). p53 cDNA was amplified by PCR, in three overlapping shorter fragments covering the entire coding cDNA sequence (GenBank accession number NM_000546.4) and the untranslated exon 1, and one longer amplicon, using the primers shown in Table 1, using FastStart Taq DNA Polymerase Kit (Roche). PCR products were purified using QIAquick PCR purification kit (Qiagen) and then directly sequenced using an ABI PRISM 3730 automated DNA sequencer (Applied Biosystems).

Primer ID	**Exon**	**Sequence 5'- 3'**	**Tm (°C)**	**Lenght (bp)**	**Amplicon size (bp)**
p53 **F1**	1	TGGATTGGCAGCCAGACT	60,36	18	
p53 **R1**	5	GGGGGTGTGGAATCAACC	61,01	18	**491**
p53 **F2**	5	TCAACAAGATGTTTTGCCAACT	59,65	22	
p53 **R2**	8	GCGGAGATTCTCTTCCTCTGT	59,97	21	**482**
p53 **F3**	8	GGTAATCTACTGGGACGGAACA	60,24	22	
p53 **R3**	11	CTATTGCAAGCAAGGGTTCAA	60,25	21	**498**
p53 **F1**	1	TGGATTGGCAGCCAGACT	60,36	18	
p53 **R3**	11	CTATTGCAAGCAAGGGTTCAA	60,35	21	**1,317**

Table 1: sequence, melting temperature (Tm) and lenght (bp) of forward (F) and reverse (R) primers used for amplification of p53 transcripts and p53 amplicon size (bp).

- Quantitative Real Time PCR (qRT-PCR)

One μg of total RNA was used to synthesize the first strand cDNA using Transcriptor First strand cDNA Synthesis Kit (Roche). BMI-1 expression was evaluated by qRT-PCR, performed on a Light Cycler 480 Roche with Taqman assay using 50 ng of cDNA for each sample. Thermal cycling conditions were as follows: denaturation of the cDNA at 95°C for 10 min for activation of polymerase enzyme, followed by 40 cycles of amplification of the target cDNA at 95°C for 10 sec, 60°C for 10 sec, 72°C for 15 sec. Each sample was analyzed in triplicate. Relative mRNA expression values were normalized using *GAPDH* as reference gene and calculated on the basis of the $E^{-\Delta\Delta Cp}$ method.

- MTS assay

Cell viability was assessed by using colorimetric Methanethiosulfonate (MTS) test (Promega). This assay is composed of solutions of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS] and an electron coupling reagent phenazine methosulfate (PMS). MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium. The absorbance of the formazan product at 490 nm was measured directly from 96-well assay plates. The conversion of MTS into the aqueous soluble formazan product is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture. Cells were cultured in 96-well plates at 50,000 cell/100 μl with different concentrations of drug, for different times and incubated at 37°C. Then, MTS (0.33 mg/ml) was added to each well and the cells were incubated for an additional 3 h. Following incubation, the optical density of the wells was read with microplate reader set a test wavelength of 490 nm. Cellular viability was calculated as a percentage of the viable cells compared to the untreated controls (DMSO 0.1%).

- Cell viability assay

In ex-vivo primary leukemia cells the effects on cell viability was assessed by counting viable and non-viable cell numbers by the Trypan blue dye exclusion method. Cells were seeded in 6-well plates at 500,000 cell/1 ml with increasing concentrations of drug for 24 hours and incubated at 37°C. Cellular viability was calculated as a percentage of the viable cells compared to the untreated controls (DMSO 0.1%).

- Flow cytometry

Flow cytometry was performed to analyze the apoptosis of cells after Nutlin-3a treatment.

Apoptosis

FITC Annexin V is a sensitive probe for identifying apoptotic cells, binding to negatively charged phospholipid surfaces with a higher affinity for phosphatidilserine than most other phosphoplipids. FITC Annexin V is used to quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis. For analysis of apoptosis, ALL cells were seeded in 6-well-plate at 500,000 cell/1 ml and treated with increasing concentrations of Nutlin-3a or vehicle control (DMSO 0.1%), for different times. After treatment, cells were washed in 1X Binding Buffer and stained with FITC-Annexin V/propidium iodide (PI). Apoptosis was determined using PI exclusion assay. PI is a fluorescent vital dye that stains DNA, detected in the orange range of the spectrum using a 562-588 nm band pass filter. Annexin V binds to cells early in apoptosis, and continues to be bound through cell death. PI is used in two-color Annexin V flow cytometric assays to distinguish cells that are in the earlier stages of apoptosis (Annexin V positive, PI negative) from those that are in the later stages of apoptosis or already dead (Annexin V positive, PI positive). Apoptosis was determined by flow cytometry.

- Microarray

Gene expression profiling was performed using Affymetrix GeneChip Human Gene 1.0 ST platform that ensures the coverage of 36,079 transcripts. Raw data were normalized by using the RMA algorithm and filtered. Genes differentially expressed were selected by analysis of

variance (ANOVA) (p-value threshold=0.05, Partek Genomics Suite). The most significantly involved process networks were defined by GeneGo software.

- Western blotting

Western blotting was performed using primary monoclonal antibodies directed toward MDM2 (Sigma Aldrich AB-166) at 1:1000 dilution; p53 (1C12) (Cell Signaling #2524) at 1:500 dilution; BMI1 (D20B7) (Cell Signaling #6964) at 1:1000 dilution; caspase 3 (Cell Signaling #9662) at 1:1000 dilution; caspase 7 (Cell Signaling #9492) at 1:1000 dilution; p21/WAF1/Cip1 (Millipore 05-345) at 1:500 dilution; actin (Sigma Aldrich A 2066) at 1:1000 dilution. ALL cell lines and ALL mononuclear cells were lysated in Lysis Buffer (50 mM TrisHCl pH 7.5, 150 nM NaCl, 1% Triton, 1mM EDTA, 0.1% SDS with protease and phosphatase inhibitor cocktail). The protein concentration of each sample was determined by Bradford assay (BioRad) and an equal amount of protein was transferred to Nitrocellulose membranes. The membranes were blocked with 5% milk in PBS at room temperature for 1 hour, followed by incubation with primary antibodies at 4°C over-night and secondary antibodies at room temperature for 1 hour. The membranes were washed three times for 15 minutes at room temperature with TPBS (PBS with 0,5% Tween-20) between the incubations, developed using the chemiluminescent detection reagent and scanned with chemiluminescence system.

3. RESULTS

MDM2 overexpression in leukemia

The web-based public database Oncomine (https://www.oncomine.org/) was queried for Mdm2 expression in the available leukemia datasets based on the comparison leukemia versus normal using a criterion of a 2 fold change and a p-value of 1E-4. Using these stringent criteria, we found that *Mdm2* transcript is highly overexpressed in B-ALL if compared to normal peripheral blood samples (Fig.18), providing the rationale for its inhibition using MDM2 inhibitors.

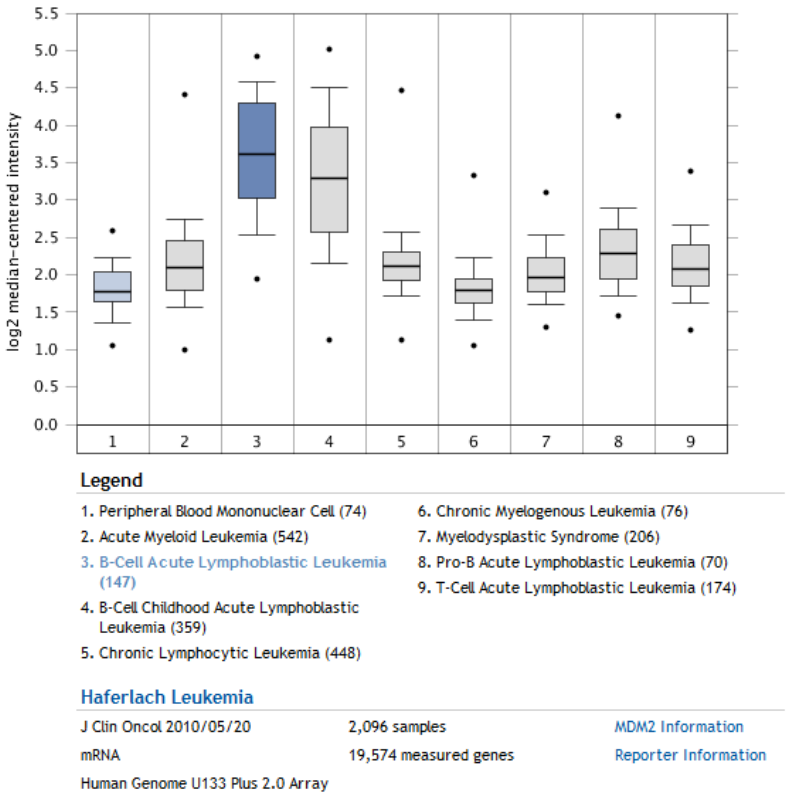

Figure 18: Oncomine expression analysis of Mdm2 levels between peripheral blood normal monunuclear cells and different subtypes of leukemia (including AML, B-ALL, T-ALL). Expression values are log transformed and median centered per array. Differential expression is identified by a permutation test and P values are calculated by t test and corrected for multiple comparisons by the method of false discovery rates. The group with the highest p-value is highlighted in blue. *Mdm2* transcript levels are extracted from the study of Haferlach et al[83]. Dot points indicate the minimum and the maximum values in each dataset.

CDKN2A copy number state in leukemia cell lines

The *CDKN2A* copy number state of *BCR-ABL1* positive and negative cell lines was verified on http://cancer.sanger.ac.uk/cancergenome/projects/cell_lines/ website.
Data available from the main Catalogue of somatic mutation in cancer (Cosmic) website report that all these leukemic cell lines harbor homozygous deletion of *CDKN2A* (Tab.2).

CELL LINE	GENE	POSITION	CNV	TYPE
BV-173	CDKN2A	9:21976871..22005395	0	Loss
SUP-B15	CDKN2A	9:21976871..22005395	0	Loss
NALM-6	CDKN2A	9:20300989..22140864	0	Loss
NALM-9	CDKN2A	9:20300989..22140864	0	Loss
REH	CDKN2A	9:20677556..23118474	0	Loss
K562	CDKN2A	9:20756547..26590984	0	Loss

Table 2: *CDKN2A* Copy Number Variant (CNV) in *BCR-ABL1*-positive and negative cell lines reported on http://cancer.sanger.ac.uk/cancergenome/projects/cell_lines/ web site. This table indicates the homozygous deletion of *CDKN2A* gene at 9:20756547..26590984 position.

P53 mutation screening in ALL cells

BCR-ABL1-positive (BV-173, SUP-B15 and K562) and negative (NALM-6, NALM-19 and REH) cell lines were investigated for *TP53* mutations by Sanger Sequencing.
BV-173, SUP-B15, NALM-6 and NALM-19 cells showed wild-type p53. On the contrary, a p53 mutation with a heterozygous substitution of C > T was identified in REH cells at position:17:7578389 (Fig.19). This mutation resulted in a substitution of arginine in cysteine (R181C), causing functional inactivation of p53 protein. Finally, K562 cells were p53-null.
Our results of p53 mutational screening are confirmed by p53 sequences reported in International Agency for Research on Cancer (IARC) database.

The IARC TP53 Mutation Database compiles all TP53 mutations that have been reported in the published literature since 1989. The following datasets are available: *TP53* somatic mutations in sporadic cancers; TP53 germline mutation in familial cancers; common *TP53* polymorphisms identified in human populations; functional and structural properties of p53 mutant proteins; *TP53* gene status in human cell-lines; mouse-models with engineered TP53; experimentally-induced mutations.

Moreover, wild type p53 sequences were observed in ALL patients.

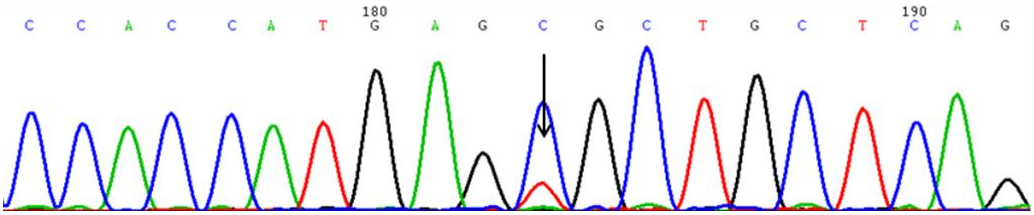

Figure 19: Electropherogram of *p53* mutation in REH cells identified by Sanger Sequencing. The arrow indicates heterozygous substitution of C > T, at position:17:7578389.

Inhibition of the p53-MDM2 interaction by MDM2 inhibitor induces growth arrest in Ph^+ and Ph^- leukemia cell lines with wild-type p53

In order to investigate the effects of Nutlin-3a treatment, firstly we examined cell viability of leukemic cells. Ph^+ and Ph^- cell lines were incubated with increasing concentrations (0.1-10 μM) of the active enantiomer (Fig.20A) of Nutlin-3 and with its inactive enantiomer (Fig.20B) for 24, 48 and 72 hours (hrs). The figure 20 represents an example of this experiment conducted in BV-173 cell line. Only the active enantiomer reduced BV-173 viability in dose and time-dependent manner. The figure 20 and 21 represents the example of Nutlin-3 treatment in Ph^+ and Ph^- ALL cells, BV-173 and NALM-6, respectively.

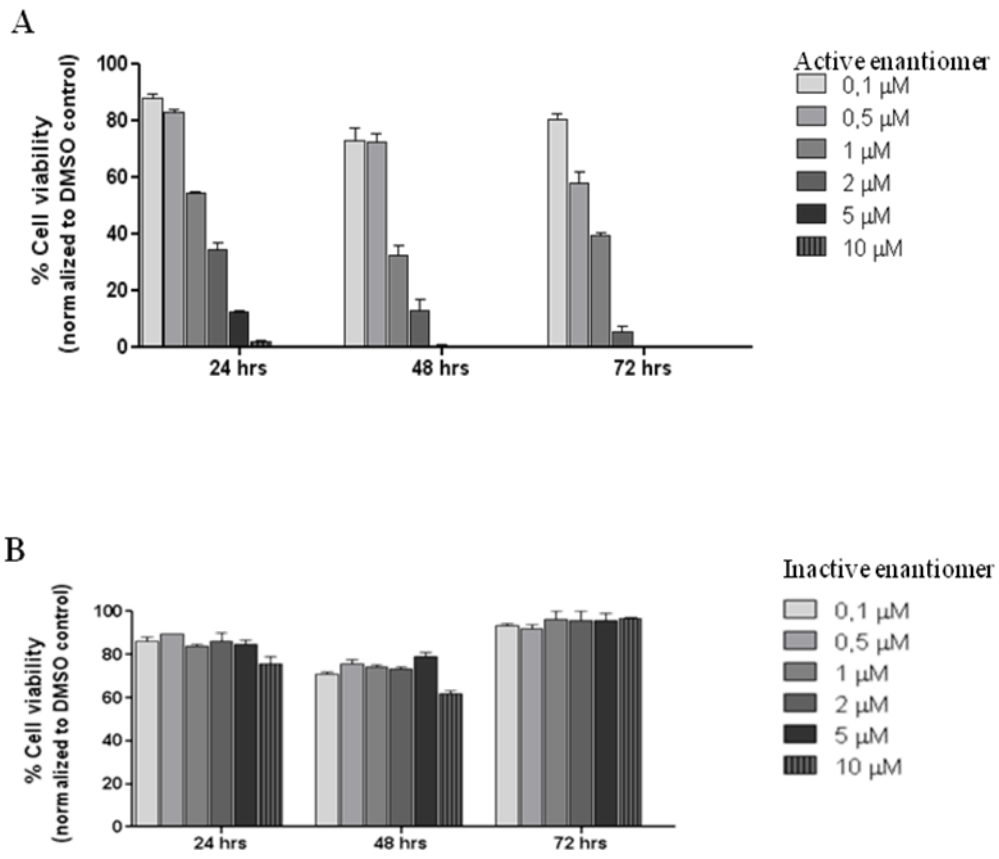

Figure 20: Cell viability in Ph^+ ALL cell line, BV-173, after incubation with active **(A)** or inactive **(B)** enantiomer of Nutlin-3 at increasing concentration (0.1 µM-10 µM) for 24 hrs, 48 hrs and 72 hrs.

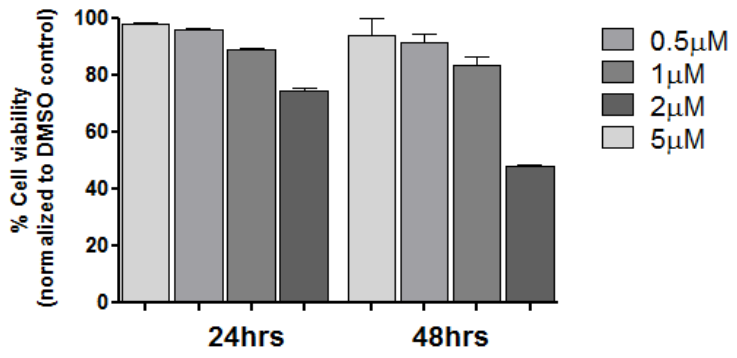

Figure 21: Cell viability of Ph^- ALL cell line, NALM-6, after Nutlin-3a treatment at increasing concentration (0.5 µM-5 µM) for 24 hrs and 48 hrs.

MDM2 inhibition resulted in a dose and time-dependent antiproliferative and cytotoxic activity with IC_{50} at 24 hrs ranging from 1.349 μM for BV-173 and 1.621 μM for SUP-B15 (Fig.22A), to 2.7 for NALM-6 and 3.7 μM for NALM-19 (Fig.22B). By contrast, no significant changes in cell viability were observed in K562 p53-null (Fig.22A) and REH p53-mutated cells (Fig.22B) after incubation with MDM2 inhibitor.

These results showed that Nutlin-3 efficiently inhibited growth of Ph^+ and Ph^- ALL cells, with wild type p53. REH and K562 cells, which have mutant p53, did not respond to the MDM2 inhibitor, confirming that the p53 pathway can be activated by Nutlin-3 only in cells with wild-type p53.

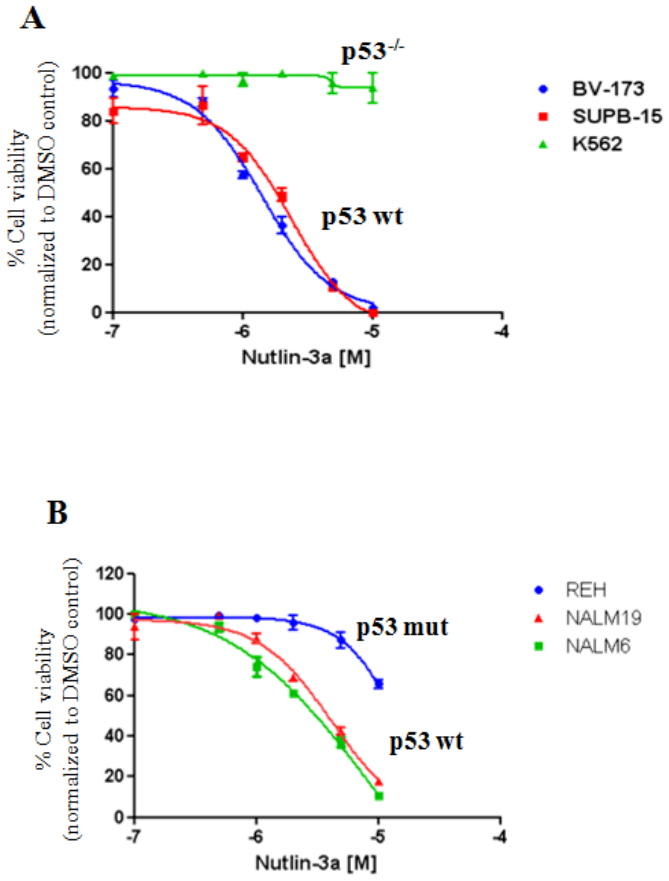

Figure 22: Cell viability of Ph^+ **(A)** and Ph^- **(B)** leukemia cell lines, with wild-type or mutated p53, after Nutlin-3a treatment. At 24 hrs, IC_{50} was 1.349 μM for BV-173 and 1.621 μM for SUP-B15 (A), 2.789 μM for NALM-6 and 3.781 μM for NALM-19 **(B)**. K562 p53-null **(A)** and REH **(B)**, p53-mutated cell lines did not respond to the Nutlin-3a.

Inhibition of the p53-MDM2 interaction by MDM2 inhibitor induces apoptosis in Ph^+ and Ph^- leukemia cell lines with wild-type p53 and in Ph^+ ALL patients

To understand the cell viability reduction as a consequence of Nutlin-3a treatment in ALL cells, we analyzed the apoptosis induction after MDM2 inhibitor treatment in Ph^+ and Ph^- ALL cell line and in primary Ph^+ cells. These experiments demonstrated that Nutlin-3a induced a time and dose-dependent apoptosis in ALL cells with wild type p53. The figures 23 and 24 show two examples of apoptosis induction in Ph^+ and Ph^- ALL cells, BV-173 and NALM-6, respectively. By contrast, ALL cells with mutation in p53, such as REH cells shown in figure 25, did not respond to Nutlin-3a and consequently, p53 pathway is not activated.

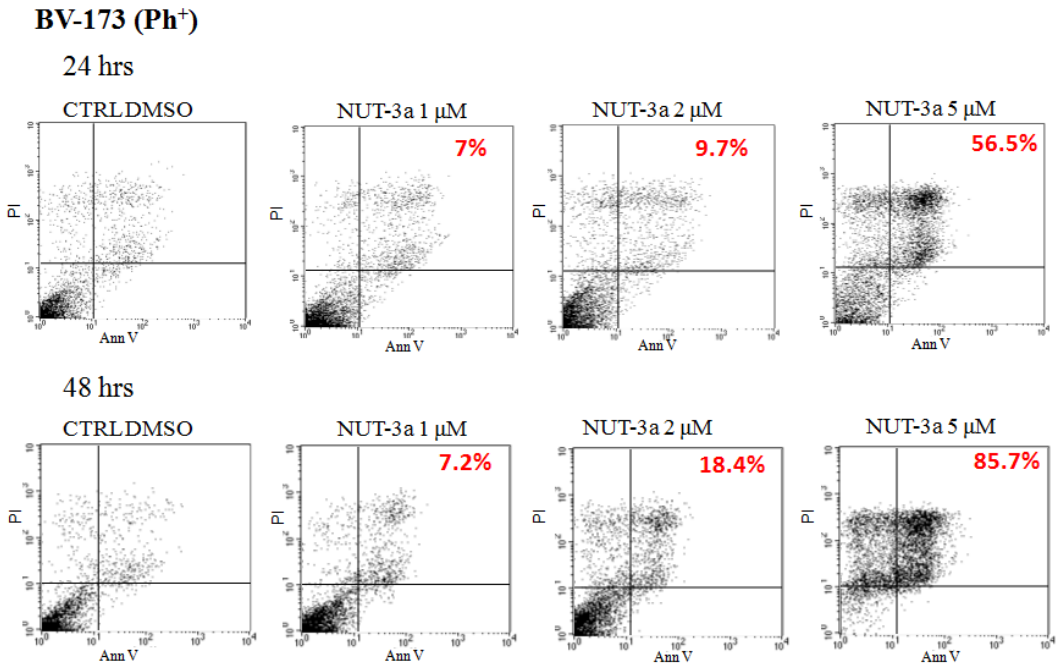

Figure 23: Induction of apoptosis in BV-173, Ph^+ cell line, after 24 hrs and 48 hrs of Nutlin-3a incubation at 1µM, 2 µM and 5 µM concentration, or DMSO control 0.1%. The percentage of cells undergoing apoptosis was detected using Annexin V (Ann V) and propidium iodide (PI) staining and normalized to DMSO control.

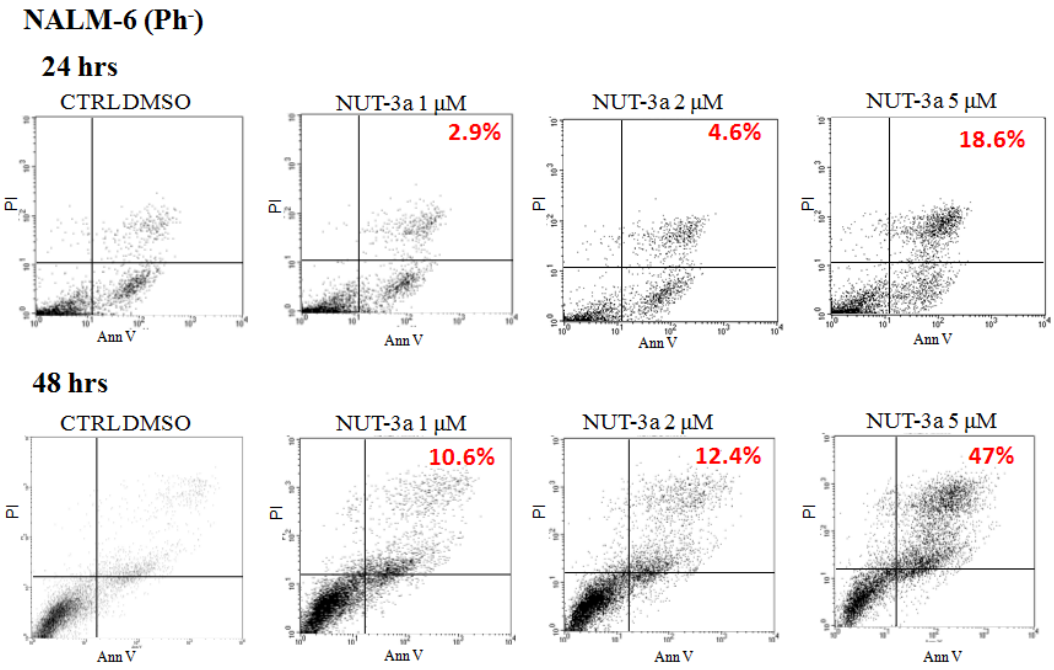

Figure 24: Induction of apoptosis in NALM-6, Ph- cell line, after 24 hrs and 48 hrs of Nutlin-3a incubation at 1µM, 2 µM and 5 µM concentration, or DMSO control 0.1%. The percentage of cells undergoing apoptosis was detected using Ann V and PI staining and normalized to DMSO control.

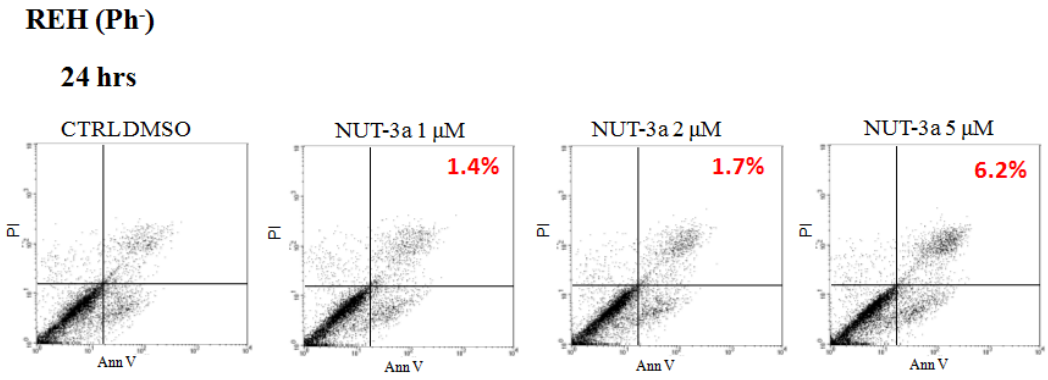

Figure 25: Induction of apoptosis in REH, Ph- cell line, after 24 hrs and 48 hrs of Nutlin-3a incubation at 1µM, 2 µM and 5 µM concentration, or DMSO control 0.1%. The percentage of cells undergoing apoptosis was detected using Ann V and PI staining and normalized to DMSO control.

Inhibition of the p53-MDM2 interaction by MDM2i induces growth arrest of primary Ph$^+$ ALL cells

Next, we analyzed the effect of Nutlin-3a on cell viability in ALL cells. Primary cells isolated by Ph$^+$ ALL (patients 2, 4, 5, 6) and Ph$^-$ ALL (patients 1 and 3) patients were incubated with increasing concentration of Nutlin-3a (1µM, 2µM e 5µM) and with DMSO vehicle (0.1%). (Fig.26) for 24 hrs.

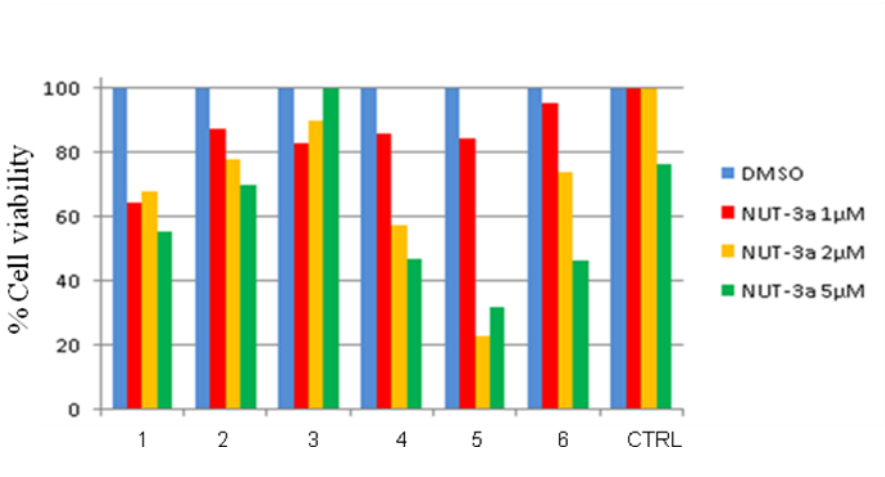

Figure 26: Cell viability in primary cells isolated by Ph$^+$ and Ph$^-$ ALL patients compared to DMSO 0.1% control, in response to increasing concentration (1µM, 2 µM and 5 µM) of 24 hrs of Nutlin-3a treatment. The control sample is represented by a patient in disease remission.

These results demonstrated that, generally, Nutlin-3a induces a reduction of cell viability in ALL patients compared to DMSO 0.1% control. Cell viability reduction is dose-dependent in almost all treated patients, except for patient 3 in which an increase of viability in concomitance with the incubation of increasing dose of the MDM2 inhibitor has been observed. All these patients showed p53 wild-type sequence.

Therefore, the different drug sensitivity may be attributed to other molecular causes that will be further investigated. The control sample is represented by peripheral blood mononuclear cells from a patient in disease remission. In this sample no viability reduction after MDM2 inhibitor treatment was observed, confirming the lack of induction of apoptosis Nutlin-3a-mediated in normal cells.

For some of these samples it has been possible to evaluate the efficacy of Nutlin-3a in modulation of protein expression. After treatment, the MDM2 expression was reduced; by contrast, p53 levels rised in p53 wild-type cells. Consequently, p53 induced the activation of its downstream pathway, like demonstrated by an increased expression of proapoptotic proteins and key regulators of cell cycle arrest, like p21 (Fig.27A), but not in resistant cells with mutation in p53 (Fig.27B).

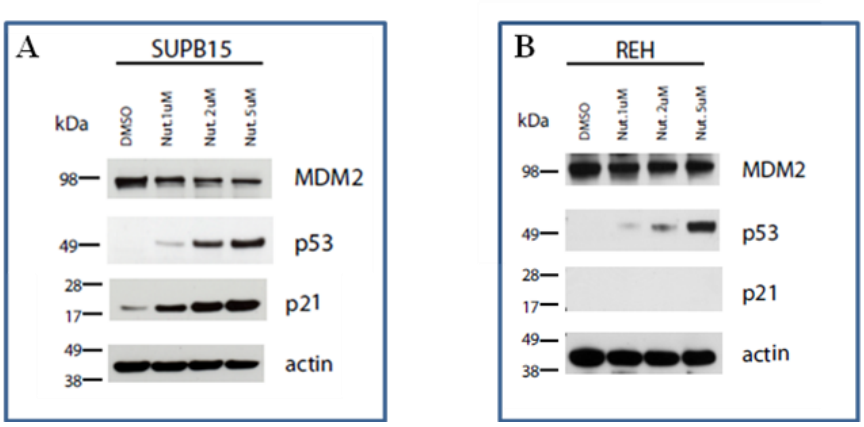

Figure 27: MDM2, p53 and p21 expression after 24 hrs of exposure to MDM2 inhibitor at 1 µM, 2 µM, and 5 µM concentration or to DMSO vehicle in sensitivity Ph^+ **(A)**, and in resistant Ph^- ALL cells **(B)**. Actin was used for normalization.

Combination of Nutlin-3 and TKIs induces dose-dependent reduction in cell viability in BV-173 Ph^+ ALL cell line

The combination effect of Nutlin-3a and TKIs, Imatinib and Nilotinib, was evaluated on BV-173 Ph^+ ALL cell line viability (Fig.28). Treatment with increasing concentration of Nutlin-3a alone (1 µM and 2 µM) (Fig.28A), reduced the BV-173 viability of 15% and 33%, respectively, while single treatment with Imatinib (IM) (at 1 µM concentration) or Nilotinib (AMN) (at 100 nM concentration) reduced the BV-173 viability of 59% and 43%, respectively. The results showed that combination of increasing concentration of Nutlin-3a (1 µM and 2 µM) and Imatinib (at 1 µM concentration) reduced the BV-173 viability of 65% and

66%, respectively; instead, Nutlin-3a (1 μM and 2 μM) and AMN (100 nM) reduced the BV-173 viability of 63% and 71%, respectively (Fig.28B).

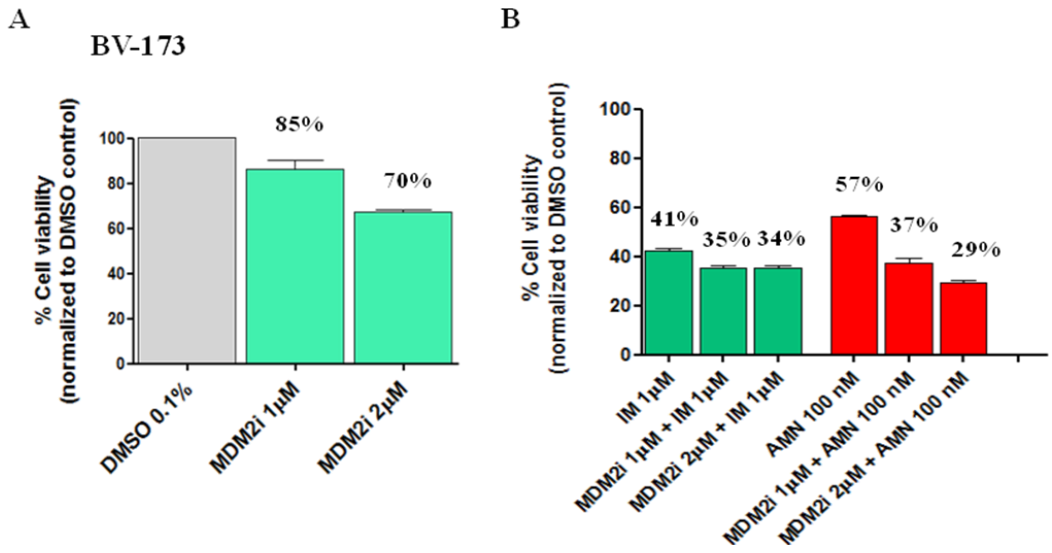

Figure 28: Cell viability in BV-173, Ph^+ ALL cell line, treated with Nutlin-3a **(A)** at 1 μM and 2 μM concentration, with Imatinib (IM) (1 μM), with Nilotinib (AMN) (100 nM), alone, or Nutlin-3a (1 μM and 2 μM) in combination with IM (1 μM), or with AMN (100 nM) **(B)**.

Nutlin-3a induces reduction in cell viability in primary blast cells from Ph^+ ALL patients with the T315I BCR-ABL kinase domain mutation

The dose-dependent reduction in cell viability was confirmed in primary blast cells from Ph^+ ALL patients with the T315I Bcr-Abl kinase domain mutation found to be insensitive to the available TKIs (Fig.29). Nutlin-3a treatment, at 1 μM and 5 μM concentrations, was performed on primary blast cells of two Ph^+ ALL patients. In particular, in one case Nutlin-3a reduced cell viability of 27% and 73% at 1 μM and 5 μM concentrations, respectively; in a second case Nutlin-3a reduced cell viability of 65% and 82% at 1 μM and 5 μM concentrations, respectively.

Thus, the results demonstrated that Nutlin-3a reduced cell viability in dose-dependent manner.

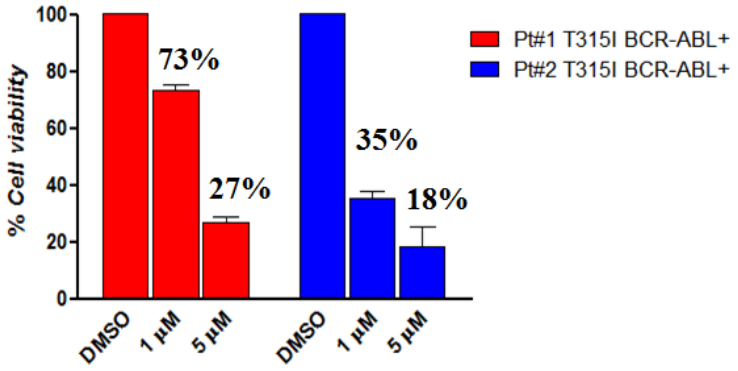

Figure 29: Cell viability of primary blast cells isolated by two Ph^+ ALL patients with T315I Bcr-Abl mutation after 24 hrs of MDM2 inhibitor treatment at 1 µM and 5 µM concentration. In one case Nutlin-3a reduced cell viability of 27% and 73% at 1 µM and 5 µM concentrations, respectively; in second case Nutlin-3a reduced cell viability of 65% and 82% at 1 µM and 5 µM concentrations, respectively.

Gene expression signature associated with response to MDM2 inhibitor

In order to better elucidate the implications of p53 activation and to identify biomarkers of clinical activity, microarray analysis was performed, comparing sensitive cell lines (BV-173 and SUPB-15) after 24 hrs of exposure to 2 µM Nutlin-3a and their untreated counterparts (DMSO 0.1%). A total of 621 genes (48% down-regulated versus 52% up-regulated) were differentially expressed ($p < 0.05$) (Fig.30). They include genes involved in cell cycle and apoptosis control (e.g. *Histone H1, TOP2, GAS41, H2AFZ*) and in the down-regulation of the Hedgehog signaling (e.g. *BMI1, BMP7, CDKN1C, POU3F1, CTNNB1, PTCH2*) with a strong repression of stemness genes and re-activation of *INK4/ARF* (Tab.3). Both *GAS41* (growth-arrest specific 1 gene) and *BMI1* (a polycomb ring-finger oncogene) are repressors of *INK4/ARF* and *p21* genes and their aberrant expression has found to contribute to stem cell state in tumor cells[84,85]. Additionally, experimental reduction of BMI1 protein levels have been demonstrated to result in apoptosis in tumor cells and increases susceptibility to cytotoxic agents and radiation therapy[86].

In our data, *BMI-1* and *GAS41* were strongly down-regulated (fold-change -1.35 and -1.11, respectively; p-value 0.02 and 0.03, respectively) after *in vitro* treatment as compared to control cells (Tab.3), suggesting that these genes have a potential as new biomarkers of activity.

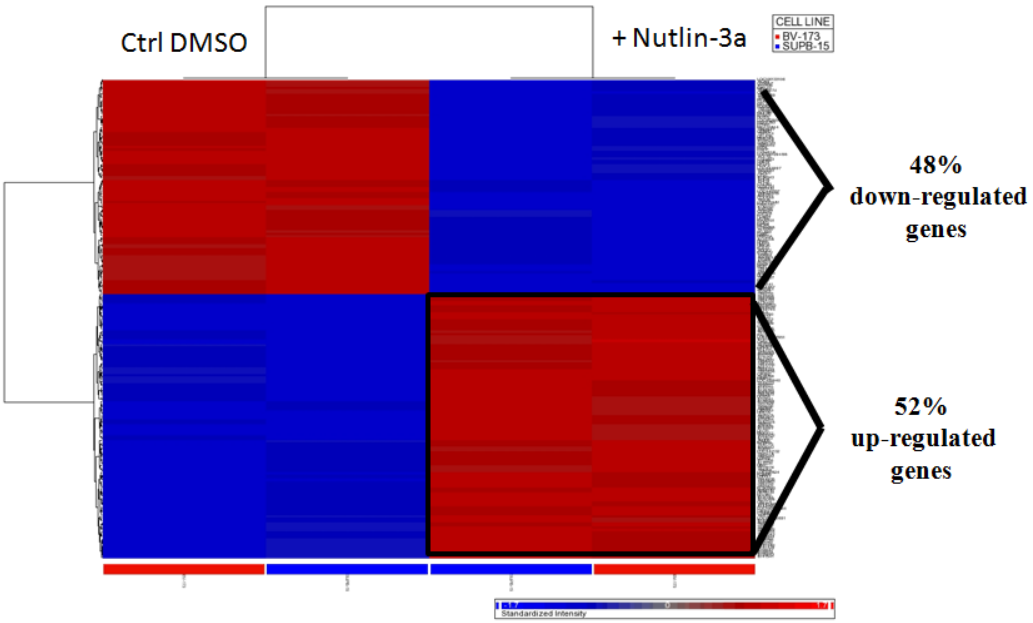

Figure 30: Heat-map showing differentially expressed genes between control (DMSO) and sensitive cells treated with 2 µM of Nutlin-3a for 24 hrs.

Gene Symbol	Object Type	Description	Signal	p-value
BMI1	Generic binding protein	Polycomb complex protein BMI-1	-1,1053	0,0337587
GAS41	Generic binding protein	YEATS domain-containing protein 4	-1,34902	0,0159932
H2AFZ	Generic binding protein	Histone H2A.Z	-1,10123	0,0098745
H1FX	Generic binding protein		-1,42939	0,0084268
SETD1A	Generic enzyme	Histone-lysine N-methyltransferase SETD1A	-1,12061	0,0112585
TBL1XR1	Generic binding protein	F-box-like/WD repeat-containing protein TBL1XR1	-1,08508	0,0311275
SUPT3H	Generic enzyme		1,11364	0,0493653

Table 3: genes involved in transcription and chromatin modification. Both *GAS41and BMI1* were down-regulated after *in vitro* treatment (fold-change -1.35 and -1.11, respectively; p-value 0.02 and 0.03, respectively). They are repressors of *INK4/ARF* and *p21* genes.

Nutlin-3a induces a down-regulation of *BMI-1* in ALL patients

To validate microarray data analysis and given the importance of BMI-1 in the control of apoptosis, we investigated its pattern in primary blast cells of 5 ALL patients.

ALL cells were treated with Nutlin-3a at 5 μM concentration for 24 hrs, and subsequently *BMI-1* expression was evaluated (Fig.31).

BMI-1 gene expression was down-regulated in 3 of 5 ALL patients after Nutlin-3a treatment, if compared with their untreated counterparts (DMSO 0.1%). This result confirmed a marked decrease of *BMI-1* in leukemia cells.

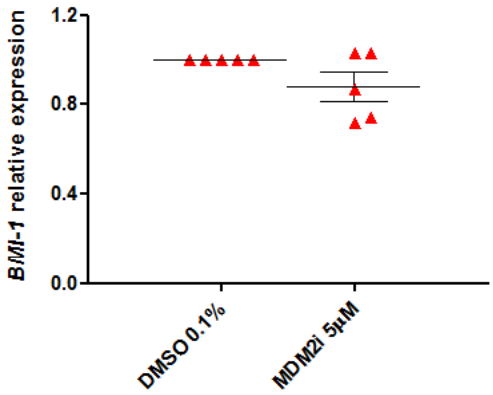

Figure 31: *BMI-1* relative expression in primary cells isolated by 5 ALL patients after 24 hrs of Nutlin-3a treatment at 5 μM concentration.

Nutlin-3a decreases BMI-1 protein levels in ALL with wild-type p53

BMI-1 protein expression decreased in ALL cell lines and primary blast cells with wild-type p53. The figure 32A represents an example of this experiment conducted in sensitive BV-173 cell line. Noteworthy, the BMI-1 levels remained constant in REH resistant cells (Fig.32B). BMI-1 expression is markedly reduced in sensitive cells and it may be used as a biomarker of response.

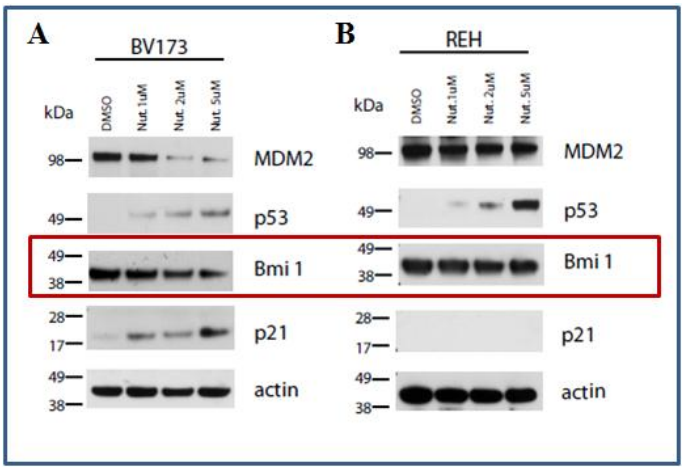

Figure 31: MDM2, p53, p21 and BMI-1 expression after 24 hrs of exposure to MDM2 inhibitor at 1 µM, 2 µM, and 5 µM concentration or to DMSO vehicle in sensitivity Ph^+ (A), and in resistant Ph^- ALL cells (B). Actin was used for normalization.

5. DISCUSSION

The *BCR-ABL* fusion gene, generated by a reciprocal t(9;22) chromosomal translocation causing the Philadelphia chromosome, is the molecular signature of CML and is also observed in 30–40% of ALL. The BCR-ABL fusion protein is a tyrosine kinase that is constitutively activated and confers survival and proliferation advantages to hematopoietic cells, thus contributing to leukemogenesis[3]. Imatinib and second TKIs specifically inhibit the BCR-ABL tyrosine kinase showing unprecedented efficacy for the treatment of these leukemias[11]. However, BCR-ABL transcripts remain detectable by qRT-PCR in the majority of responding patients and resistance may develop in significant portions of patients with CML in advanced stages or with Ph^+ ALL, mostly due to the emergence of mutations in the BCR-ABL kinase domain, including the most frequent E255K, T315I and F317L mutations, that may inhibit binding of TKIs to the kinase domain[18].

Novel treatment strategies able to overcome or synergize with current TKI-based therapies are required to improve cure rates. An attractive approach is represented by the small-molecule MDM2 antagonists, which target the p53-MDM2 interaction, leading to the activation of p53-mediated apoptosis in tumors with overexpressed MDM2, but wild-type p53[75,77].

The human p53 tumor suppressor protein, the product of the *TP53* gene, is one of the most important molecules in human cancers. It is known as the "guardian of the genome", which plays a central role in the regulation of cell cycle, apoptosis, DNA repair, senescence and angiogenesis in response to diverse stresses[54]. Deleted or mutated *TP53* has been shown in ~50% of human tumors. Although 50% of all human tumors express wild-type p53, many are thought to have inadequate p53 function due to abnormalities in p53 regulation or defective signaling in the p53 pathway[55].

One mechanism for suppressing p53 uses its negative regulator murine double minute 2 gene product, MDM2, which negatively modulates p53 by binding directly to and decreasing p53 stability[49]. Overexpression of MDM2 in animal models increases tumor formation. Somatic mutations in the MDM2 gene have been identified in adenocarcinoma of the lung, and MDM2 overexpression or amplification has been frequently observed in multiple malignancies[70]. In addition, a germline single nucleotide polymorphism (SNP309 T>G; rs2279744) in the promoter region of MDM2 has been identified. The presence of the G allele increases MDM2 gene expression and is associated with an earlier age of onset of human

cancers[71]. MDM2 is inhibited by the tumor suppressor $p14^{ARF}$, which can bind to and prevent MDM2-mediated ubiquitination of p53 and activate p53 responses[31].

ARF is an alternate reading frame protein expressed from the *INK4a* locus. Hyperproliferative signals lead to the increased expression of ARF, which inhibits MDM2 by blocking its E3 ubiquitin ligase activity, uncoupling the p53–MDM2 interaction and sequestering MDM2 in the nucleolus, thereby segregating it from nucleoplasmic p53[31,32].

In normal cells, tight feedback regulation of the ARF/MDM2/p53 axis controls p53 activity through a host of protein modifications (eg, phosphorylation, acetylation, sumoylation) as well as ubiquitination and deubiquitination and limits the effects of p53 activation[31,33].

Recently, *ARF* deletions have been described to frequently occur in patients with Ph^+ ALL and contribute to resistance to targeted therapy in ALL induced by BCR-ABL[28].

Because the interaction between MDM2 and p53 is a primary mechanism for inhibition of the p53 function in cancers retaining wild-type p53, targeting the MDM2-p53 interaction by small molecules to reactivate p53 has emerged as a promising new cancer therapeutic strategy.

Small-molecule inhibitors have been identified that specifically target the E3 ligase activity of MDM2. The first potent and selective small-molecule MDM2 antagonists, the Nutlins, were identified from a class of cis-imidazoline compounds. These inhibitors could displace p53 from MDM2 in vitro with nanomolar potency (IC_{50}=90 nM for Nutlin-3a, the active enantiomer of Nutlin-3). Crystal-structure studies demonstrated that Nutlins bind to the p53 pocket of MDM2 in a way that remarkably mimics the molecular interactions of the crucial amino acid residues from p53. Nutlins have been shown to enter multiple types of cultured cells and inhibit the p53–MDM2 interaction in the cellular context with a high degree of specificity, leading to stabilization of p53 and activation of the p53 pathway. Proliferating cancer cells were effectively blocked in G1 and G2 phases, and underwent apoptosis when exposed to low micromolar concentrations of Nutlins[77].

Based on these previous findings, in this study we aimed to investigate the efficacy of MDM2 inhibitors for ALL cells. We demonstrated that Nutlin-3a induces growth arrest and apoptosis in Ph^+ and Ph^- leukemia cells, with wild-type p53, in dose and time dependent manner. By contrast, no significant changes in cell viability and apoptosis were observed in p53-null and p53-mutated cell lines after incubation with MDM2 inhibitor, confirming that the p53 pathway can be activated by Nutlin-3 only in cells with wild-type p53.

We evaluated also the combination of Nutlin-3a and TKIs demonstrating that the combination between Nutlin-3a and Imatinib or Nilotinib, induced dose-dependent reduction in Ph^+ ALL cell viability, indicating a synergistic effect between these drugs.

Given the clinical importance of *BCR-ABL1* mutations, we tested the efficacy of Nutlin-3a in primary blast cells isolated by ALL patients with T315I mutation that confers resistance to available TKIs. We observed that Nutlin-3a induced cell viability reduction, supposing a possible alternative treatment in resistant patients to TKIs treatment.

In this study we demonstrated that treatment with Nutlin-3a in ALL cell lines and also in primary blast cells isolated by ALL patients induced the activation of apoptosis mechanism mediated by p53 pathway with consequent increase of proapoptotic proteins and key regulators of cell cycle arrest.

Gene expression profiling analysis, conducted in Nutlin-3a sensitive cells, permitted to better elucidate the implications of p53 activation and to identify possible biomarkers of clinical activity. In particular, we studied BMI-1, involved in control of apoptosis and in regulation of *CDKN2A* and *p21* genes[85]. BMI-1 expression is markedly reduced in sensitive cells and it may be used as a biomarker of response. Evaluation of its expression before and after treatment in clinical settings will better gain insight into its role.

Our findings provide a strong rational for further clinical investigation of Nutlin-3a in Ph^+ and Ph^-ALL.

In conclusion, small-molecule MDM2 antagonists, which target the p53-MDM2 interaction, leading to the activation of p53-mediated apoptosis, could be able to overcome or synergize with current TKIs-based therapies in Ph^+ ALL patients with wild-type p53. Furthermore, Nutlin-3 may provide a novel therapeutic tool for the therapy of Ph^- ALL patients with wild-type p53.

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