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The inhibition of Chk1/Chk2 and Wee-1 kinases as a promising therapy for the treatment of adult Acute Lymphoblastic Leukemia

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Introduction

Acute Lymphoblastic Leukemia

Acute Lymphoblastic Leukemia (ALL) represents a biologically and clinically heterogeneous group of hematological malignancies arising from genetic insults that block lymphoid differentiation and drive aberrant cell proliferation and survival. ALL is the commonest childhood type of cancer. Today different treatment regimens result in 5-year event-free survival rates that exceed 85% in children (aged 1–21); however, relapses are frequently associated with a poor outcome and for this reason ALL remains the leading cause of cancer-related death in children and young adults (aged 21–39)[1]. In adults ALL is less common but the treatment outcomes are significantly inferior to those in children. The reasons of this difference are not fully understood but, generally, are due to a reduced prevalence of genetic alterations associated with a favorable outcome in the adult cohort. According to the phenotype, ALL is traditionally classified into precursor T, precursor B, and B-cell (Burkitt), which are then further classified according to recurrent cytogenetic abnormalities. The development of microarray technologies to profile gene expression and structural genetic alterations in a genome-wide and high-resolution fashion have revolutionized our ability to identify genetic abnormalities providing important insights into the pathways deregulated in ALL. Moreover, recently the development of next-generation sequencing (NGS) technologies has provided researchers with completely new and effective tools for the discovery of novel alterations, depicting an exhaustive picture of the leukemia genome complexity. ALL is characterized by gross chromosomal alterations and in particular by alteration in the number of chromosomes (aneuploidy) or by chromosomal rearrangements that either result in the expression of fusion genes, or deregulation of genes by juxtaposition to strong enhancers[2]. In ALL aneuploidy is relatively frequent, reaching the 20-25% of pediatric cases (hypodiploidy 5% and hyperdiploidy 20%) and 10% in adult patients (hypodiploidy 2% and hyperdiploidy 8%). In both adult and pediatric patients hyperdiploidy (>50 chromosomes) is generally associated with good prognosis and usually isolated to specific chromosomes (4, 6, 10, 14, 17, 18, 21 and X). However hypodiploidy (<44 chromosomes) in ALL is generally associated with poor outcome and with a significant tendency of progressively worse outcome with decreasing chromosome

number [1]. Chromosomal rearrangements creating chimeric fusion genes are common events in both children and adult ALL. These chromosomal rearrangements involve crucial elements of the hematopoietic lineage like transcription factors, epigenetic modifiers, cytokine receptors, and tyrosine kinases. Common rearrangements in B-lineage ALL are the t(12;21)(p13;q22) encoding *ETV6-RUNX1* (*TEL-AML1*), t(9;22)(q34;q11.2) resulting in formation of the "Philadelphia" chromosome encoding BCR-ABL1*,* t(1;19)(q23;p13) encoding *TCF3-PBX1* (*E2A-PBX1*), rearrangements of *MLL* (*KMT2A*) at 11q23 to several partners, and rearrangement of the cytokine receptor gene *CRLF2* at the pseudoautosomal region 1 (PAR1) at Xp22.3/Yp11.3. T-ALL is characterized by activating mutations of *NOTCH1* and rearrangements of different transcription factors like *TLX1* (*HOX11*), *TLX3* (*HOX11L2*), *LYL1*, *TAL1*, and *MLL*[3]. Different somatic genetic alterations in addition to gross chromosomal alterations are relative frequent in childhood and adult ALL [4–6]. The most frequently mutated genes, particularly in B-ALL, are genes encoding for transcriptional regulators of lymphoid development. These include *PAX5, IKZF1*, and *EBF1* that encode DNA binding transcription factors required for lymphoid development. Among them the most frequent are somatic deletions or sequence mutations of *PAX5* and *IKZF1* genes, while less common are translocations of *PAX5* and deletions of *EBF1*[5]. These somatic mutations results in loss of function or expression of dominant-negative alleles and impaired lymphoid maturation that contributes to leukemogenesis. Several other pathways are frequently altered in ALL and include tumor suppression and cell-cycle regulation (*TP53*, *RB1*, and *CDKN2A/ CDKN2A*); cytokine receptor, tyrosine kinase and Ras signaling (*ABL1, ABL2, CRLF2, CSF1R, EPOR, FLT3, IL2RB, IL7R, JAK1/2/3, NTRK3,* and *PDGFR*B) and epigenetic modification (*EZH2, CREBBP, SETD2, MLL2* [*KMT2D*], and *NSD2* [*WHSC1*])[7](Figure 1).

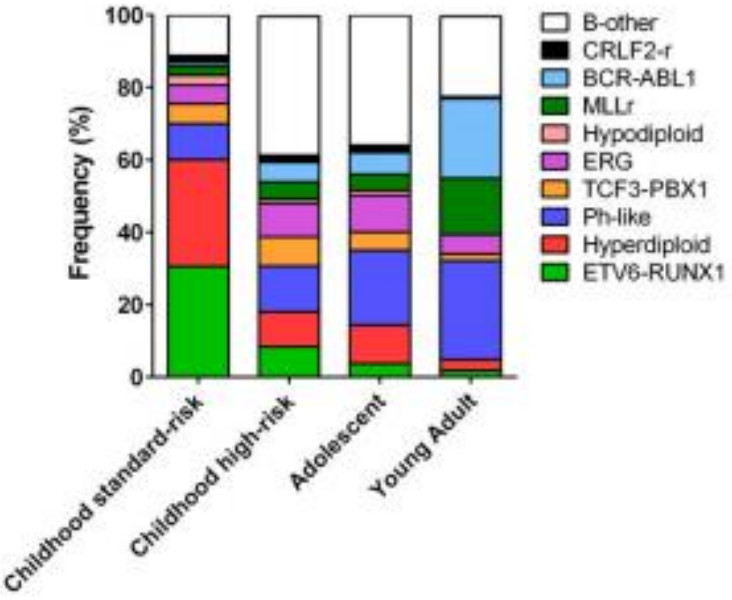

Figure 1: Prevalence Chromosomal rearrangements and frequently mutated pathways in ALL across age groups. The prevalence of the different chromosomal rearrangements and the frequency of somatic mutations deeply vary among the age groups. High hyperdiploidy and ETV6-RUNX1 are each present in 25% to 30% of childhood ALL cases but occur in <3% of young adults (age 21-39 years). The BCR-ABL1–positive ALL comprises 2% to 5% of childhood vs one-quarter of adult ALL [3].

Overview of standard treatments in adult ALL patients

Progress in the treatment of ALL in children and, with fewer efficacies in adults, has been remarkable, from a disease being lethal for decades to current cure rates exceeding 90% in childhood. More specific diagnostic procedures, like immunephenotypes, cytogenetics, molecular genetics and large scale genomics analyses allowed the definition of new ALL sub-entities which, in some cases, have been translated into novel specific therapies [8]. Despite the above mentioned improvements in the treatment of ALL, several leukemia subtypes continue to have a very poor prognosis. In the treatment of adult ALL patients, both national and international therapies are chemotherapy-based, and are usually structured in induction-consolidation-maintenance [9,10]. The goal of these therapies, and in particular of those related to the induction phase, is the achievement of the complete (hematologic) remission (CR), leukemic cells not detectable by light microscopy (less the 5% in the bone marrow), or the achievement of the molecular complete remission, minimal residual disease (MRD) negativity. These two clinical parameters are usually evaluated within 6-16 weeks since the beginning of the chemotherapy [8]. Based on pediatric-inspired therapies, the overall survival of standard risk adult ALL patients has improved from 38% to 50-70% with chemotherapy alone, and the outcome for high-risk ALL patients from 20-30% to more than 50%. The actual induction regimes are modification of the Berlin-Frankfurt-Munster (BFM) regimen for pediatric patients or of the hyper-fractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone (hyper-CVAD) regimen. The first regimen is an 8-week induction consisting of vincristine, prednisone, daunorubicin, cyclophosphamide, 6-mercaptopurine, L-asparaginase, intrathecal methotrexate, and CNS irradiation[11]. The hyper-CVAD regimen is based on two alternating chemotherapy cycles, one of hyper-fractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone (A), and one of high-dose methotrexate (MTX) and cytarabine [12]. The goal of consolidation chemotherapy is to eliminate any remaining, subclinical disease at the level of the bone marrow. The agents used for the consolidation phase are the same of the induction phase. There is a high heterogeneity in term of treatments, but mainly all the protocols include high doses of MTX and L-asparaginase. The last step of all regimes is the

maintenance chemotherapy whose goal is to maintain the remission of the disease and prevent the relapses. The common regimens are based on daily chemotherapy of 6-Mercaptopurine and weakly doses of MTX or vincristine[10]. A side of the conventional chemotherapy the actual treatments for ALL patients are based on targeted therapies. These therapies specifically target tumor cells that harboring particular genetic or genomic alterations. The introduction in the treatment of Philadelphia-positive ALL patients of the tyrosine kinase inhibitors (TKIs), imatinib, against the oncoprotein BCR-ABL1 have increased the RC rate from 60-70% in the era pre-imatinib, to the actual rate of 80-90 %[13]. During the past years novel TKIs, second-(dasatinib, nilotinib) and third-generation (ponatinib), have been developed to obtain faster and deeper molecular remission and to target mutations that frequently occurs during TKIs treatments and that are responsible for treatment failure. The targeted therapy is not limited to the use of TKIs but includes also immunologically based treatments using monoclonal antibodies or activated T cells. B-ALL cells specifically express different superficial antigens (CD19, CD20, CD22, CD33 and CD57) that can be routinely used in cytofluorimetry to define and to confirm a specific diagnosis. Due to the abnormal expression on leukemic blasts, these antigens can be used as targets for specific therapies. Anti-cancer monoclonal antibodies (mAbs) like the anti-CD20, rituximab, can mediate anti-tumor effects by different of mechanisms including signaling against proliferation, direct induction of apoptosis, complement dependent cytotoxicity (CMC) and antibody dependent cellular cytotoxicity (ADCC). Other mAbs, called antibody-drug conjugate (ADC), can deliver cytotoxic compounds on tumor cells that specifically express an antigen on their cell surface The inotuzumab ozogamicin, anti-CD22 linked to agents calicheamicin, is an example of this class ADC[14]. Antibodies have recently been used not only to selectively kill tumor cells but also to stimulate the response of the immune system against leukemic cells. In particular novel bi-specific antibody, called BiTE (Bi-specific T cell engagers)[15] like the CD19-CD3 blinatumomab, have been developed to enhance the interactions between CD8+ lymphocytes /NK cells with CD19+ leukemic cells[16–18]. Despite the great response rates with BiTE or ADC strategies responses appear short-lived and most patients relapse. Novel therapeutic strategies using activated T cells showed a more durable

response[19,20]and today, CD-19-directed T cells, modified with a chimeric antigen receptor (CAR), are one of the most promising technologies for CD-19 expressing B-ALL patients[21].

DNA Damage Response (DDR)

Despite all the above mentioned therapies, tumor cells respond to the genotoxic compounds or more generally to DNA damages, activating different intracellular pathways in order to survive[22]. In the eukaryotic cells the mechanisms of response to DNA damages is generally termed DNA Damage Response (DDR) (Figure 2). The crucial function of the DDR system is to maintain genomic stability and prevent transmission of incorrect genetic information to daughter cells during cell division. The DDR system includes different regulators involved in the recognition of DNA damage (DNA damage sensors), in the recruitment proteins on the site of DNA damages (DNA damage mediators) and in the response to DNA damages (DNA damages effectors) [23]. The most important consequences of the DDR activation are: i) the regulation of the cell cycle, ii) the activation of the mechanisms of DNA repair and iii) the induction of the apoptosis.

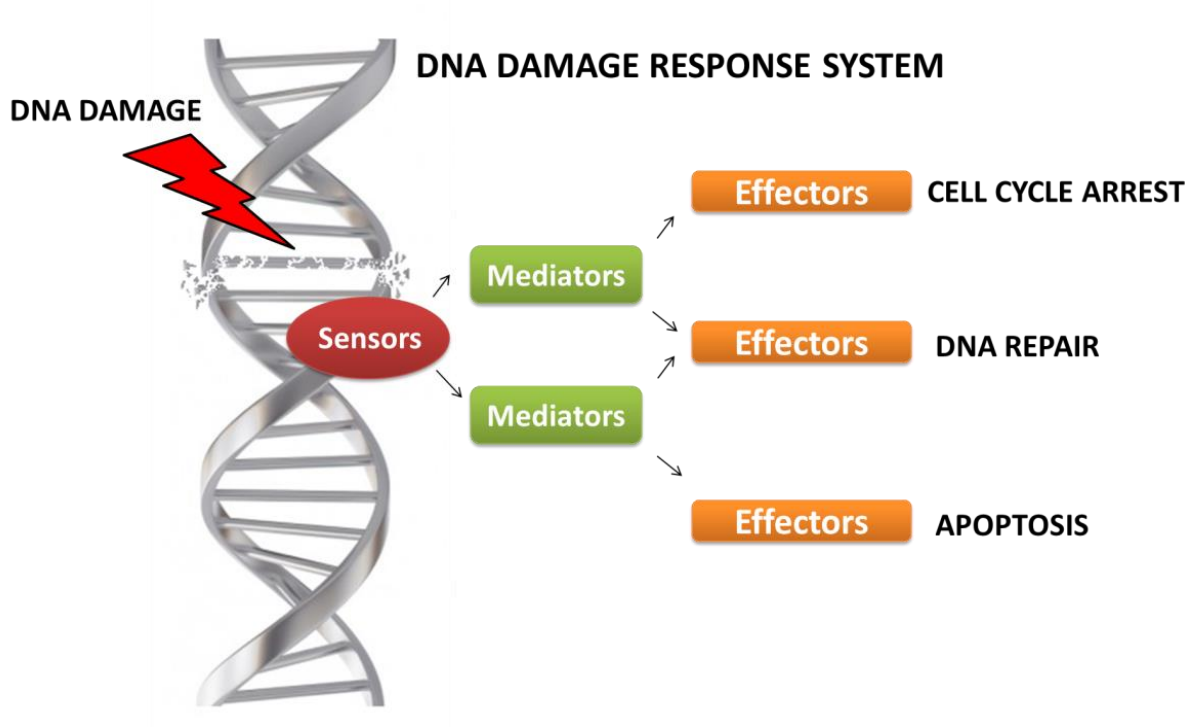

Figure 2: Schematic representation of the DNA Damages Response (DDR) pathway. DNA damages are sensed and repaired in multi-protein complexes. Signaling caused by this damage

results in the activation of different mediators of the damage response and then results in cell cycle arrest and a choice between repair or progression to apoptosis.

DNA Damage Sensors and Mediators

The first step of the DDR is the identification of the DNA damages. Different deleterious attacks from extrinsic agents as well as intrinsic sources, such as reactive oxygen species (ROS), can induce different DNA damages. One of the most deleterious lesions that can mine the genomic stability are double-strand breaks (DSBs), whose high cytotoxicity is the basis for conventional genotoxic agents, such as ionizing radiation (IR) and other compounds, like the topoisomerase II inhibitors, currently used in the treatment of different kind of cancers[24]. Three kinases, members of the phosphoinositide three-kinase-related kinase (PIKK) family, the DNA-dependent protein kinase (DNA-PK), the ataxia-telangiectasia-mutated (ATM) and the ATM and Rad3-related (ATR), have relevant biological roles in this initial phase of the DDR. In particular both DNA-PK and ATM are involved in the response DSBs while ATR is mostly involved in the response to DNA replication stress and in particular to damages that involved only one strand of the DNA structure (single strand breaks, SSBs)[25,26]. The Mre11-Rad50-Nbs1 (MRN) complex is fundamental for the response to DSBs and for the activation of ATM. The principal function of MRE11 is to bind DNA and, thanks to an exo- and endonuclease activity, to synapse DNA ends [27]. The main function RAD50 is to maintain DNA ends in close proximity thanks to an ATPase activity [28]. Finally NBS1 recruits DNA repair and different checkpoint protein (ATM itself) in the site of DNA damages [29]. During normal cell cycle or more generally in absence of DNA damages, ATM is an inactive dimer. In presence of DNA damages ATM dissociates in monomers and rapidly auto-phosphorylate on multiple residues which are fundamentals for ATM stability and activation. ATM recruitment has been shown to require its binding to the C-terminus of NBS1, an interaction fundamental also for the kinase activity of ATM. When ATM is associated to the site of damages, it rapidly phosphorylate the histone variant H2AX (ser139). This is a key event of the ATM/ATR transduction pathways and is necessary to amplify the signal of DNA damages and to facilitate the recruitment of different mediators of the DDR.

Single strand DNA (SSD) is physiologically generated during the duplication of the DNA. Indeed, during the S phase of the cell cycle, replication block is generated to allow the polymerase to duplicate the two strains of DNA. The first event for the generation of the replication blocks is

the activation of the replicative elicase, MCM (Mini-Chromosome Maintenance), that ahead of the polymerase unwind the double chain of DNA, generating SSDs. Different insults can main the stability of the replication forks, like the exposure to UV ray, causing the break of one strand of the DNA and, consequently, generating SSBs. In the contest of stalled replication forks or during the resolution of a DSBs, specific proteins, termed RPA (Replication protein A), bind the SSDs. The activation of ATR is strictly associated with the constitution of the SSDs-RPA complex [30]. This complex stimulates the binding to the damage sites of second critical group of protein, the RAD17/RFC2-5 clamp loader complex. Consequently, to the site of damage is recruited the RAD9/HUS1/RAD1 (9–1–1) heterotrimer that in turn recruits TopBP1 which activates ATR[31]. Once activated both ATM and ATR regulate the cell cycle progression allowing cells to resolve DNA damages before continue the cell replication (Figure 3).

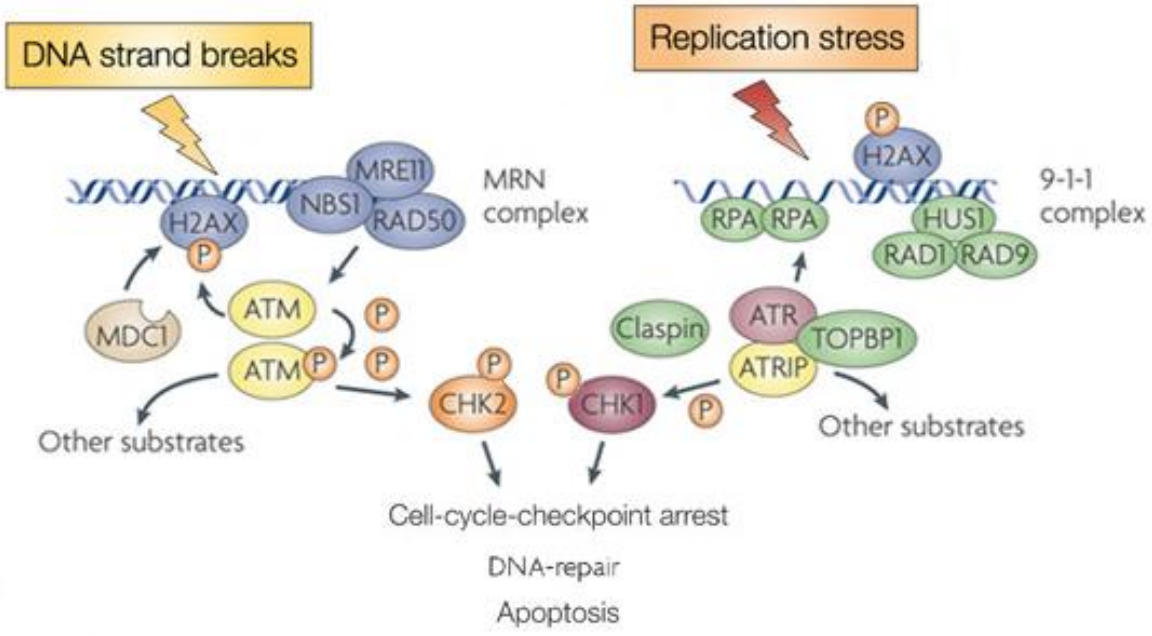

Figure 3: DNA damages Sensor and Mediators in the response to DSBs and SSBs. Damage to the DNA triggers the recruitment of specific damage sensor protein complexes. On the one hand, the MRN (MRE11–RAD50–NBS1) complex is required for the activation of ataxia telangiectasia mutated (ATM) in response to double-strand breaks (DSBs). On the other hand the ATM- and Rad3-related (ATR)-interacting protein (ATRIP) complex, formed by ATR-ATRIP-TOPBP1, is recruited to sites of single-strand breaks and activates ATR. The activation of ATM

and ATR promotes respectively the activation of two different effectors, Chk2 and Chk2 (Figure modified from [32])

DNA Damage Effectors

Different effectors are direct substrates of both ATM and ATR following the activation of the DDR. In particular all the downstream proteins activated by ATM/ATR are involved in cell cycle regulation (checkpoint kinases) and in the repair of DNA damages. The direct substrate of ATR and ATM are the Checkpoint kinase 1 (Chk1) and 2 (Chk2), respectively. Chk1 kinase is activated by ATR through the phosphorylation on serine 317 (ser317) and on serine 345 (ser345). Rapidly Chk1 auto-phosphorylates on serine 296 stabilizing its structure and creating a binding site for the interaction with its direct substrates, the phosphatases Cdc25 (Cdc25A/B/C). The activation of Chk2 is enhanced by ATM through the phosphorylation on threonine 68 (thr68) and followed by several auto-phosphorylation events. Chk2 shares the substrate homology with Chk1 and inhibits Cdc25A/B/C phosphatases in a similar way[33]. In eukaryotic the cell cycle is finely regulated by the oscillation in the activity of different cyclin-dependent kinases, CDKs, which are positively regulated by proteins, called cyclins, and negatively regulated by CDK inhibitors (CDKI) and by event of inhibitory phosphorylation[34,35]. The transition from a phase of the cell cycle to another is regulated by different cell cycle checkpoints and in particular by the G1/S (transition through the G1 phase to the S phase), the intra S and the G2/M checkpoints (transition to the G2 phase and entry in the mitosis). The activation of the G1/S checkpoint is mainly regulated through the activity of the tumor-suppressor p53 which have been showed to be one of the direct substrate of ATM/ATR activation via the phosphorylation on serine 15 (ser15). Different sequential phosphorylations, contribute to p53 stabilization and prevent the ubiquitination and consequently degradation enhanced by the negative regulator of p53, Mdm2[36]. The regulation of p53 in the G1/S checkpoint is also related to the activation of two direct substrates of both ATM and ATR, respectively the checkpoint kinase 2 (Chk2) and the checkpoint kinase 1 (Chk1), that promote the activatory phosphorylation of p53 on serine 20 (ser20)[37,38]. Once fully activates, p53 promote the transcription of different genes involved in cell cycle regulation, like the CDKN1A (Cyclin-Dependent Kinase Inhibitor 1A (P21, Cip1)), and in the induction of apoptosis, like BAX/PUMA/NOXA[39]. The transition through the S phase is mainly regulated by a specific phosphate, Cdc25A[40]. This protein is necessary to remove the

inhibitory phosphorylation on tyrosine 15 (tyr15) and threonine 14 (thr14) on Cyclin-depend kinase 2 (CDK2, Cdc1). During normal replication CDC25A activates CDK2 promoting the formation of the complex CDK2-CyclinE/CyclinA necessary for entry into the S phase and for the DNA synthesis. In the presence of DNA damages both Chk1 and Chk2 phosphorylate Cdc25A on serine 136 (ser136) promoting its ubiquitination by SCF/TrCP ubiquitin ligase complex and degraded by proteasome. The inhibition of Cdc25A causes an S phase delay allowing the repair of DNA damages before cell cycle progression continues. Similar to the regulation of the S phase also the transition from the G2 to the M phase is strictly related to the activation of specific phosphates and in particular to the activation of both Cdc25B and Cdc25C. During checkpoint activation Cdc25B is phosphorylated at serine 323 (ser323), by Chk1, and bound by 14-3-3 that blocks its catalytic activity [41,42]. Both Chk1 and Chk2 negatively regulate Cdc25C via phosphorylation of a serine reside (ser216), this event create a site for the binding to 14-3-3 protein resulting in its cytoplasmic sequestration and G2/M checkpoint activation. The events that follow Cdc25C sequestration are analogue of the once that follows Cdc25A degradation. The segregation of this phosphatase in the cytoplasm prevents its accumulation into the nucleus and consequently the inactivation of a protein complex crucial for the transition through the G2/M phase, the CDK1 (Cdc2)-CyclinB complex. This complex is finely regulated not only by Chk1 or Chk2 but also by two proteins of the Wee1 family, Wee1 and Myt1. While both kinases can inhibit CDK1 through the phosphorylation on Tyrosine 15 (Tyr15), Myt1 can also phosphorylate on Threonine 14 (thr14), which has been shown to negatively regulate CDK1 as well. Thus after the activation of the G2/M checkpoint Chk1, Chk2 and Wee1 cooperate to negative regulate the CDK1 to prevent the formation of the complex with the cyclin B[43]. [43].Although the regulation of Wee1 during normal cell cycle have been established[44], the mechanisms by which Wee1 is activated in response to DNA damages in human is still not fully understood[45]. During normal cell division Polo kinase 1(PLK1) phosphorylates Wee1 promoting its degradation and, consequently, the beginning of the mitosis. After DDR activation both ATM and ATR promote the inhibitory phosphorylation of PLK1, leading to the nuclear accumulation of Wee1[46].

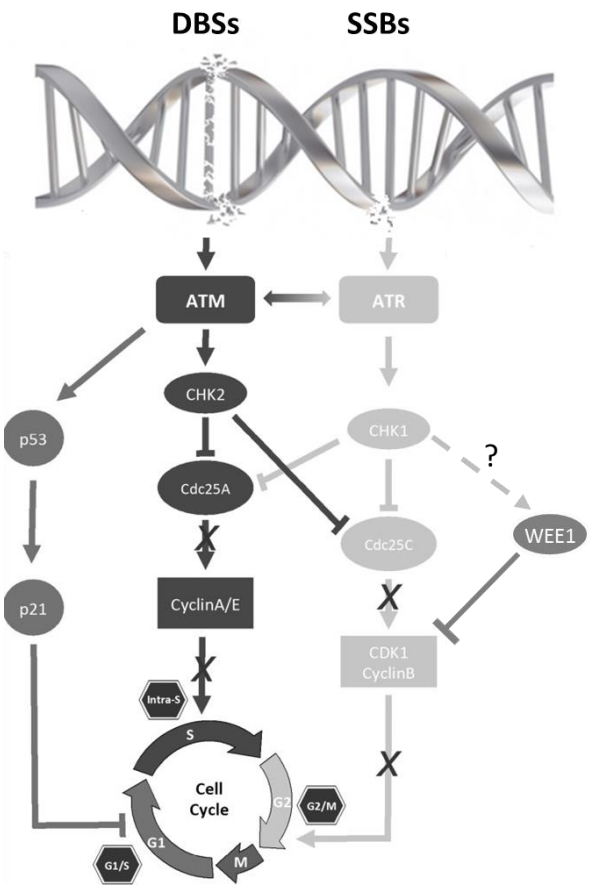

Figure 4: Activation of cell cycle checkpoints. Cell cycle progression is arrested primarily through ATM and ATR dependent phosphorylation of p53, Chk1 and Chk2. The G1/S checkpoint is primarily mediated through a p53-dependent increase in p21, a cyclin-dependent kinase inhibitor. Key targets of Chk1/2 include the Cdc25 phosphatases that control the activity of specific cyclin–CDK complexes, which in turn regulate progression through S-phase and the G2/M checkpoint. Phosphorylation of Cdc25A/B/C phosphatases by Chk1/2 inhibits their activity ensuring that CDK–cyclin complexes are not activated. The inhibition of the CDK1/CyclinB complex, crucial for the G2/M transition, is enforced by the activity of Wee1 kinase. This kinase directly phosphorylates CDK1 keeping this kinase inactive.

The consequences of the activation of the DDR system are not limited to the regulation of cell cycle progression but are also related to the induction of DNA repair. The major pathways of DNA repair are the Homologous Recombination repair (HRR) and the Non-Homologous End Joining (NHEJ), which may operate in several variants. The choice between these two mechanisms depends on the cell cycle phase. Indeed the NHEJ is a rapid mechanism active during all the phases of the cell cycle but in inactive during mitosis where the HRR system predominates, due to the presence of sister chromatin as template for the repair of the damages[47]. HRR is a slow, high accurate, process which involves many proteins including: the MRN complex, RPA, RAD51, RAD52, RAD54, BRCA1, BRCA2 and other proteins. A key protein in the HRR is RAD51. Cells deficient for RAD51 accumulate DSBs after replication or at stalled replication forks. Chk1 has been found to directly interact with different element of the HRR and in particular with RAD51. In response to DNA damages, Chk1 phosphorylates RAD51 on threonine 309 (thr309), promoting its recruitment at the site of damage[48]. Also Chk2 promote the HR system and in particular through the phosphorylation of BRCA2 on threonine 3387 (thr3387), which is critical for RAD51 localization to DSBs and through the phosphorylation of BRCA1 on serine 988 (ser988) [49]. The core machinery for NHEJ is typically considered to include the Ku heterodimer (Ku 80/70), the DNA dependent protein kinase catalytic subunit (DNA-PKcs), DNA Ligase IV, XRCC4 (X-ray repair cross-complementing protein 4), and the XRCC4-like factor (XLF, or Cernunnos)[50,51]. This process begins with the binding of the KU70/KU80 heterodimer to the DNA ends. This event then recruits DNA-PKcs to form the DNA-PK holoenzyme. The DNA ends are resected by the Artemis and/or MRE11/RAD50/NBS1 (MRN) nucleases, followed by XRCC4/DNA Ligase IV/XLF before the ligation starts by DNA Ligase IV[52]. In NHEJ critical is the role of ATM, by protecting the DNA ends from degradation. This protection involves a variety of proteins associated with the DSB repair complex, including 53BP1, histone γH2AX and the MRN complex[53].

Checkpoint kinase inhibitors

Although the checkpoint kinases in normal cells are crucial for the maintenance of genetic stability, in cancer development these kinases protect tumor cells from the replication stress[54]. In tumor cells the high proliferative rate, induced by oncogenes, or an hypoxic microenvironment can induce replication stress and consequently the hyper-activation of the ATR/CHK1 in S phase cells[55,56]. On these bases the use of checkpoint kinase inhibitors, even in single agent, can drastically mine the mechanisms by which tumor cells sustain the replicative stress and thus promote cancer cells death.[57] Moreover, due to the elevated proliferation rate oncogene-driven that characterized different types of tumors, the use of checkpoint kinase inhibitor could be a promising strategy to selectively target tumor cells and to limit off-target toxicity on normal tissue. In addition, due to the central role in the response to DNA damages, different checkpoint kinase inhibitors (Chk-i) have been developed to specifically inhibit the mechanisms by which tumor cells respond to DNA damaging agents. Initially this class of compounds have been developed for the treatment of p53 mutated tumors because of their impair functionality of the G1/S checkpoint, and then their applicability have been extended also to p53 wild type tumors[58,59]. These compounds have been developed to potentiate the efficacy of different chemotherapeutic compound especially for the treatment of solid tumors[60]. Indeed the number of publications and clinical trials evaluating the effectiveness of this compound in solid tumors is in constantly growth but only few studies have been performed for the evaluation of the efficacy of the Chk-Is in hematological malignances. The following section summarizes the main studies that have been done to evaluate the efficacy of different Chk1/Chk2/Wee1 inhibitors in leukemia (Figure 5).

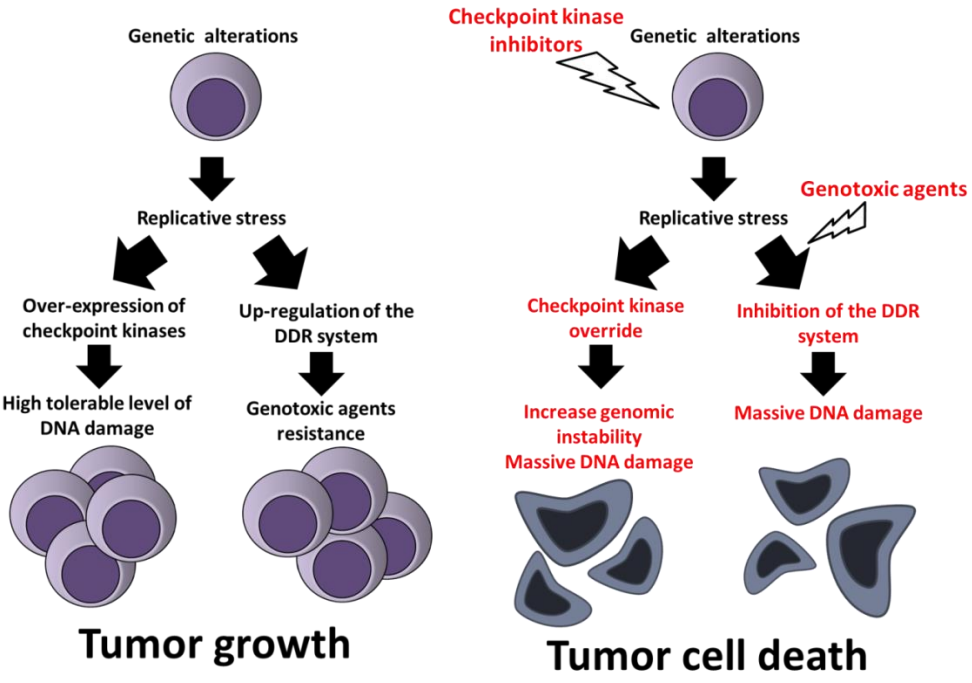

Figure 5: The DDR pathway in cancer cell. The left cartoon shows that the up-regulation of the DDR pathway increase the tolerable level of DNA damages and induces chemo-resistance to genotoxic agents. The right cartoon shows that the inhibition of the DDR pathway can increase the intrinsic genetic instability inducing massive DNA damages. Moreover the inhibition of the DDR using different inhibitors sensitizes cells to the toxic effect of chemotherapy.

Checkpoint kinase 1 (Chk1)/Checkpoint kinase 2 (Chk2) inhibitors

In the last decade the number of publications evaluating the preclinical and clinical efficacy of small molecule inhibitors of Chk1 is constantly growth [61] as well as the number of molecules against this kinase[62,63]. The first inhibitor of Chk1 was the **UCN-1** (known as staurosporine). This molecule have been shown to inhibit not only Chk1 but also other different kinases (Chk2, CDK1, CDK2, PKC 7and MK2) and to promote the G2/M checkpoint override upon treatment with DNA damaging agents such as cisplatin or topoisomerase inhibitor. UCN-01 was tested in a number of clinical trials however the low specificity of the compound caused many harmful side-effects and this have not allowed its progression beyond phase II of clinical trials [64,65].

MK-8776 (SCH900776) is a potent and selective Chk1 inhibitor in clinical development. It rapidly, less than two hours, induced gH2AX accumulation and suppressed Chk1 functionality (shown by the reduction of the auto-phosphorylation site of serine 296). The efficacy of this inhibitor was assessed not only in single agent but also in combination with different genotoxic compounds showing chemotherapy sensitization by increasing the level of DSBs. Many other studies confirmed the great efficacy of this compound for the treatment of different kind of tumors and today the MK-8776 entered in phase II clinical trials in combination with chemotherapy [62,66–68]. The efficacy of the compound was also evaluated on hematological malignances. Day and colleagues[68] demonstrated that MK-8776 synergistically potentiated the histone deacetylase (HDAC) inhibitor (HDACI) vorinostat on both AML cell lines and primary cells. Moreover they showed that efficacy of the combination was independent on the mutational status of p53 and that the synergistic interactions were associated with inhibition of Chk1 activity, interference with the intra-S-phase checkpoint, disruption of DNA replication, and down-regulation of proteins involved in DNA replication and repair [68].

AZD7762 is an ATP competitive Chk1/Chk2 inhibitor. This compound was evaluated in different trials as a chemo-sensitizer agent for conventional chemotherapy. It has been described that lung cancer cells expressing high levels of Chk1 were hypersensitive to AZD7762. This suggests a correlation between Chk1 inhibitor-mediated sensitivity and elevated amounts of Chk1 [89].

Different further studies were conducted to investigate the efficacy of ASD7763 in combination with different compound. Indeed it has been reported that combination of AZD7762 with gemcitabine and ionizing radiation deeply sensitized pancreatic cells to radiation[69]. The efficacy of the compound has been evaluated also in hematologic malignances, e.g in different myeloma multiple (MM) cell lines. In the combination studies the researchers showed that AZD7762in combination with alkylating agents (melphalan) promoted apoptosis and mitotic catastrophe of p53-mutated MM cells[70]. Moreover the Didier et al.[71], showed that *AZD7762* enhances genotoxic treatment efficacy in immature KG1 leukemic cell line and in AML primary leukemic cells. In this study they also correlated the sensitivity to the checkpoint kinase inhibitors with patients with a complex karyotype highlighting that this group of patient was displays major genomic instability and chemo-resistance [71].

PF-0477736 is a selective and competitive inhibitor for the Chk1 ATP site. Its specificity is one hundred times stronger for Chk1 than for Chk2.The efficacy of this compound have been well established against different kinds of tumors. In ovarian cancer it has been showed that tumor cells strongly respond to treatment with PF-0477736 but generate metastasis and chemo-resistant clones [72]. The efficacy of PF-0477736 has been evaluated also in leukemia. Sarmento et al. [73] demonstrated that the T-ALL primary samples express higher level of Chk1 kinase in comparison to normal thymocyte. The treatment with PF-0477736 promoted apoptotic cell death, Chk1 inhibition and consequently impaired replication and abrogation of G2/M checkpoint in T-ALL cells. Interestingly, this inhibitor in vitro did not significantly affect the viability of normal thymocyte cells[73].

LY2603618, a potent and selective inhibitor of Chk1, is the first second generation checkpoint kinase inhibitor that have been evaluated in a clinical trial [74].King and colleagues [75] reported that the treatment with LY2603618 produced a cellular phenotype similar to that reported for depletion of Chk1 by RNAi. Moreover they reported that the inhibition of Chk1 caused impaired DNA synthesis, elevated H2A.X phosphorylation and premature entry into mitosis. Finally they showed that LY2603618 was able to override the G2/M checkpoint activated after the exposure to doxorubicin, resulting in cells entering into metaphase with

poorly condensed chromosomes[75].It has been reported that LY2603618 potentiated the effect of different DNA damage drugs like pemetrexed and cisplatin in vitro. This result was confirmed in vivo using a tumor xenograft approaches and were the bases for a phase I clinical trials evaluating the effectiveness of LY2603618 in combination with pemetrexed and cisplatin in patients with advanced cancer [74].

LY2606368 is a novel Chk1/Chk2 inhibitor which has been reported to causes as a single agent DBSs while simultaneously removing the protection of the DNA damage checkpoints. King and colleagues reported that LY2606368 increases extensive DNA damage in the S phase cell population highlighting the possible mechanism of death through replication catastrophe[76].

Wee1 inhibitors

Many Wee1 inhibitors have been developed to override checkpoint signaling and, consequently, to improve the sensitivity of tumor cells to the toxic effect of different genotoxic agents. Several studies have shown their efficacy in the treatment of different kinds of tumors not only in combinatorial studies but also as single agent. The PD0166285 is a non-selective kinase inhibitors, which targets Wee1 but also Chk1, Myt1, c-Src, PDGFR-, fibroblast growth factor receptor-1, and epidermal growth factor receptor tyrosine kinases [77,78]. This inhibitor has been shown to successfully inhibit CDK1 phosphorylation (tyrosine 15 and threonine 14) and to abrogate G2/M checkpoint after IR irradiation in vitro (Figure 6).

MK-1775 (AZD1775) is currently mostly studied Wee 1 inhibitor. Several studies have shown that this particular inhibitor selectively sensitizes p53-deficient cancer cells to the toxic effect of gemcitabine, carboplatin, 5-fluorouracil and cisplatin [79–82]. The sensitizing activity of MK-1775 selectively on p53 deficient cells has been showed also after irradiation. Although all the above mentioned studies, recent finding highlighted that the effectiveness of this compound in different types of tumor is independent by the functional status of the tumor-suppressor p53 [83]. Today several studies on hematological malignancies, mostly on acute myeloid leukemia, have showed the efficacy of this compound, not only as single agent [84] but also in combination with different compound like HDAC (e.g., vorinostat) [85,86] or like cytarabine (AraC). Finally, different studies showed the synergistic efficacy between Chk1 and Wee1 inhibitors on different tumor cell lines and on primary cells [87–89].Today the efficacy of MK-1775, either as a single agent or in combination with different DNA-damaging agents, is currently evaluated in different clinical trials (https://clinicaltrials.gov). The first study conducted on patients with refractory solid tumors evaluating the maximum-tolerated dose (MTD), the pharmacokinetics, and the modulation of phosphorylated forms of CDKs and of histone H2AX (γH2AX) in paired tumor biopsies showed that, in line with the per-clinical studies, MK-1775 decreased CDK1 phosphorylation (tyrosine 15) and increased H2AX phosphorylation[90].

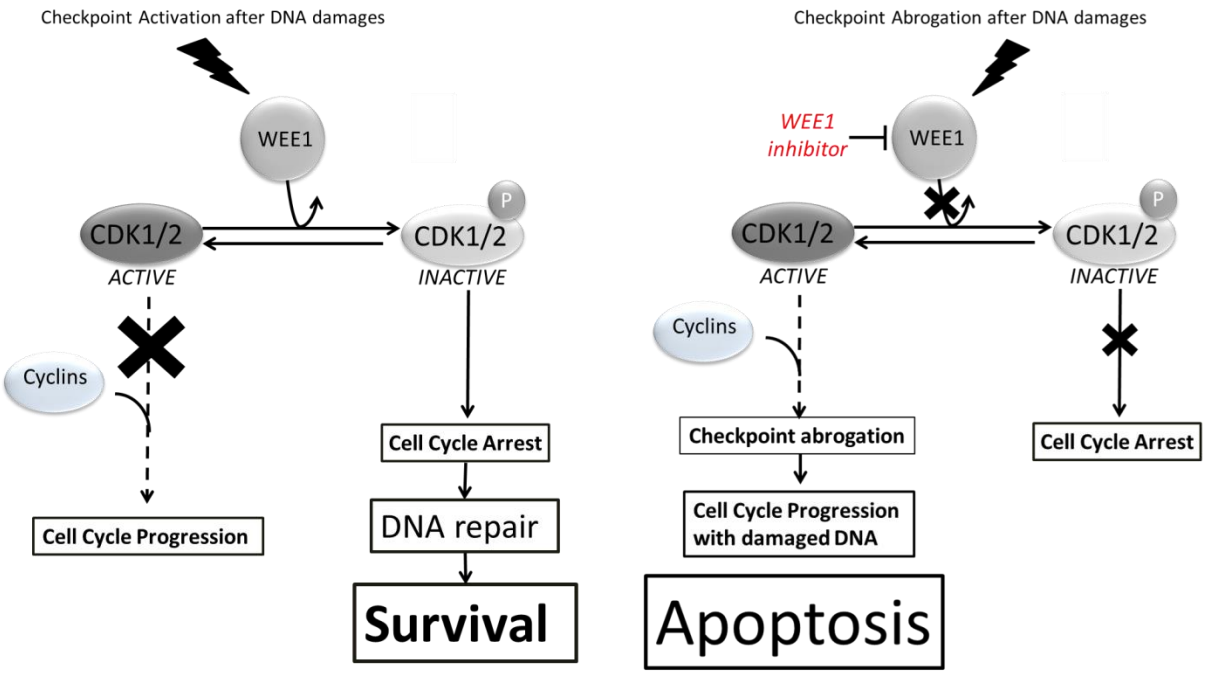

Figure 6: Schematic representation of the role of Wee1 in the activation of the G2/M checkpoint and the consequence of its inhibition using Wee1 inhibitors.

Aims

The overall aim of this study was to evaluate the efficacy of different checkpoint kinase inhibitors for the treatment of ALL as *single agent* or in combination with various compounds normally used in the clinic for the treatment of ALL patients.

The specific aims were the followings:

1. Different elements of the DNA damages response pathways are up-regulated in many tumors. Indeed, the activation of different oncogenes frequently causes replicative stress and tumor cells, in order to sustain the high replicative stress, up-regulate different elements of the DDR. Different studies have shown that the up-regulation of these keys elements of the DDR is fundamental to sustain the high genetic instability. Starting from this background the first aim was to evaluate the efficacy of a Chk1/2 inhibitor, PF-0477736, as single agent on a panel of different B-/T-ALL cell lines, on primary leukemic cells isolated from peripheral blood and bone marrow of adult ALL patients, and on a mouse model.

2. Different checkpoint kinase inhibitors have been developed to specifically sensitize tumor cells to the cytotoxicity of genotoxic agents. The second aim of the study was to evaluate the chemo-sensitizer efficacy of LY2606368, a selective Chk1/Chk2 inhibitor, on leukemic cell lines and primary cells. The efficacy of LY2606368 was firstly evaluated as single agent and then in combination with different compounds like tyrosine kinase inhibitors, imatinib or dasatinib, and the purine nucleoside analogue clofarabine.

3. The final aim of this study was to evaluate the effect of the inhibition of WEE1 kinase, using MK-1775 inhibitor, as single agent and in combination with different compounds like tyrosine kinase inhibitors (two isomer of bosutinib), purine nucleoside analogue (clofarabine), and Chk1/Chk2 inhibitor (PF-00477736) on B-/T-ALL cell lines and on primary leukemic cells isolated from adult B-ALL patients.

Results I

PF-00477736

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In vitro and in vivo single-agent efficacy of Checkpoint Kinase inhibition in acute lymphoblastic leukemia

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Abstract

Background: Although progress in children, in adults ALL still carries a dismal outcome. Here, we explored the in vitro and in vivo activity of PF-00477736 (Pfizer), a potent, selective ATP-competitive small-molecule inhibitor of checkpoint kinase 1 (Chk1) and with lower efficacy of checkpoint kinase 2 (Chk2). Methods: The effectiveness of PF-00477736 as single agent in B-/T-ALL was evaluated in vitro and in vivo studies, as single agent The efficacy of the compound in term of cytotoxicity, induction of apoptosis, changes in gene and protein expression was assessed using different B-/T-ALL cell lines. Finally the action of PF-00477736 was assessed in vivo using leukemic mouse generated by a single administration of the tumorigenic agent N-

ethyl-N-nitrosourea. Results: Chk1 and Chk2 are over expressed concomitant with the presence of genetic damage as suggested by the nuclear labeling for γ-H2A.X (Ser139) in 68% of ALL patients. In human B- and T- ALL cell lines inhibition of Chk1/2 as a single treatment strategy efficiently triggered the Chk1-Cdc25-Cdc2 pathway resulting in a dose and time-dependent cytotoxicity, induction of apoptosis and increased DNA damage. Moreover, treatment with PF-00477736 showed efficacy ex-vivo in primary leukemic blasts separated from 14 adult ALL patients and in vivo in mice transplanted with T-ALL, arguing in favor of its future clinical evaluation in leukemia. Conclusions: In vitro, ex-vivo and in vivo results support the inhibition of Chk1 as a new therapeutic strategy in acute lymphoblastic leukemia and they provide a strong rationale for its future clinical investigation.

Background

Acute lymphoblastic leukemia 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. Survival rates for children are approximately 80%-85% with current risk-oriented treatment protocols in contrast to less than 40% for adults[91–93]. Although the outcome of children with relapsed ALL is heterogeneous, many achieve lasting second remissions. In contrast, survival after relapse in adult ALL is short[94,95]. New therapeutic strategies are therefore needed to improve remission rates in adults and to prevent relapse both in adult and pediatric ALL patients.

Recently, in order to enhance DNA-damaging effects inflicted by cytotoxic drugs or radiation, different checkpoint kinase (Chk) inhibitors have been developed and assessed alone or in combination with DNA damaging agents in preclinical studies and in PhaseI/II trials for cancer therapy[96–104]. Some of them inhibit both Chk1 and Chk2, other have a higher specificity for Chk1. The biological background underlying the development of Chk1 inhibitors and preliminary data deriving from early clinical trials have been recently well reviewed in[105]. Following DNA damage multiprotein complexes recruit the transducers ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR) which activate Chk2 and Chk1, respectively, despite an existing extensive crosstalk between the ATR-Chk1 and ATM-Chk2

pathways. Once activated by phosphorylation at S317/S345 in response to DNA single strand breaks, Chk1 phosphorylates the phosphatase Cdc25A at the G1/S and S phase checkpoints preventing cells from entering S phase and Cdc25A and Cdc25C at the G2/M checkpoint avoiding the entry in mitosis[106–109]. If Chk1 is induced by single strand breaks, Chk2 activation is largely restricted to DNA double strand breaks via ATM[38]. Once activated, they mediate cell cycle delay, DNA repair, and apoptosis in response to DNA damage. Moreover, Chk1 is required for proper mitotic spindle assembly and maintenance of chromosomal stability during mitosis[110].

PF-00477736 is a potent, selective ATP-competitive small-molecule inhibitor of Chk1, synthesized by Pfizer, which is selective for Chk1 and shows general selectivity over other kinases[96]. Preclinically, PF-00477736 enhanced docetaxel activity in tumor cells and xenografts by abrogating the mitotic spindle checkpoint, as well as the DNA damage checkpoint[100]. The checkpoint abrogating activity and cytotoxic activity attributed to PF-00477736 in combination with chemotherapy agents (e.g gemcitabine and carboplatin) showed selectivity for p53-defective cancer cell lines over p53-competent normal cells *in vitro*[96] . In xenografts, PF-00477736 enhanced the antitumor activity of gemcitabine in a dose-dependent manner. PF-00477736 combinations were well tolerated with no exacerbation of side effects commonly associated with cytotoxic agents[96].

In this study, we investigated the activity of PF-0477736, as a single agent in B-/T-ALL by: assessment of the *in vitro* efficacy in ALL cell lines and primary blast cells; assessment of the *in vivo* efficacy in mouse models; identification and validation of potential biomarkers of functional inhibition. Results demonstrated that *in vitro* treatment of B-/T-ALL cell lines and primary blast cells with PF-0477736 resulted in inhibition of cell viability, induction of DNA damage and apoptosis. Moreover, *in vivo* studies confirmed the efficacy of Chk1 inhibition, suggesting that this therapeutic strategy may be promising in leukemia.

Results

Chk1 and Chk2 are overexpressed in acute lymphoblastic leukemia

Chk1/2 transcript levels were assessed by qPCR in eight cell lines from B/T- ALL (BV-173, SUP-B15, REH, NALM-6, NALM-19, MOLT-4, RPMI-8402 and CCRF-CEM)(Fig. S1) and in blast cells from 54 adult newly-diagnosed ALL cases including 41 (76%) *BCR-ABL1*-positive and 13 (23%) *BCR-ABL1*-negative cases. Normal bone marrow precursor cells isolated from leukemia patients in complete remission were also analyzed by the same method. In this cohort higher transcript levels of Chk1 but not Chk2 were found in leukemia cell lines and newly diagnosed ALL cases compared to normal bone marrow mononuclear cells (p value < 0.001) (Fig. 1). The web-based public database Oncomine[111] (https://www.oncomine.org/) was queried for Chk1/2 expression in the available leukemia datasets based on the comparison leukemia versus normal using a criterion of a 2 fold change for both Chk1 and Chk2 expression and a p-value of 1E-4. Using these stringent criteria, we found that both Chk1 and Chk2 transcripts are highly overexpressed in B-ALL and T-ALL if compared to normal bone marrow samples (Fig. 2S). We then investigated by immunohistochemistry formalin-fixed paraffin-embedded (FFPE) tissue samples collected at diagnosis from 60 ALL patients (36 B-ALL and 24 T-ALL) for protein expression of Chk1, phosphorylated Chk1 (Ser345), Chk2, phosphorylated Chk2 (Thr68), Cdc25C, phosphorylated Cdc25C (Ser 2016) and phosphorylated H2A.X (Ser139) (γ-H2A.X). Results are detailed in Table 1 and viewed in Fig. 1C. Among B/T-ALL, a diffuse positivity for Chk1, Chk2, CDdc25c and the phosphorylated forms of Chk1 (Ser345) and CDCdc25c (Ser216) was observed; these were detected in 51/54 (96%), 55/57 (94%), 57/57 (100%), 45/56 (80%) and 38/55 (70%) of the cases respectively, whereas 15/55 (27%) of ALLs stained for phosphorylated form of Chk2 (Thr68) (Fig. 1D). Interestingly, in our ALL series, genomic damage was suggested by the nuclear labeling for γ-H2A.X molecule in 40/59 (68%) of samples (Fig. 1D). In thymuses, normal lymphoblasts did not show protein expression of Chk1, Chk2, Cdc25C and their phosphorylated counterparts (Fig. 1D). In B follicles of the reactive lymph node, in spite of the Chk1 weak positivity, diffuse Chk2 and Cdc25C nuclear staining, no phosphorylation in their amino acid Ser345, Thr68 and Ser216 respectively, was detected (Fig. 1D). Notably, in thymuses and reactive B follicles, only scattered γ-H2A.X^{+} cells were present, indicating a low level of genomic damage in physiologic conditions compared to ALLs (Fig. 1D).

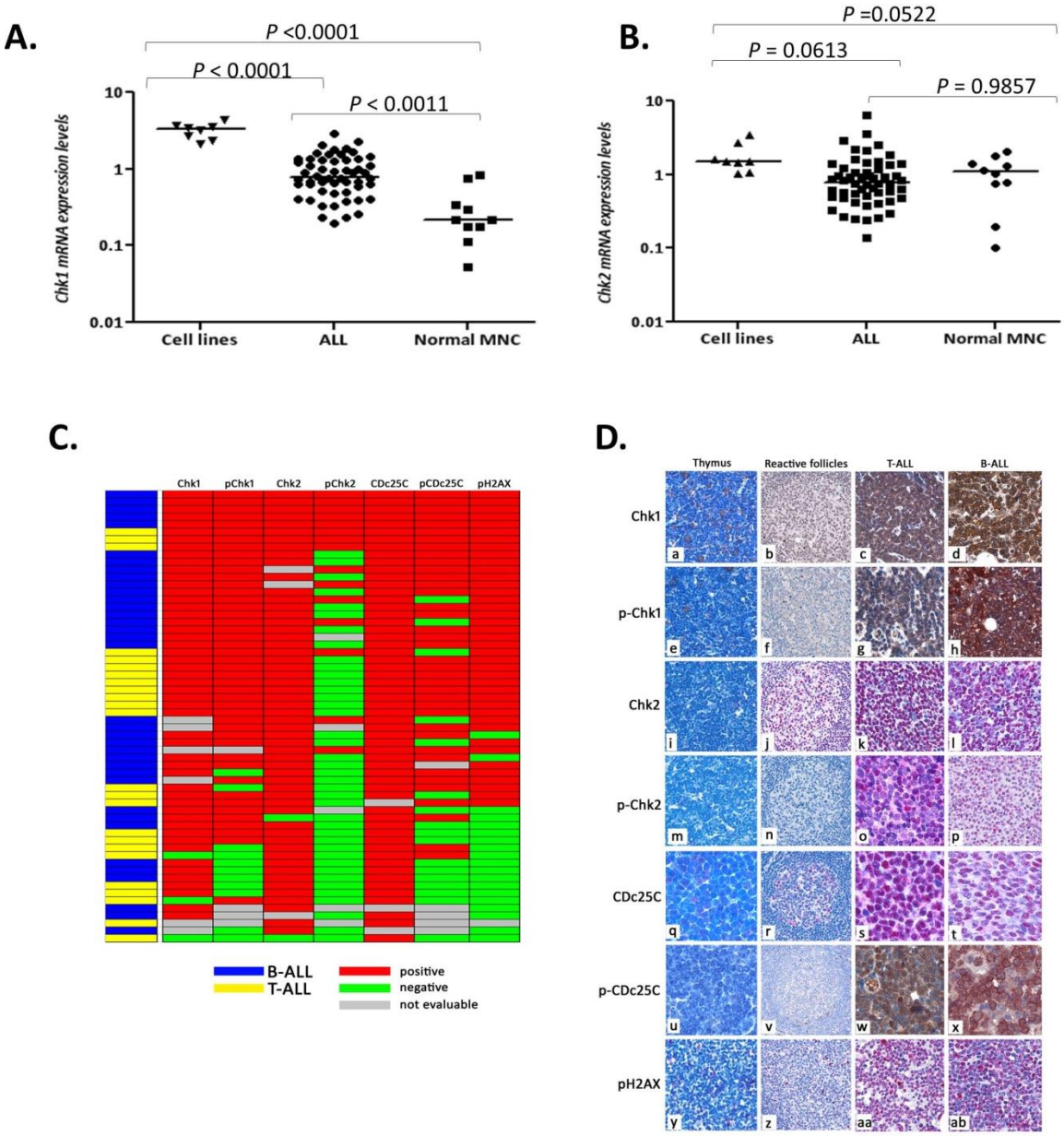


Figure 1. Chk1 (A) and Chk2 (B) mRNA expression levels in leukemia cell lines (BV-173, SUP-B15, REH, NALM-6, NALM-19, MOLT-4, RPMI-8402 and CCRF-CEM), blast cells from fifty-four adult newly-diagnosed ALL cases and in normal bone marrow mononuclear cells (MNC). Results are expressed as Log10 2exp[-(ΔΔCt)] and they are the mean of at least two different replicates. C. Immunohistochemical profile of the 60 ALL (36 B-ALL and 24 T-ALL); a red box indicates expression of the marker, whereas a green box signifies negativity (samples were considered positive if 30% or more of the cells were stained with an antibody); gray box indicates a not evaluable reaction due to a core loss in TMA. The figure allows the assessment of the co-

expression of the DNA damage markers in individual samples D. Immunohistochemical expression of Chk1, phosphorylated Chk1 (Ser345), Chk2, phosphorylated Chk2 (Thr68) and phosphorylated H2A.X (Ser139) (γ-H2A.X) in thymus, reactive follicle (RF), T-ALL and B-ALL. The figure highlights the close similarity between expression profiling of B- and T-ALL, characterized by a high percentage of cases $Chk1^{+}$, $pChk1^{+}$, $Chk2^{+}$, Cdc25C, pCdc25C, $pH2A.X^{+}$ and, in a lesser extent , $pChk2^{+}$, and its distinction with the expression profiling of the non tumoral populations as maturing thymocytes, that were substantially negative for these seven proteins and as B lymphocytes of reactive follicles, that resulted Chk1 weakly $^{+}$, $Chk2^{+}$, $Cdc25C^{+}$ but negative for the corresponding phosphorylated forms and pH2A.X. a) thymus: thymocytes $Chk1^{-}$ (x400); b) RF: B lymphocytes in mantle zone (MZ) and germinal center (GC) showing weak Chk1 positivity (x100); c) strong expression of Chk1 in T-ALL and d) B-ALL (x400). e) thymus: thymocytes $pChk1^{-}$ (x400); f) RF: B lymphocytes in MZ and GC $pChk1^{-}$; g) strong positivity of pChk1 in T-ALL and h) B-ALL (x400); i) thymus: normal lymphoblasts $Chk2^{-}$ (x400); j) RF: B lymphocytes in MZ and GC showing diffuse Chk2 positivity (x100); k) strong nuclear staining for Chk2 in T-ALL and l) B-ALL (x400). m) thymus: thymocytes $pChk2^{-}$ (x400); n) RF: B lymphocytes in MZ and GC $pChk2^{-}$ (x100); o) diffuse expression of pChk2 in T-ALL and p) B-ALL (x400); q) thymus: normal lymphoblasts negative for Cdc25C (x400); r) RF: B lymphocytes in mantle zone (MZ) and germinal center (GC) showing weak Cdc25C positivity (x100); s) strong nuclear staining for Cdc25C in T-ALL and t) B-ALL (x400); u) thymus: thymocytes $pCdc25C^{-}$ (x400); v) RF: B lymphocytes in MZ and GC $pCdc25C^{-}$ (x100); w) diffuse expression of pCdc25C in T-ALL and x) B-ALL (x400); y) thymus: normal lymphoblasts negative for pH2A.X (γ-H2A.X) (x400); z) RF: MZ B and GC lymphocytes substantially pH2A.X (γ-H2A.X) negative, with only occasional GC positive cells (x100); aa) strong nuclear staining for pH2A.X (γ-H2A.X) in T-ALL and ab) B-ALL (x400).

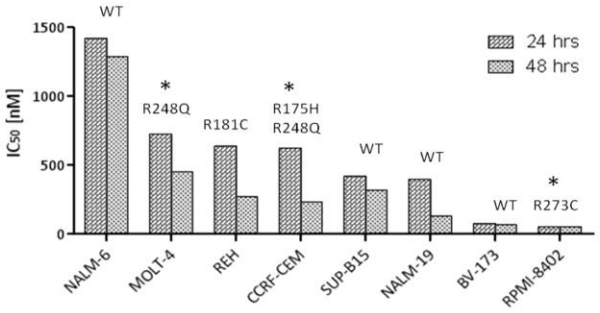
PF-00477736 reduces cell viability in a dose-dependent manner

B-/T-ALL cell lines were incubated with increasing concentrations of drug (5-1000 nM) for 24 and 48 hours. PF-00477736 inhibition resulted in dose and time-dependent cytotoxicity with RPMI-8402 (T-ALL) being the most sensitive (IC_{50} = 57.4 nM at 24 hours), while NALM-6 (B-ALL) the most resistant (IC_{50} = 1423.0 nM at 24 hours). *In vitro* sensitivity does not correlate with leukemia cell type (B-ALL *vs* T-ALL), *TP53* mutation status (BV-173, SUPB-15, NALM-6 and NALM-19 cells were p53 wild-type whereas REH, MOLT-4, RPMI-8402 and CEM cells were p53 mutated) (Fig. 2A) and with baseline levels of Chk1/2 and ATR/ATM phosphorylation, indicative of intrinsic genetic stress (Fig. 2B).

PF-00477736 induces apoptosis at 24 and 48 hours in B-/T-ALL cells

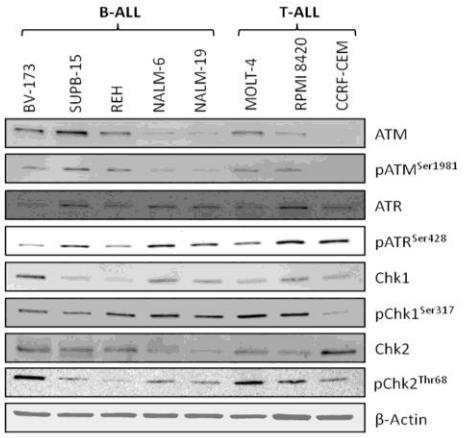
In order to assess whether cytotoxicity was correlated to increased susceptibility to apoptosis, B-/T-ALL cell lines were incubated with increasing concentrations of drug (0.1, 0.5 and 1 µM and 0.05, 0.1 and 0.2 µM only for BV-173 and RPMI-8402) for 24 and 48 hours. Consistent with the viability results, Annexin V/PI staining analysis showed a significant increase of apoptosis at 24 and 48 hours in B- and T-ALL cells proportional to drug-dose and drug-exposure time (Fig. 3S-A). The induction of apoptosis by PF-00477736 was also assessed by detection of poly (ADP-ribose) polymerase (PARP) cleavage by Western blot analysis[112]. The PARP-1 cleavage band at 89 kDa was observed in lysates from leukemia cell lines after 24 hours of drug exposure, while it was undetectable in cells treated with only DMSO 0.1% (Fig. 2C).

A.



	IC_{50} [nM] 24 hrs	IC_{50} [nM] 48 hrs
NALM-6	1423.00	1288.00
MOLT-4	725.80	455.50
REH	637.90	274.00
CCRF-CEM	628.10	236.50
SUP-B15	425.30	317.50
NALM-19	397.40	136.60
BV-173	81.59	71.46
RPMI-8402	57.37	57.97

B.



C.

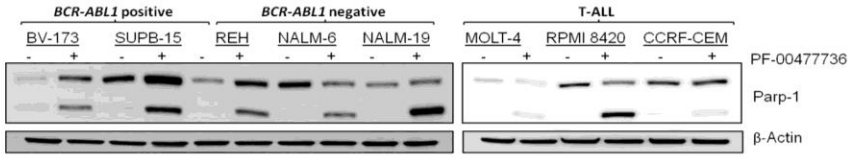


Figure 2. A. IC50 values for B- and T-ALL cell lines at exposure durations of 24 and 48 hours (hrs) to PF-0477736. For each cell line the mutational status of *TP53* gene is shown. Abbreviations: WT, wild-type; hrs: hours. B. Assessment of the expression and activation of the major components of the ATR-Chk1 and ATM-Chk2 pathways in B-/T-ALL cell lines at baseline. The homogeneity of the protein loaded was determined by using as an internal control (β-actin). C. Reduction of PARP-1 cleavage in leukemia cell lines detected by western blotting. PARP-1 cleavage was detected as a marker of apoptosis. B-actin was detected on the same membrane for loading normalization.

PF-00477736 perturbs the cell cycle profile in B-/T-ALL cells

The effect of the inhibition of Chk1 pathway on cell cycle progression was evaluated in RPMI-8402, BV-173, SUP-B15 and NALM-6 cell lines. Cells were incubated with increasing concentration of PF-00477736 for 6 and 24 hours and then stained with propidium iodide to quantify the DNA amount. The effect of PF-00477736 on the cell cycle progression was very weak after 6 hours of incubation (data not showed) and very heterogeneous among the different cell lines after 24 hours. In RPMI-8402, BV-173 and SUP-B15 cell lines the treatment induced a progressive reduction of the number of cells in S and G2/M phase and a concomitant increment of cell debris. In the less sensitive cell line, NALM-6, the inhibition of Chk1 progressively increased the percentage of cells in G2/M phase and reduced the percentage of cells in G1 phase (Fig. 3S-B).

PF-00477736 efficiently targets Chk1 pathway and induces DNA damage

In order to assess whether PF-00477736 efficiently targeted Chk1 pathway, we examined the changes in the downstream phosphorylation of the phosphatase Cdc25C and cyclin-dependent kinase, Cdc2. Moreover, Chk1 itself and γ-H2A.X, which is a marker of stalling replication forks and checkpoint abrogation-induced apoptosis, have been assessed. Functional analyses were initially performed on the most sensitive (BV-173) and the most resistant (NALM-6) B-ALL cell lines, as determined by the viability and apoptosis analyses. Specifically, BV-173 cells were treated with 0.05, 0.1 and 0.2 μM and NALM-6 with 0.1, 0.5 and 1 μM of PF-00477736 for 24 hours (Fig. 3A). In the second analysis BV-173 and NALM-6 cells were treated with the dose of PF-00477736 that goes near to the IC_{50} (0.1 μM for BV-173 and 1 μM for NALM-6) for 18, 24, 30 and 48 hours (Fig. 3B). Thereafter, the same molecular targets have been assessed on all B- and T-ALL cell lines using the dose that goes near to the IC_{50} (Fig. 4S). Active Chk1 phosphorylates Cdc25C at serine 216 throughout interphase and upon G2 checkpoint activation. This leads to the nuclear export of Cdc25C and its subsequent cytoplasmic sequestration by 14-3-3 protein, which prevents the activation of the downstream target of Cdc25C, the cyclin B/Cdc2 kinase that is responsible for G2/M transition[113]. Upon inhibition of Chk1, we observed decreased levels of phosphorylated Cdc25c (Ser 216) and Cdc2 (Tyr 15) as well as reduced levels of their

total forms in a dose and time-dependent manner, suggesting that PF-00477736 efficiently targets Chk1 pathway. In contrast, the phosphorylated forms of Chk1 (Ser317 and Ser345) were dose-dependently increased suggesting that treatment amplifies genomic damage which in a feedback loop increases Chk1 phosphorylation. Although the increment of the phosphorylated forms of Chk1 (ser317 and ser345) as a consequence of genomic damage amplification, the complete inhibition of Chk1 functionality was confirmed by the progressive reduction of the auto-phosphorylated form of Chk1 (ser296) in a early time point analysis. Indeed even after 3 hours of treatment with PF-00477736 in both BV-173 and NALM-6 cell lines the auto-phosphorylated form of Chk1 (ser296) was drastically reduced in comparison with the untreated counterpart (Fig. 4S-B).The enhancement of genomic damage was also confirmed by the time-dependently increase of γ-H2A.X both in western blot and immunofluorescence analysis. The phospho-H2A.X accumulates and forms characteristic nuclear foci where the DNA is damaged [114] . Both NALM-6 and BV-173 cell lines show an increased number of γ-H2A.X foci when treated with PF-00477736 compared to untreated controls (Fig. 3C). However, evaluating the mean fluorescence intensity of γ-H2A.X positive cells, BV-173 cells seem to be more damaged than NALM-6. Actually, BV-173 treated cells have 5-fold change difference of mean value intensity of γ-H2A.X compared to BV-173 untreated cells, while NALM-6 treated cells have 1.4-fold change difference of mean value intensity of γ-H2A.X compared to their untreated counterparts. In addition to these changes in the overall amount of γ-H2A.X foci among the two different cell types we observed the presence of hyper-γ-H2A.X-positive cells specifically in BV-173 cells treated with PF-00477736. The so called hyper-γ-H2A.X-positive cells loose the typical foci signal of γ-H2A.X and emit a much higher and diffuse signal of γ-H2A.X positivity. Almost 6.5% of the γ-H2A.X-positive cells are hyper-γ-H2A.X-positive in BV-176 treated cells compared to 1.8% in the untreated controls. These data suggest a higher induction of DNA damage upon PF-00477736 treatment in BV-173 than NALM-6 cells as expected by the fact that BV-173 cells are more responsive to PF-00477736. However, the increase of hyper-γ-H2A.X-positive cells only in BV-176 treated cells would suggest the existence of a specific effect that causes a hyper DNA damage only in a restricted, but significant, population of leukemic cells. Interestingly, upon treatment we observed a strong reduction of Chk1. As already

hypothesized this may be the result of cleavage by caspase during apoptosis induced by genotoxic stress[70,115]. Persisting protein levels of Cdc25, pChk1 ser345, pCdc2 (tyr15) and Cdc2 after 48 hours of exposure to PF-00477736 differentiated less sensitive leukemia cells from more sensitive ones(Fig. 3C).

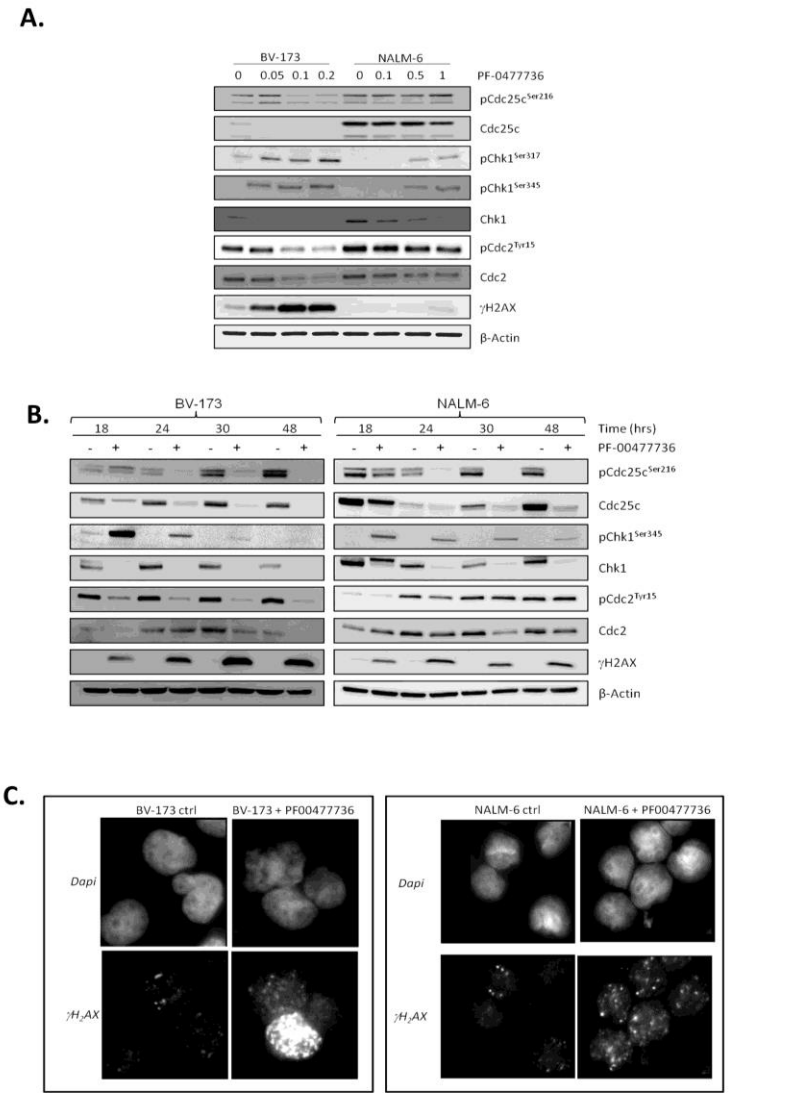


Figure 3. A. Western Blot analysis in BV-173 and NALM-6 cell lines after exposure to increasing concentrations of PF-00477736 (0.05, 0.1 and 0.2 uM for BV-173 and 0.1, 0.5 and 1uM for NALM-6) or DMSO 0.1% (-). B.. Western Blot analysis in BV-173 and NALM-6 cell lines after exposure to PF-00477736 (+) at the concentration closest the IC50 or DMSO 0.1% (-) at 18, 24, 30 and 48 hours. C. Western Blot analysis in all leukemia cell lines after exposure to PF-00477736 (+) at the concentration closest the IC50 or DMSO 0.1%.

Gene expression profiling results

In order to identify gene expression changes specifically correlated with Chk1 inhibition and to better elucidate the mechanism of action of Chk1 inhibitor, GEP analysis was performed by microarray on treated B/T ALL cell lines (BV-173, SUP-B15, REH, NALM-6, NALM-19, MOLT-4, RPMI8402 and CCRF-CEM) and on their untreated counterparts (DMSO 0.1%) after 24 hours of drug exposure. Treatment resulted in a differential expression of 941 genes ($p < 0.05$): 528 (56%) were down-modulated and 413 (44%) were up-regulated in treated leukemia cells compared to untreated cells (Supplementary Material 1S and Fig. 4). To identify peculiar critical pathways affected by Chk1 inhibition, the differentially expressed genes were analyzed in terms of biological function using the Metacore pathway-mapping software (GeneGo Inc) which categorizes genes in pathway maps, Gene Ontology (GO) cellular processes and cellular and molecular process networks. Consistent with a Chk1 mechanism of action, the three top scored maps (map with the lowest p value) were "Cell cycle: estrogen receptor 1 (ESR1) regulation of G1/S transition" including *c-Jun/c-Fos, Cyclin A*, *CARM1*, Skp2/TrCP/*FBXW*, *SKP2*, c-Fos, *CDK4, NCOA3, c-Jun, CDK2* genes (Fig. 5S); "Apoptosis and survival: Granzyme A signaling" including *PHAP1*, *Ku70/80, Ku80, Ku70, Histone H3, NDPK A, SET*, Histone H2B, Histone H1 genes (Fig. 6S) and "DNA damage: ATM/ATR regulation of G1/S checkpoint" including *PCNA,* growth arrest and DNA damage 45 alpha (*GADD45a*)*, Chk2, Cyclin A, NF-kB,* Cyclin-dependent kinase 4 (*CDK4), FANCD2, Claspin, CDK2* genes (Fig. 7S). GO cellular processes highlighted 617 genes involved in cellular metabolic process (Supplementary Material 2S). Finally, the three top scored process networks impaired by the treatment with Chk1 inhibitor were: the DNA damage checkpoint, the S phase of cell cycle and the apoptosis, confirming the specificity of treatment on its target pathways (Supplementary Material 3S). Thirty-five genes had a false discovery rate less than 0.05 (Table 1S) and the three most differentially expressed genes were: DNA damage-inducible transcript 3 (*DDIT3*, fold-change 3.32, *p-value* 6.06E-05), Kruppel-like factor 6 (*KLF6,* fold-change 2.17, *p-value* 8.41E-05) and FBJ murine osteosarcoma viral oncogene homolog (*FOS*, fold-change 2.40, *p-value* 1.97E-04). Due to its implication in the regulation of the cell cycle and apoptosis, western blot analysis against c-jun was performed to better comprehend its biological role in response to PF-00477736. C-jun is a component of the transcription factor

activating protein-1 (AP-1) which is involved in cell cycle progression through the G1 phase by the regulation of the cyclin D1[116]. As soon after treatment with Chk inhibitor c-jun protein levels increased in all B-ALL cell lines but not in all T-ALL cell lines in which the response to the treatment was very heterogeneous (Fig. 8S-A). The data found in the gene expression profile analysis were validated using qPCR. Four genes (two up-regulated,PLK3 and Gadd45a; two down-regulated, Chk2 and CDK4), that have been chosen on their biological relevance in the Chk1 pathway, were validates on B-/T-ALL cell lines treated for 24 hours with or without PF-00477736 (IC50) (Fig.8S-C).

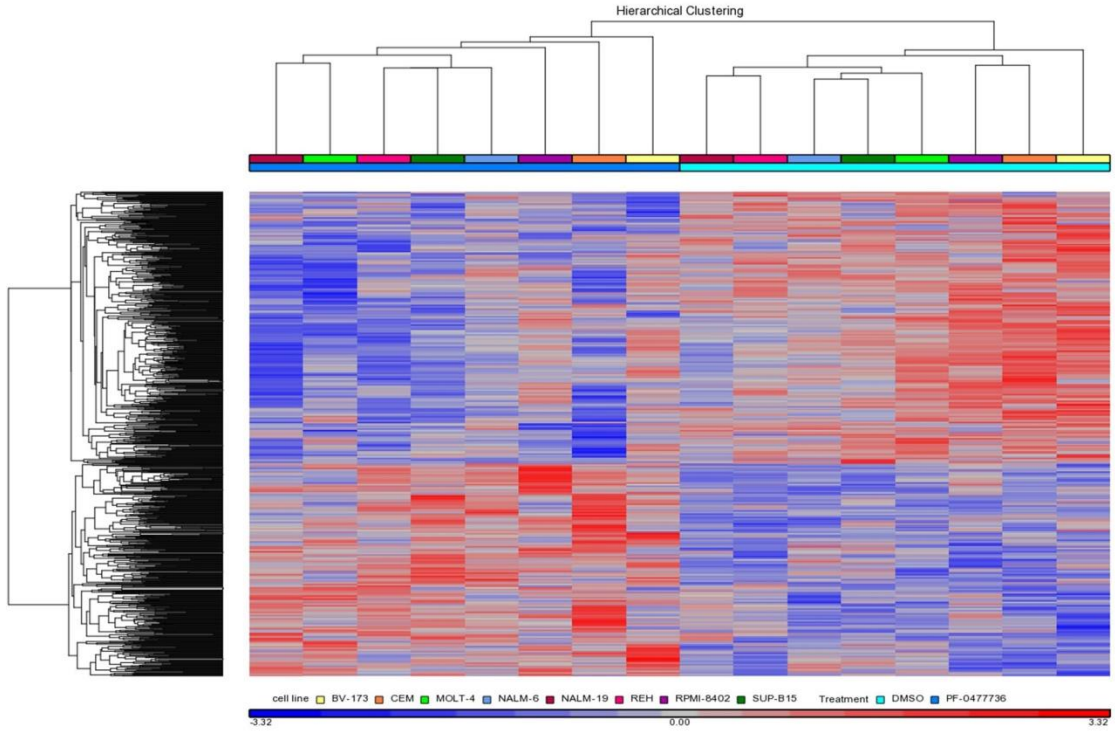


Figure 4. Heatmap of differentially expressed genes. Lists of differentially expressed genes with a *t*-test *P*-value < 0.05 were generated for each cell line. A hierarchical clustering method in Partek Genomics Suite was used to construct both the gene tree and the sample tree. Data are shown in a matrix format: each raw represents a single gene, and each column represents a cell line. Red indicates overexpressed genes and blue indicates underexpressed genes (see legend).

PF-00477736 reduces viability in primary ALL blast cells

The efficacy of PF-00477736 was confirmed in primary blast cells from 14 newly diagnosed cases including 10 *BCR-ABL1*-positive (71%) cases and 4 *BCR-ABL1*-negative ALL cases (29%). Leukemic cells were incubated with increasing concentrations of drug (0.1, 0.5 and 1 µM) for 24 hours. Based on the viability results, three groups of patients were identified: very good responders, 36% (5/14) with IC_{50} at 24 hours ranging from 0.1 and 0.5 µM; good responders, 43% (6/14) with IC_{50} at 24 hours ranging from 0.5 and 1 µM; poor responders, 21% (3/14) with IC_{50} at 24 hours higher than 1 µM (Table 2S and Fig. 5A). PF-00477736 did not reduce viability in primary cultures of normal bone marrow mononuclear cells, demonstrating that it selectively targets leukemia cells (Fig. 5B). Moreover, in contrast to leukemia cells, treatment did not induce protein phosphorylation changes in Chk1 (Ser 345), Cdc25c (Ser 216) and Cdc2 (Tyr 15) neither increased levels of phospho-H2A.X (Fig.8S-B).

PF-00477736 impairs survival of leukemic mice

We extended the *in vitro* and *ex-vivo* studies by assessing the efficacy of Chk inhibitor in mice transplanted with T-ALL.

Leukemic mice were generated by the usage of the tumorigenic agent ENU. The obtained leukemia has been immunophenotyped and characterized as a T-ALL. Leukemic blasts coming from the spleen of leukemic animals were transplanted into C57BL/6Ly5.1 recipient mice. Three days after transplantation, a time sufficient for leukemic blasts to home the bone marrow of the host, we started to treat the animals. A concentration of 40mg/kg of PF-00477736 given with a q3dx4 schedule seems not to be toxic for the animals, however it significantly affects the overall survival of the treated animals (5 mice) in comparison to the untreated ones (7 mice) (p-value=0.0009). Actually control animals (intraperitoneally injected with PBS) die all at day 17 post-transplantation while the animals treated with PF-00477736 live longer (up to 59 days post transplantation). The leukemia used for the experiments shown here was very aggressive

(untreated animals die with massive infiltration of the spleen and the liver by leukemic blasts at day 17) suggesting a reason why the effect of the Chk inhibitor is significant though quite variable among treated mice (Fig. 5C).

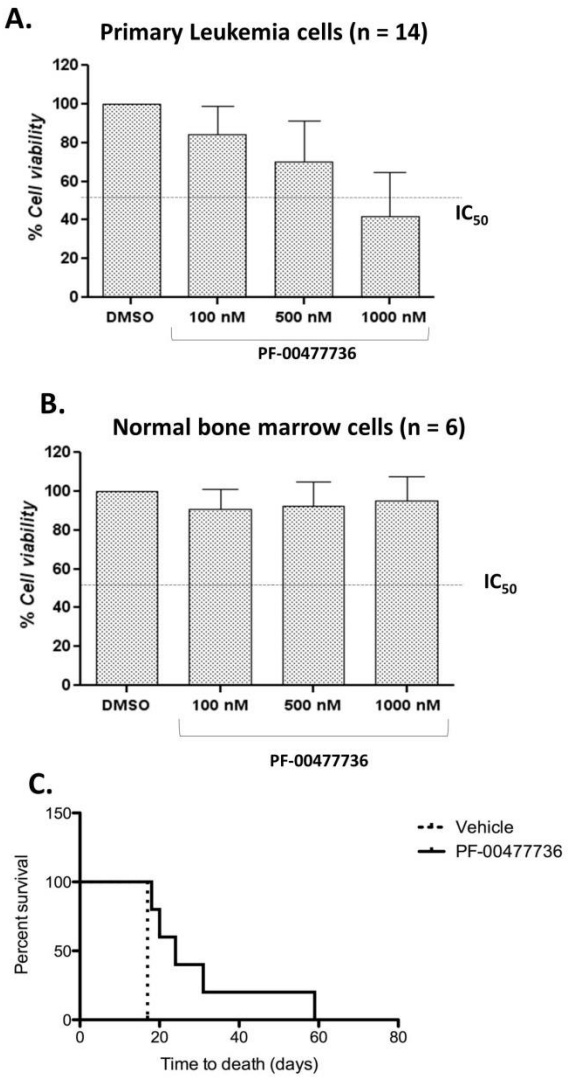


Figure 5. A. Cell viability results in primary ALL blast cells after 24 hours of exposure to PF-0477736 at 100, 500 and 1000 nM. B. Cell viability results in normal bone marrow mononuclear cells after 24 hours of exposure to PF-0477736 at 100, 500 and 1000 nM. C Survival curves of leukemic mice treated with PF-0477736 and control mice injected with vehicle. PF-0477736 treatment significantly increases the overall survival of mice transplanted with murine lymphoid leukemia (*p*-value = 0.0009).

Discussion

Several studies in different tumors have investigated the role of Chk inhibition in combination with conventional chemotherapy demonstrating that this combination enhances tumor cell death [6, 10]

Here, we evaluated the *in vitro* and *in vivo* effects of the administration of PF- 00477736 as a single agent and not in combination with other chemotherapeutic agents in cell lines and primary blast cells from of acute lymphoblastic leukemia based on the hypothesis that the intrinsic genomic instability of leukemic clones as demonstrated by the nuclear labeling for γ-H2A.X molecule in 68% of ALL patients may be itself sufficient to bring the cells to apoptosis. According to this hypothesis, it was possible to highlight how the administration of PF- 0477736 as single agent was able to reduce cell viability in all leukemia cell lines treated in this study (BV -173, SUP- B15 , REH , NALM -6 , NALM -19 , MOLT -4, RPMI8402 and CCRF -CEM) and to induce apoptosis (Fig. 6). Different leukemia cell lines showed different sensitivity to PF-00477736 with RPMI-8402 (T-ALL) being the most sensitive (IC_{50} = 57.4 at 24 hours), while NALM-6 (B-ALL) the less sensitive (IC_{50} = 1,423.0 at 24 hours) cell line. Interestingly, the sensitivity to Chk1 inhibitor was not related to the mutational status of the tumor suppressor p53.

The results obtained on cell viability and induction of apoptosis have been confirmed by genome-wide studies evaluating global gene expression changes upon treatment and by functional studies performed by western blot analysis. Interestingly, by GEP analysis the majority of genes down-regulated were involved in the DNA damages response and in particular in DNA repair mechanisms, while the genes up-regulated were involved in chromatin assembly, nucleosome organization, DNA packaging and apoptosis. The efficacy of Chk inhibition has been evaluated and confirmed in term of reduction of cell viability in primary ALL blast cells but not in normal bone marrow precursor cells. Furthermore, assessing the efficacy of Chk inhibition in mice transplanted with T-lymphoid leukemia, we demonstrated that PF-0477736 increases the survival of treated mice compared with mice treated with vehicle ($p = 0.0016$).

In conclusion, *in vitro*, *ex-vivo* and *in vivo* results support the inhibition of Chk1 as a new therapeutic strategy in acute lymphoblastic leukemia and they provide a strong rationale for its future clinical investigation. The checkpoint kinase inhibitor have been synthetized to increase the effectiveness of conventional chemotherapy, preventing cells to arrest cell cycle and to repair the DNA damages caused by the exposure to genotoxic agents. Here we highlight that even the treatment with a checkpoint kinase inhibitor alone associated with the high genetic instability, can be enough to kill cancer cells. Indeed we believe that leukemic cells, thanks to a higher activation of the ATR-Chk1/ATM-Chk2 pathways, can better tolerate the high genetic instability. Switching off these mechanisms of survive we can kill leukemic cell by overcoming the cell cycle checkpoint, by inhibiting the mechanisms of DNA damages repair and by inducing massive damages that cannot be tolerate (Fig. 6).

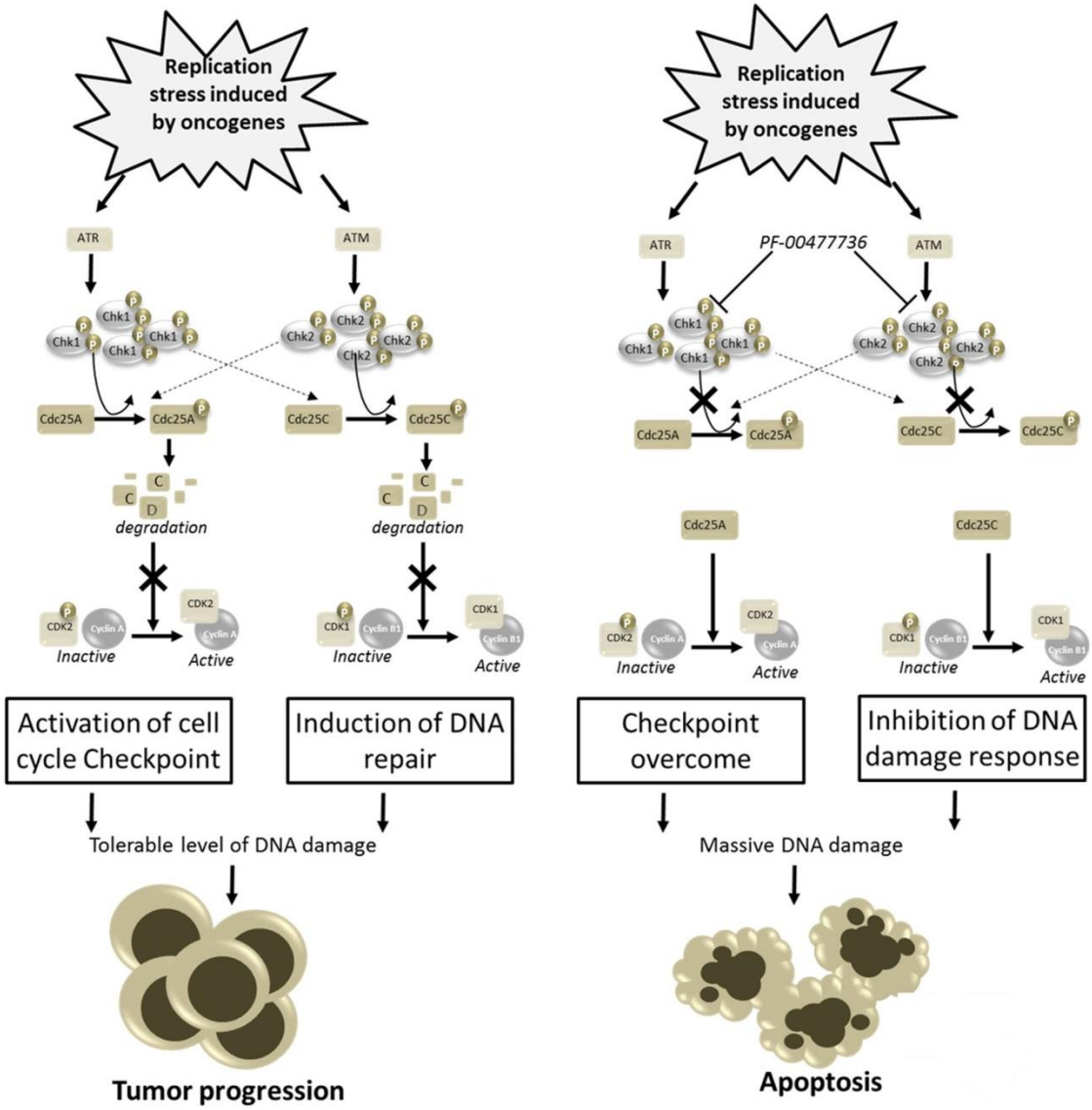


Figure 6. Cartoon drawing the rational of the study: in leukemia cells oncogenes could lead to replication stress, activation of ATR/Chk1 pathway that in conjunction with elevated proliferation promote tolerable level of DNA damage, genomic instability and tumor progression. Inhibition of Chk1/Chk2 could increase DNA damage leading to apoptosis

Conclusions

Inhibition of Chk1/2 represents a novel therapeutic strategy to overcome genetic instability and to promote selective killing of leukemia cells in ALL.

Methods

Leukemia cell lines

Human B- (BV-173, SUP-B15, REH, NALM-6, NALM-19),T-ALL (MOLT-4, RPMI-8402, CCRF-CEM) cell lines were obtained from Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Germany). Cells were cultured in RPMI-1640 medium (Invitrogen, Paisley, UK) with 1% l-glutamine (Sigma, St. Louis, MO), penicillin and streptomycin (Gibco, Paisley, UK) supplemented with 10%-20% fetal bovine serum (Gibco) in a humidified atmosphere of 5% CO2 at 37°C. Online databases have been interrogated to molecularly characterize leukemia cell lines: International Agency for Research on Cancer (IARC) TP53 database (http://www-p53.iarc.fr/) and the Catalogue of Somatic Mutations in Cancer, (COSMIC, http:// http://www.sanger.ac.uk/genetics/CGP/cosmic/). The Chk1 inhibitor PF-00477736 was purchased by Sigma-Aldrich (Sigma-Aldrich Co. St. Louis, Missouri 63103 United States).

Reagents

The Chk inhibitor PF-00477736 was purchased by Sigma-Aldrich (Sigma-Aldrich Co. St. Louis, Missouri 63103 United States). qPCR analysis Chk1 and Chk2 mRNA expression was evaluated in 41 (76%) newly diagnosed BCR-ABL1-positive (median age 56 years, range 26-81; ratio male/female: 20/21) and 13 (23%) newly diagnosed BCR-ABL1-negative ALL cases (median age 41 years, range 18-69; ratio male/female: 5/8). One microgram of RNA was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). PCR analysis was performed using HS00967506_m1 (Chk1) and HS00200485_m1 (Chk2) assays (Applied Biosystems) and the Fluidigm Dynamic Array 48 x 48 system, a real-time quantitative polymerase chain reaction (qPCR) assay which enables to automatically assemble 48 samples and 48 assays to create individual TaqMan reactions of a final volume of 6.75 nanolitres each (Fluidigm, San Francisco, CA, http://www.fluidigm.com/). RNA integrity was confirmed by PCR

amplification of the GAPDH mRNA (Hs99999905_m1), which is expressed ubiquitously in human hematopoietic cells. Results were expressed as 2exp(-ΔΔCt). GraphPad Prism 5 software (GraphPad, Avenida de la Playa La Jolla, CA USA) was used to plot the data. The basal mRNA expression of Chk1 and of Chk2 was evaluated in all the cell lines using the same assays (Applied Biosystems: HS00967506_m1 Chk1 and HS00200485_m1 Chk2) used for the primary samples and the statistical validity of the results were confirmed using ANOVA multiple comparisons test (GraphPad Prism 5 software) In addition, reverse transcription and quantitative-PCR analysis for GADD45a, PLK3, CDK4 and CHK2 in treated cell lines and on their untreated counterpart were performed as upper described using the following assays from Applied Biosystems: Hs00169255_m1 (Gadd45a), Hs00177725_m1 (PLK3), Hs00262861_m1 (CDK4) and HS00200485_m1 (Chk2).

TP53 mutation screening

Total cellular RNA was extracted using the RNeasy total RNA isolation kit (Qiagen, Valencia, CA). One microgram of total RNA was reverse transcribed using the M-MLV Reverse Transcriptase (Invitrogen, San Diego, CA). Three overlapping shorter amplicons [amplicon 1 (491 bp): exons 1-5; amplicon 2 (482 bp): exons 5-8; amplicon 3 (498 bp): exons 8-11)] covering the entire TP53 coding sequence (GenBank accession number NM_000546.4) were amplified with 2U of FastStart Taq DNA Polymerase (Roche Diagnostics, Mannheim, Germany), 0.8 mM dNTPs, 1 mM MgCl2, and 0.2 M forward and reverse primers (Table 3S) in 25 µl reaction volumes. PCR products were purified using QIAquick PCR purification kit (Qiagen) and then directly sequenced using an ABI PRISM 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA) and a Big Dye Terminator DNA sequencing kit (Applied Biosystems). All sequence variations were detected by comparison using the BLAST software tool (www.ncbi.nlm.nih.gov/BLAST/) to reference genome sequence data (GenBank accession number NM_000546.4).

Immunohistochemistry

Studies evaluating Chk1, phosphorylated Chk1 (Ser345), Chk2, phosphorylated Chk2 (Thr68) CDc25C, phosphorylated Cdc25C (Ser216) and phosphorylated H2A.X (Ser139) (γ-H2A.X) proteins expression were performed on FFPE samples corresponding to three thymuses, three reactive lymph nodes and to primary tumours collected from 60 ALL patients at diagnosis, including 36 B-ALL (median age 50 years, range 5-86; ratio male/female: 18/18) and 24 T-ALL (median age 38 years, range 2-76; ratio male/female: 15/9). All the cases were retrieved from the archives of the Haematopathology Unit, Department of Experimental, Diagnostic and Specialty Medicine - DIMES, University of Bologna. The study was conducted according to the principles of the Helsinki declaration after approval of the Internal review Board. Two different tissue-microarrays (TMAs) were constructed from these paraffin-embedded blocks as previously reported. TMAs sections were investigated by antibodies raised against fixation resistant epitopes. The antibody reactivity and sources as well as the antigen retrieval protocols, dilutions and revelation systems are detailed in Table 4S. A cut-off of staining > 30% of the examined cells was assigned as positive score, according to formerly defined criteria[117]. Immunohistochemical preparations were visualized and images were captured using Olympus Dot-slide microscope digital system equipped with the VS110 image analysis software.

Primary cells

Primary blast cells from 14 newly diagnosed ALL cases were obtained, upon written informed consent, from bone marrow and peripheral blood samples by density gradient centrifugation over Lymphoprep (Nycomed UK, Birmingham). 10 (71%) samples were from adults with BCR-ABL1-positive ALL (median age 51 years, range 25-74 years; median blast percentage 92%, range 60-100%) and 4 (29%) from patients with BCR-ABL1-negative ALL (median age 34 years, range 18-43 years; median blast percentage 93%, range 90-100%). Main patient's characteristics are given in Table 1S.

Normal bone marrow progenitors were harvested from bone marrow aspirations performed in lymphoma patients undergoing initial staging procedures, which then resulted negative for lymphoma infiltration. Normal peripheral blood progenitors were harvested from healthy donors.

Cell viability assay

In order to assess the cell viability after treatment with PF-00477736 (Pfizer), ALL cell lines were seeded in 96-well plates at 50,000 cell/100 µl/well with increasing concentrations of drug (0.005-2 µM) for 24 and 48 hours and incubated at 37°C. Cell viability was assessed by adding WST-1 reagent (Roche Applied Science, Basel, Switzerland) to the culture medium at 1:10 dilution. Cells were incubated at 37°C and the optical density was measured by microplate ELISA reader at λ450 after 3 hours. The amount of the formazan formed directly correlates to the number of metabolically active cells. All viability experiments were performed in triplicates and repeated in least two separated experiments. 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 (0.1, 0.5 and 1 µM) 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%).

Annexin V staining of apoptotic cells

According to the WST-1 results, three different increasing concentrations of PF-00477736 were used to treat leukemia cells lines in order to detect and discriminate apoptotic, necrotic and dead cells. Cell lines were seeded in 12-well plates at 500,000 cell/1 ml with increasing concentrations of drug (0.1, 0.5 and 1 µM and 0.05, 0.1 and 0.2 µM only for BV-173 and RPMI-8402) for 24 and 48 hours and incubated at 37°C. Following the treatment, cells were harvested and stained with Annexin V/Propidium Iodide (PI) according to the manufacturer's instruction

(Roche). The percentage of Annexin V-PI positive cells was determined within 1 × 104 cells of the population by flow cytometry (Facs CantoII, BD Biosciences Pharmingen, San Jose, California, USA). The mean percentage of Annexin V-PI positive cells and standard error measurement was calculated from at least two separate experiments.

Western Blot analysis

To gain insight into the molecular mechanisms responsible for cell death following treatment, functional analyses by western blot were performed. Leukemia cell lines were plated in 6-well plates at 500,000 cell/1 ml with increasing concentrations of drug for 24 hours and incubated at 37°C. After the treatment, the cells were collected and lysate using a specific buffer made of KH2PO4 0,1 M (pH 7,5), Igepal 1% (NP-40), β-glicerofosfato 0,1 mM and complete protease inhibitor cocktail 1X (Roche Diagnostics).For each sample 30ug of protein were fractioned on Mini-Protean TGX stain-free precasted gels, blotted to nitrocellulose membranes (Bio-Rad Trans-blot turbo transfer pack) and incubated overnight with the following antibodies: ATM (#2873S), phosphorylated ATM (Ser1981)(#5883S), ATR (#2790S), phosphorylated ATR (Ser428)(#2853S), Chk1 (#2345S), phosphorylated Chk1 (Ser317)(#2344S), phosphorylated Chk1 (Ser296)(#2349) and Chk1 (Ser345)(#2348S), Chk2 (#2662S), phosphorylated Chk2 (Thr68)(#2661S), Cdc25c (#4688S), phosphorylated Cdc25C (Ser216)(#9528S), Parp-1 (#9542), Cdc2 (#9112S), phosphorylated Cdc2 (Tyr15)(#4539S), phosphorylated H2A.X (Ser139) (γ-H2A.X) (#2577S), from Cell Signaling. Antibody to β-actin came from Sigma (St. Louis, MO). Finally all these antibodies were detected using the enhanced chemiluminescence kit ECL (GE) and the compact darkroom ChemiDoc-It (UVP).

Immunofluorescence analysis

NALM-6 and BV-173 cells were seeded to poly-D lysine-coated slides, fixed with 4% PFA (Paraformaldehyde) and stained at 37°C with a mouse anti-H2A.X-Phosphorylated Alexa 647 conjugated antibody (BioLegend). Then they were treated with DAPI (4.6 diamidino-2-phenylindole; Sigma Aldrich) and the slides were mounted with Mowiol (Calbiochem). Images

were acquired with wide field fluorescence microscope Olympus BX61 fully motorized driven by Metamorph software and the analysis was performed using the freeware ImageJ software.

Gene expression profiling

Gene expression profiling on treated and untreated cells (DMSO 0.1%) after 24 hours of exposure to PF-0077736 were performed using Affymetrix GeneChip Human Gene 1.0 ST platform (Affymetrix Inc. Santa Clara, California, USA) and following manufacturers' instructions. 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) using the Partek Genomics Suite software (Partek Incorporated Saint Louis, MO 63005, USA, http://www.partek.com). The most significantly involved process networks were defined by the Metacore software (GeneGo Inc., www.genego.com).

Cell cycle analysis

In order to evaluate the effect of PF-00477736 on cell cycle progression, NALM-6, SUPB-15, RPMI-8402 and BV-173 cell lines were seeded in a 24 wells plate at 500,000 cell/1 ml with increasing concentrations of PF-00477736 (respectively 0.1, 0.5 and 1 μM for NALM-6 and SUP-B15; 0.05, 0.1 and 0.2 μM for RPMI-8402 and BV-173). After 6 and 24 hours cells were harvested and fixed overnight using ethanol at 70%. Then cells were stained using PI/RNase Staining Buffer according to the manufacturer's instruction (BD Pharmingen) and the cell cycle profile was detected using FACSCanto II instrument. The percentage of the different cell cycle phases was performed using the DNA cell-cycle analysis software for flow cytometry data, ModFit LT (Verity).

In vivo Studies

Experiments involving mice were performed in agreement with Italian guidelines and after the approval of the Institutional Review Board of the European Institute of Oncology. Leukemic mice were generated by a single administration of the tumorigenic agent N-ethyl-N-nitrosourea

(ENU, Sigma, 50mg/kg intraperitoneally). Spleen cells from leukemic C57BL6/Ly5.2 mice were injected intravenously (2×106 cells/mouse) into non irradiated, recipient C57BL6/Ly5.1 mice. PF-0077736 was administered intraperitoneally starting from day 3 after the transplant of leukemic cells. Mice received doses of PF-0077736 (40mg/kg each dose) every three days for four treatments (q3dx4). Kaplan-Meyer rank test was used to compare the survival rate between treated and control mice.

Competing interests: Giovanni Martinelli: Novartis - BMS - Roche - Pfizer- ARIAD - MSD: Consultant; Novartis - BMS: Speaker Bureau.

Author's contributions: GM and II were the principal investigators of the study and gave final approval; II, ED and CA coordinated the research; II, AGLDR, MVFF and CA wrote the paper; II, AGLDR, MVFF, AF, AL, SR, EI, SP, CV, VG, FC, performed the laboratory work for this study; CP, MCA, AV, LE, EO contributed to sample collection; DR, PLZ, SP, PGP and GM contributed to data interpretation.

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Supplementary figures

Figure 1S

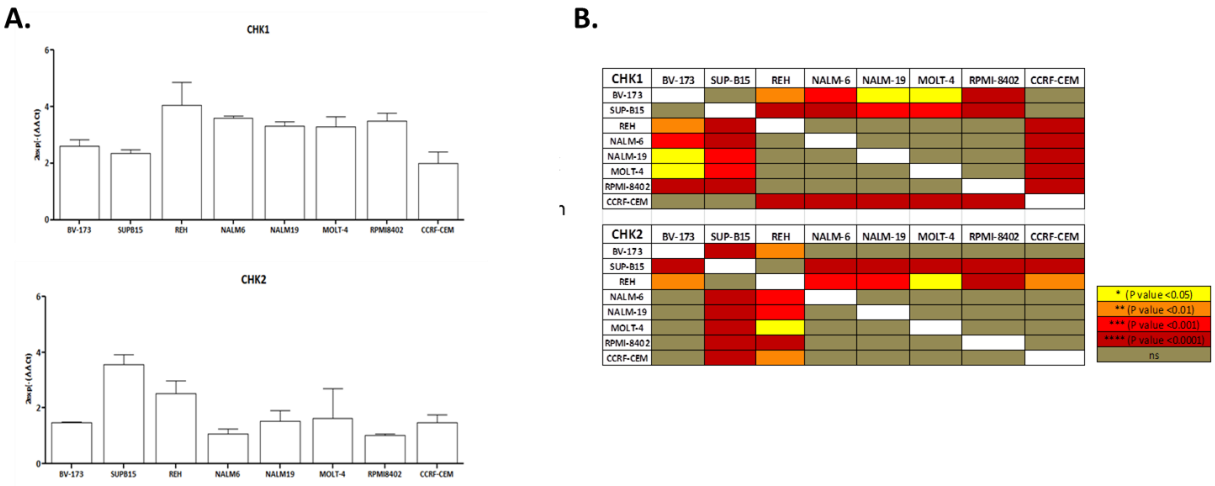

Figure 1S: Basal expression of Chk1 and Chk2 (mRNA level) in the cell lines treated, the results are expressed as 2exp(-ΔΔCt) (A). Schematic representation of the statistically significance of the ANOVA multiple comparison test in which the basal expression of each cell lines is compared to the value of the basal expression of all the other cell lines. In the legend on the right the increasing statistical significance of the analysis is showed with different color: bright yellow (p value <0.05), dark yellow (p value <0.01), bright red (p value <0.001), dark red (p value <0.0001) and gray (no statistical significance of the comparison). For the ANOVA analysis of the basal expression of Chk1 and of Chk2 for each cell lines were compared, respectively six and four different replicates (B).

Figure 2S

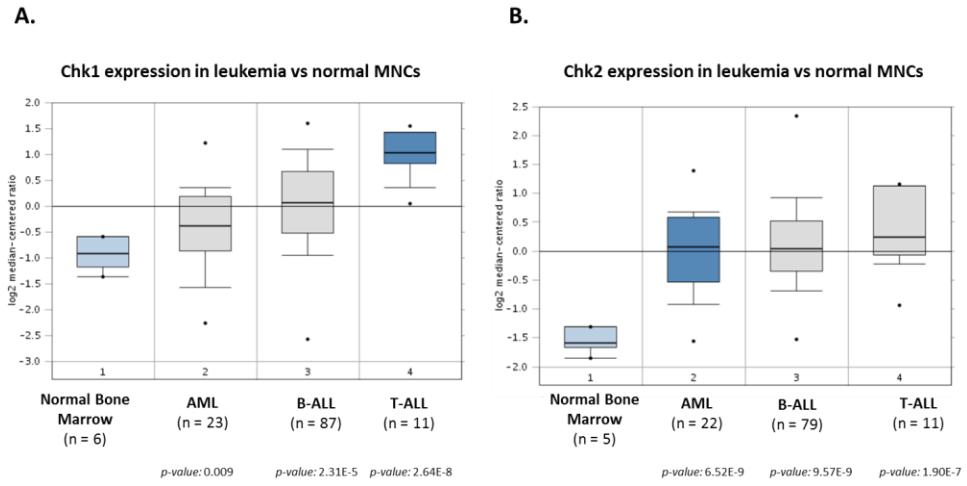

Figure 2S: Oncomine expression analysis of Chk1 (A) and Chk2 (B) levels between normal monunuclear cells (MNCs) from bone marrow and different subtypes of leukemia (AML, B-ALL, T-ALL). Expression values are log transformed and median centered per array. Differential expression is identified by a permutation 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. Chk1 transcript levels between normal MNCs and leukemia samples are extracted from the study of Andersson A et al [Leukemia. 2007 Jun;21(6):1198-203]. Dot points indicate the minimum and the maximum values in each dataset.

Figure 3S

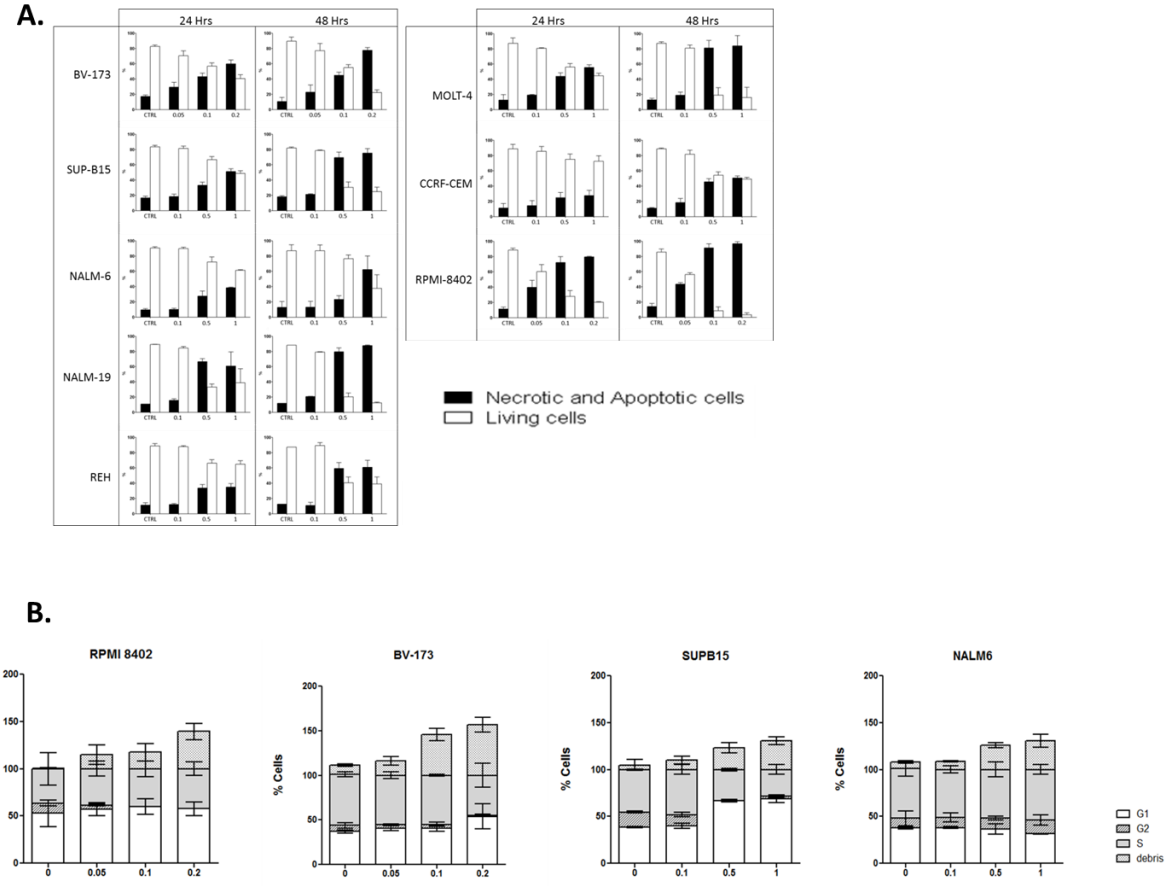

Figure 3S: Apoptosis following treatment with PF-00477736 at 24 and 48 hours in B-ALL and T-ALL cells. The Annexin V/PI staining analysis showed increase of apoptosis at 24 and 48 hours in B-ALL cell lines proportional to drug-dose and drug-exposure time. These graphs reproduce the mean of two independent experiments (A). Cell cycle analysis of RPMI-8402, BV-173, SUP-B15 and NALM-6 cell lines treated for 24 hours with increasing concentrations of PF-00477736. RPMI-8402

Figure 4S

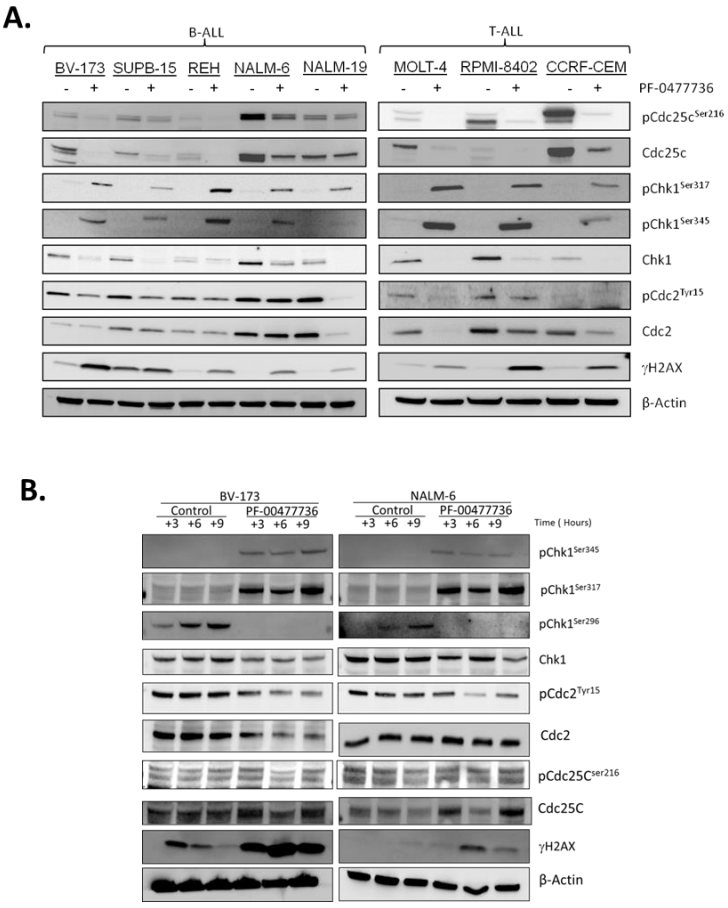

Figure 4S: Western Blot analysis in all leukemia cell lines after exposure to PF-00477736 (+) at the concentration closest the IC50 or DMSO 0.1%(-) (A). Western Blot analysis of BV-173 and NALM-6 cell lines treated with or without PF-00477736 (IC50) for 3, 6 and 9 hours (B).

Figure 5S

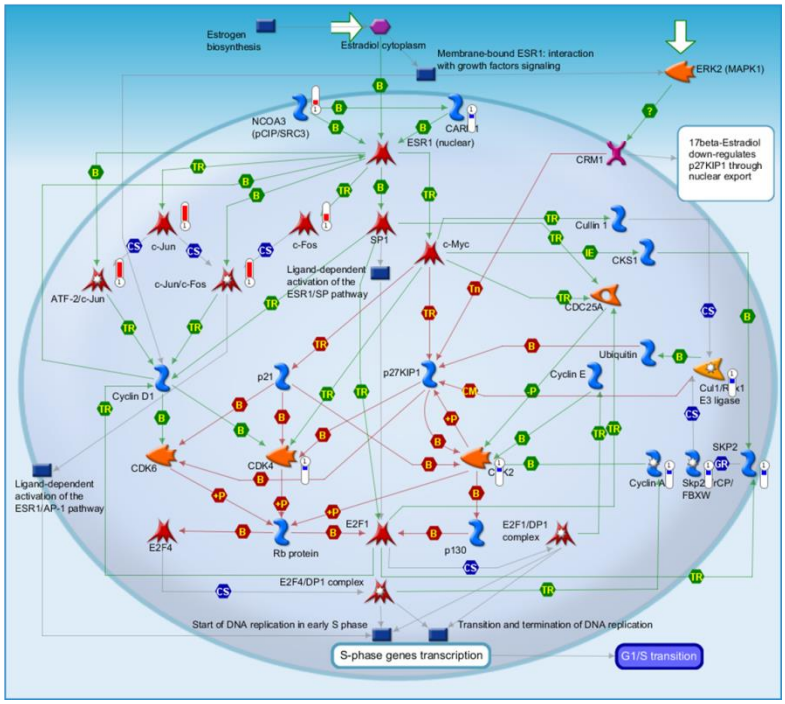

Figure 5S: Cell cycle_ESR1 regulation of G1/S transition: the top scored map (map with the lowest p-value) based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes

Figure 6S

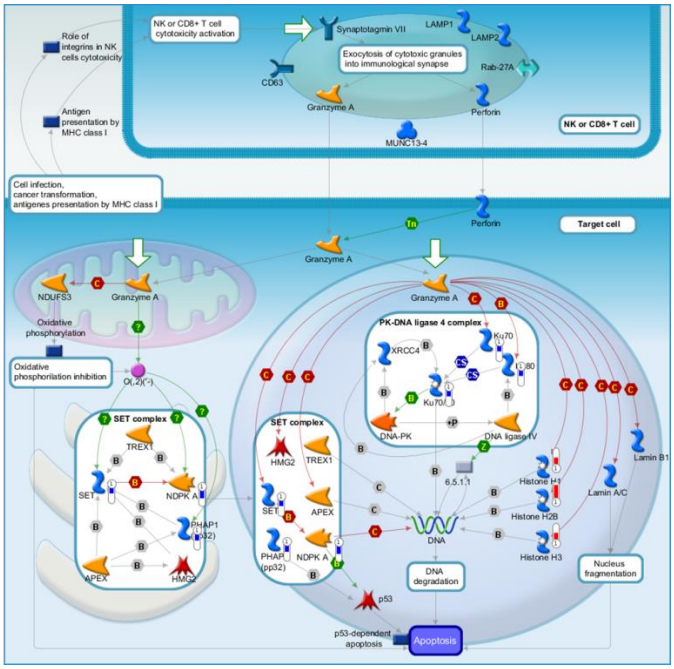

Figure 6S: Apoptosis and survival_Granzyme A signaling: the second scored map (map with the second lowest p-value) based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

Figure 7S

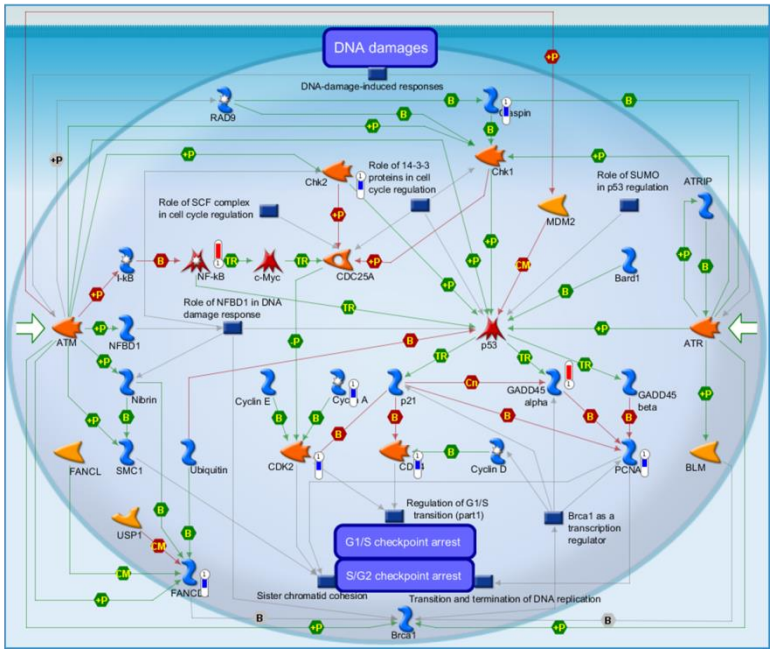

Figure 7S: DNA damage_ATM/ATR regulation of G1/S checkpoint: the third scored map (map with the third lowest p-value) based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

Figure 8S

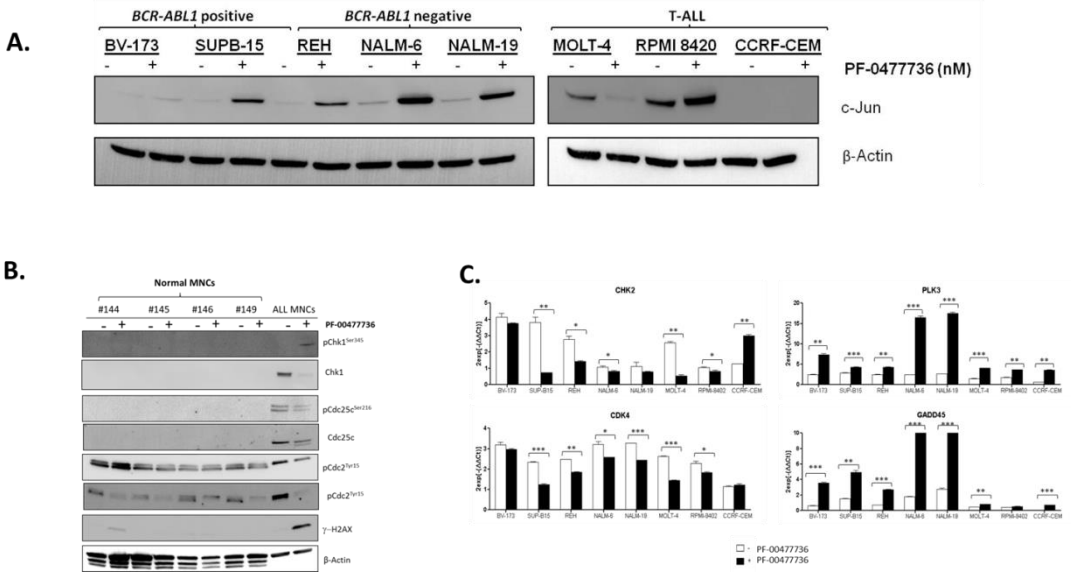

Figure 8S: Protein expression level of c-Jun in B-/T-ALL cell lines. The treatment with PF-00477736 increases the expression of c-Jun in all B-ALL cell lines but not in all T-ALL cell lines (A). Western Blot analysis of Normal MNCs and ALL MNCs after exposure to PF-00477736. Normal MNCs (#144, #145, #146 and #149) and ALL MNCs samples were exposed to PF-00477736 at the concentration of 1000 nM for 24 hours. For each sample 30 ug of protein were loaded and for housekeeping β-Actin was used (B). mRNA expression of CDK4, Chk2, Gadd45a and PLK3 in B-/T-ALL cell lines treated with or without PF-00477736(IC50 value) for 24 hours. The results are expressed as 2exp(-ΔΔCt)(C).

Table 1. Immunohistochemical results. Abbreviation: *pos* means number of positive cases/number of evaluable cases.

Tumor	**Chk1**		**pChk1**		**Chk2**		**pChk2**		**CDc25C**		**pCDc25C**		**γH2A.X**	
Type	pos	(%)	pos	(%)	pos	(%)	pos	(%)	pos	(%)	Pos	(%)	pos	(%)
ALL	51/54	(94)	45/56	(80)	55/57	(96)	15/55	(27)	57/57	(100)	38/55	(70)	40/59	(68)
B-ALL	*31/31*	*(100)*	*28/33*	*(85)*	*32/33*	*(97)*	*11/32*	*(33)*	*34/34*	*(100)*	*23/32*	*(72)*	*25/36*	*(69)*
T-ALL	*20/23*	*(87)*	*17/23*	*(74)*	*23/24*	*(96)*	*4/23*	*(17)*	*23/23*	*(100)*	*15/23*	*(65)*	*15/23*	*(65)*

Result II

LY2606368

Under minor revision (Oncotarget)

LY2606368, Chk1/Chk2 inhibitor, increases the effectiveness of conventional therapy in B-/T-Acute Lymphoblastic Leukemia.

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Keywords: CHK1, Cell Cycle, Acute Lymphoblastic Leukemia, DNA damage response, Chemo-sensitizer agent

Abstract

During the last few years many Chk1/Chk2 inhibitors have been developed for the treatment of different type of cancer. In this study we evaluated the efficacy of the Checkpoint kinase 1/2 inhibitor, *LY2606368* mesylate monohydrate, in B-/T-ALL as single agent and in combination with other drugs. The *LY2606368* deeply reduced the cell viability in a dose and time dependent manner in all the treated cell lines. The cytotoxic activity was confirmed by the increment of apoptotic cells (Annexin V/Propidium Iodide staining), by the increase of γH2A.X protein expression and by the activation of different apoptotic markers (Parp-1 and pro-Caspase3 cleavage). Furthermore, the inhibition of Chk1 deeply changed the cell cycle profile. In order to evaluate the chemo-sensitizer activity of the compound, different cell lines were treated for 24 and 48 hours with *LY2606368* in combination with other drugs (imatinib, dasatinib and clofarabine). The results from cell line models were strengthened in primary leukemic blasts

isolated from peripheral blood of adult acute lymphoblastic leukemia patients. In this study we highlight the mechanism of action and the effectiveness of *LY2606368* as single agent or in combination with other standard drugs like imatinib, dasatinib and clofarabine in the treatment of B-/T-ALL.

Introduction

Nowadays the efficacy of treatments for adult Acute Lymphoblastic Leukemia (ALL) is still very poor with the exception of particular sub-types, like the *BCR-ABL1* positive once (Philadelphia-positive), which are successfully treated with targeted drugs, like the tyrosine kinase inhibitors (TKIs)[118,119]. Although enormous progresses have been done in the treatment of ALL, several sub-types continue to have a very poor prognosis due to not adequate treatment. Moreover a large percentage of patients, initially successfully treated, develop resistance to treatment and consequently relapses. Thus, there is a need to improve therapeutic approaches. Recently, many international research groups have focused their studies on the potential efficacy of the inhibition of specific kinases involved in the regulation of the cell cycle and in the DNA damage response (DDR)[120]. In all eukaryotic cells, the cell cycle, is finely regulated by three different checkpoints (G1/S, intra-S and G2/M checkpoints) that control the transition from a specific phase of the cell cycle to another [121]. The G1/S checkpoint is mainly controlled by the tumor-suppressor p53[122] that regulates the transition through the G1 phase by enhancing the transcription of p21Waf1/Cip1 protein, a cyclin-dependent kinase (Cdk) inhibitors [123]. The transition through the S and the G2/M phase is regulated by Checkpoint kinase 1 (Chk1) and at a lower rate by Checkpoint kinase 2 (Chk2). Chk1 and Chk2 are two serine-threonine kinases involved in the response to DNA damages and, in particular, in the response to Single Strand Breaks (SSBs) and Double Strand Breaks (DSBs) of the DNA[124]. Numerous stimuli, like Ionizing Radiation (IR), X-ray exposure, replicative stress and chemotherapy drugs, activates Chk1/Chk2 response[125]. Chk1 and Chk2 are activated, via phosphorylation, respectively by ataxia telangiectasia and Rad3-related (ATR) and by ataxia telangiectasia mutated protein (ATM)[126–128]. As consequence of their activation, both

Chk1/Chk2 arrest the cell cycle, activate DNA damages repair and promote cell death via apoptosis. Although no hereditary mutations of Chk1 or Chk2 have been found to promote tumor transformation, in numerous type of cancer the expression of these kinases have been found altered. In particular in many tumors the over-expression of Chk1 has been related to the resistance to treatments and to the increase of genetic instability. Due to their biological relevance, numerous checkpoint kinases inhibitors (Chk-i) have been developed in order to potentiate the efficacy of different antineoplastic drugs and to increase the cytotoxicity of radiotherapy [57,129–131,60,132,133]. Currently although the number of publications and of clinical trials regarding the evaluation of the efficacy of Chk-i in solid tumor is in constantly growth [19,20], only few studies have been done to demonstrate their efficacy in the treatment of hematologic malignancies [132] and even a lower number specifically in ALL[133]. Our group has recently published the efficacy in single agent of PF-0477736, a small Chk1/Chk2 inhibitor, on a panel of different B-/T-ALL cell lines and on primary leukemic cells isolated from adult B-ALL patients. The results of the study showed that Chk1 kinase, but not Chk2, was significantly over-expressed in ALL patients in comparison to normal mononuclear cells and that the inhibition of Chk1/Chk2 functionality was enough to deeply reduced the cell viability and induced apoptosis on ALL primary cells and cell lines [133]. Starting from this background, in this study, we evaluated the *in vitro* efficacy of the *LY2606368* mesylate monohydrate (hereafter referred to *LY2606368*), a novel Chk1/Chk2 inhibitor, in B- and T-progenitor ALL as single agent or in combination with different drugs like TKIs and other chemotherapy drugs like purine nucleoside analogue clofarabine. The *LY2606368* is a small molecule that acts as a selective ATP competitor inhibitor of Chk1 and Chk2[135] proteins. Recently, the *in vitro/in vivo* effectiveness of the compound as a chemo sensitizer agent was assessed on different kinds of tumor models[76]. Nowadays this molecule is part of a clinical phase I study in patients with advance cancer as single agent, NCT01115790, and in combination with other chemotherapy drugs or radiotherapy (NCT02124148, NCT02555644). The chemo-sensitizer activity, in term of abrogation of the cell response to DNA damages, was evaluated combining LY2606368 with different compounds normally used in the clinic of adult ALL patients[136]. In particular Philadelphia-positive cell lines and primary leukemic cells were treated combining LY2606368

with two TKIs (imatinib and dasatinib). The efficacy of both TKIs have been well established for the treatment of ALL harboring the fusion protein BCR-ABL1[137]. Philadelphia-negative cell lines were treated with LY2606368 and with the 2'-deoxyadenosine analogue, clofarabine. Clofarabine has been showed to induce cell apoptosis due to the reduction of nucleoside triphosphate and consequently due to the inhibition of ribonucleotide reductase and DNA polymerases[138,139].

Results

LY2606368 inhibits the cell viability in B-/T-ALL cell lines

The efficacy of the compound, in term of reduction of the cell viability, was firstly evaluated on a panel of different B-/T-ALL cell lines (BV-173, SUP-B15, REH, NALM-6, NALM-19, MOLT-4, RPMI-8402 and CCRF-CEM). In order to evaluate the cytotoxicity of the compound, all the cell lines were incubated for 24 and 48 hours with increasing concentration of *LY2606368* (1-100 nM). The compound reduced the cell viability in all the treated cells in a time and dosage-dependent manner. Using specific statistical analysis, the IC_{50} values were detected for all the cell lines highlighting the BV-173 as the most sensitive cell line (6.33nM) and the REH as the less sensitive(96.7 nM). The sensitivity to the compound as *single agent* did not correlate with leukemia cell type (B-ALL *vs* T-ALL), with the mutational status of the onco-suppressor *p53* (BV-173, SUPB-15, NALM-6 and NALM-19 cells are p53 wild-type whereas REH, MOLT-4, RPMI-8402 and CEM cells are p53 mutated) (Fig. 1A; Table 1) or with the basal expression of Chk1 or Chk2 proteins (data not showed). The correlation between the mutational status of p53 and the sensitivity to the compound was evaluated because of its role in the regulation of the G1-S checkpoint and in the response of DNA damages [38,39].

LY2606368 as single agent activates the apoptotic cascade in B-/T-ALL cell lines

In order to better investigate the mechanism of action of *LY2606368* and to correlate the inhibition of cell viability with the induction of the cell death, different Annexin V/Propidium iodide (Pi) staining analyses were performed. Cells were treated for 24 and 48 hours with

increasing concentration of *LY2606368* (BV-173 and RPMI-8402: 2, 7.5 and 15 nM; NALM-6, NALM-19 and MOLT-4: 7.5, 15 and 30 nM; SUP-B15, REH and CCRF-CEM: 50, 100 and 200 nM) and the number of apoptotic and necrotic cells was evaluated using cytofluorometry. In line with the results found in the cell viability assays, the treatment enhanced cell death via apoptosis and the amount of apoptotic and necrotic cells was time and dosage-dependent in all the treated cell lines (Fig. 1C). The activation of the apoptotic cascade was confirmed by Western blot as showed by the cleavage of the apoptotic marker, poly (ADP-ribose) polymerase 1(PARP1) (Fig. 1D).

LY2606368 modifies the cell cycle profile and targets the Chk1 pathway in B-/T-ALL cell lines

In order to evaluate the consequences the inhibition of Chk1 on cell cycle progression, different cell cycle analyses were performed. To this purpose cells were treated with *LY2606368* (doses nearest to IC_{50} values) for 24 hours and then stained for 1 hour with Propidium Iodide (Pi). The inhibition of Chk1, although with heterogeneity among the different cell lines, deeply changed the cell cycle profile. In agreement with the study of King C. and colleagues[76] the treatment with *LY2606368* reduced the amount of cells in G1 and G2/M phase while increased the number of cells in early S phase (Fig.1B). Subsequently the results found on the cell cycle profile analyses were correlated with the modifications of the expression of different proteins of the Chk1 pathway. To this purpose cells were treated with *LY2606368* (doses nearest to IC_{50} values) for 24 hours and then stained for different markers. First of all the inhibition of Chk1 functionality, marker of the on-target activity of the compound, was confirmed by the reduction of the phosphorylated isoform of Chk1 (ser296 auto-phosphorylation site). The treatment with LY2606368 modified different key elements the Chk1 pathway most of them involved in the regulation of the G2/M checkpoint. In particular the alteration of the number of cells in G2/M phase seen in the cell cycle analysis was confirmed by the reduction of both basal and phosphorylated isoforms of the phosphatase Cdc25C (phospho-$Cdc25C^{ser216}$) and of the kinase cyclin-dependent, Cdc2 (phospho-$Cdc2^{Tyr15}$). No significant changes have been found on the amount of the basal forms of both Cdc25A and Cdc1. To deeply investigate the effect of Chk1 inhibition in term of induction of DNA damages, cells were stained for the following

antibodies: phospho-Chk1 ^{ser317}, marker of activation of the Chk1 pathway; γ-H2A.X, marker of DNA damages; Parp-1, marker of induction of apoptosis. In all the cell lines the induction of DNA damage after the treatment was confirmed by increment of both phospho-Chk1^{ser317} and γ-H2A.X, while the activation of apoptosis was confirmed by the cleavage of Parp-1. Finally in order to highlight if the increment of the DNA damages and the activation of the apoptosis could be related with the induction of cell death by mitotic catastrophe, the expression of the phosphorylated isoform of the histone H3 (phospho-HH3^{ser10}), marker of mitosis, was evaluated. In all cell lines, with the exclusion of RPM-8402 and MOLT-4 cells, the expression of phospho-HH3^{ser10} was reduced by the treatment excluding the hypothetic mechanism of cell death through the mitotic catastrophe (Fig 1D). This data was confirmed also by flow cytometry in REH and NALM-6 cell lines. Cells were treated for 18, 24, 30 and 48 hours and then co-stained using Pi with a primary conjugated antibody, phospho-HH3^{ser10} (FITC-conjugated). In line with the result found in the Western blot analysis, the treatment reduced the amount of cells positive for the pHH3^{ser10} in both the cell lines (Fig.S1D). The effect of *LY2606368* on cell cycle progression was also analyzed at different time points. In particular, BV-173, NALM-6 and REH cell lines were treated with *LY2606368* (respectively, with 7.5, 30 and 100 nM) for 18, 24, 30 and 48 hours and then stained with Pi (Fig.S1A). Again the consequences of the inhibition of the Chk1 pathway was also analyzed using Western blots analysis. These experiments highlighted that the effects of Chk1 inhibition were time-dependent, especially in term of the induction of DNA damages (γ-H2A.X) (Fig. S1B). The hypothetical mechanism of action of the compound in term of perturbation of cell cycle checkpoint functionality and in term of progressively accumulation of DNA damages is graphically showed in Figure 5.

Figure 1

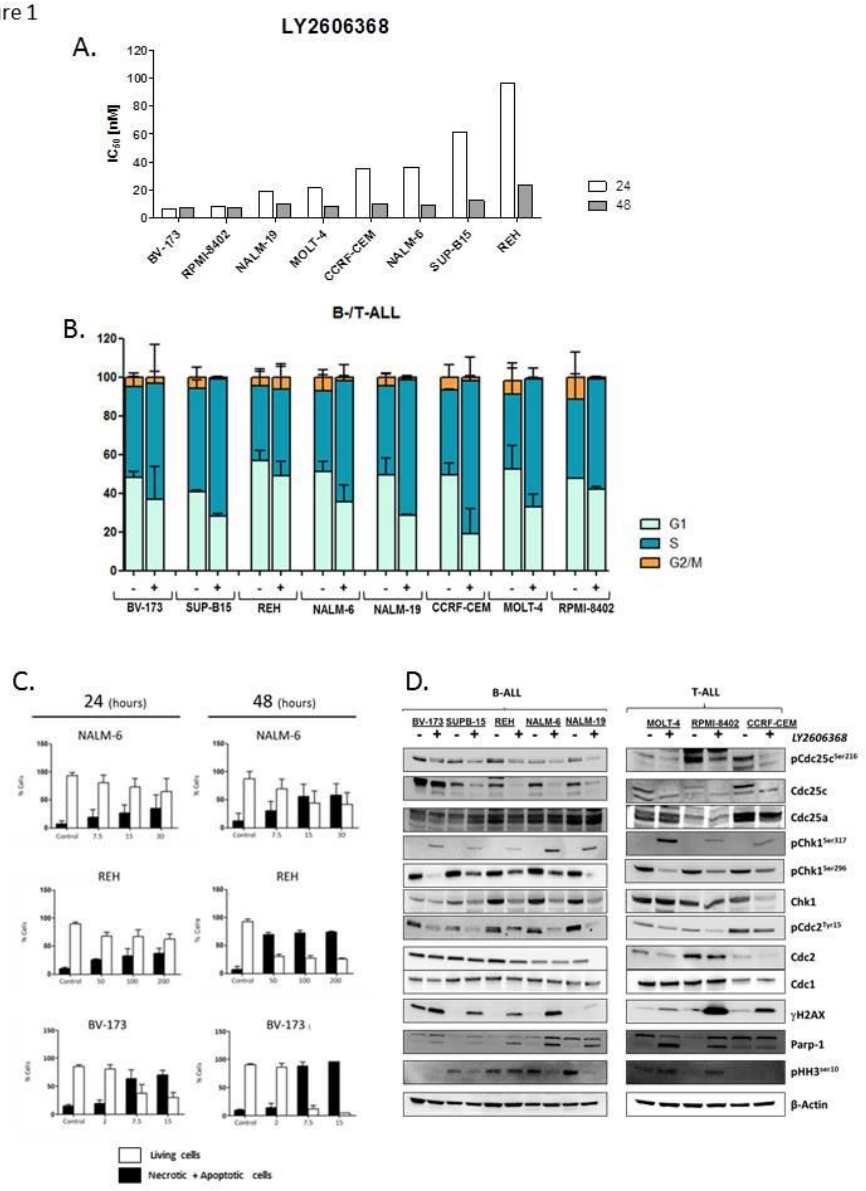


Figure 1: Effect of *LY2606368* on cell viability, induction of apoptosis, inhibition of Chk1 pathway and cell cycle profile in B-/T-ALL cell lines. Graphical representation of the IC_{50} values of the B-/T-ALL cell lines after 24 and 48 hours of incubation with *LY2606368*. The IC_{50} values were obtained from two independent experiments (A). Cell cycle profile of B-/T-ALL cell lines treated with or without *LY2606368* (IC_{50} value) for 24 hours (B). Graphical representation of apoptosis induction by *LY2606368*. BV-173, NALM-6 and REH cells were treated with increasing concentration of drug for 24 and 48 hours (C). The blots show, for each cell lines, the expression of key elements of the Chk1 pathway after 24 hours of incubation with *LY2606368* (IC50 value) (D). In the figure the samples named Control were cells treated with 0.1 % of DMSO. In the Western blot analysis the homogeneity of the protein loaded (30 μg) was determined by using an internal control (β-actin).

LY2606368 increase the cytotoxicity of different Tyrosine kinase inhibitors (TKis) in Philadelphia-positive cell lines

In order to assess the chemo-sensitizer ability of *LY2606368*, different experiments combining the Chk1/Chk2 inhibitor with different compounds, normally used in the clinic, were performed. To this purpose two Philadelphia-positive cell lines were treated for 24 and 48 hours with *LY2606368* (BV-173 7.5 nM; SUP-B15 50 nM) in combination with two different tyrosine kinase inhibitors (TKIs), imatinib (BV-173 500 nM; SUPB15 10 μM) and dasatinib (BV-173 50 nM; SUP-B15 500 nM), and the reduction of the cell viability was evaluated using WST-1 reagent. In both the treated cell lines the combinations increased the cytotoxicity of the two TKIs in comparison the effect of the single treatments (Fig. 2A).Then to deeply understand the chemo-sensitizer activity of the compound and to evaluate the effect of the combination between *LY2606368* and TKIs using sub-toxic concentrations, different combination index assays were performed. BV-173 and SUP-B15 cell lines were incubated with increasing concentration of *LY2606368* (BV-173: from 0.6 to 10 nM, dilution rate 1:2; SUP-B15: from 6.25 to 100 nM, dilution rate 1:2) and two increasing concentration of imatinib (BV-173: 250 and 500 nM; SUP-B15: 5 and 10 μM) for 24 and 48 hours. For instance in BV-173 after 24 hours of single treatment with *LY2606368* (5 nM) and imatinib (250 nM), the percentage of living cells was close respectively to the 50 % and 65 %, but in the samples with the combined drugs was less than 23 % (Fig.2C). The consequences of the co-treatment, in term of change in the expression of different key elements of the Chk1 pathway, were investigated using Western blot. BV-173 cell line was incubated with *LY2606368* (7.5 nM) and with or without the two TKIs (imatinib: 500 nM; dasatinib: 50 nM) for 24 hours. The single treatments with the TKIs as well as their combinations with *LY2606368* did not significantly change the key elements of the Chk1 pathway. However in term of induction of DNA damages (γH2A.X) the co-treatment resulted in an additive effect in both the combinations (Fig. 2B).

Figure 2

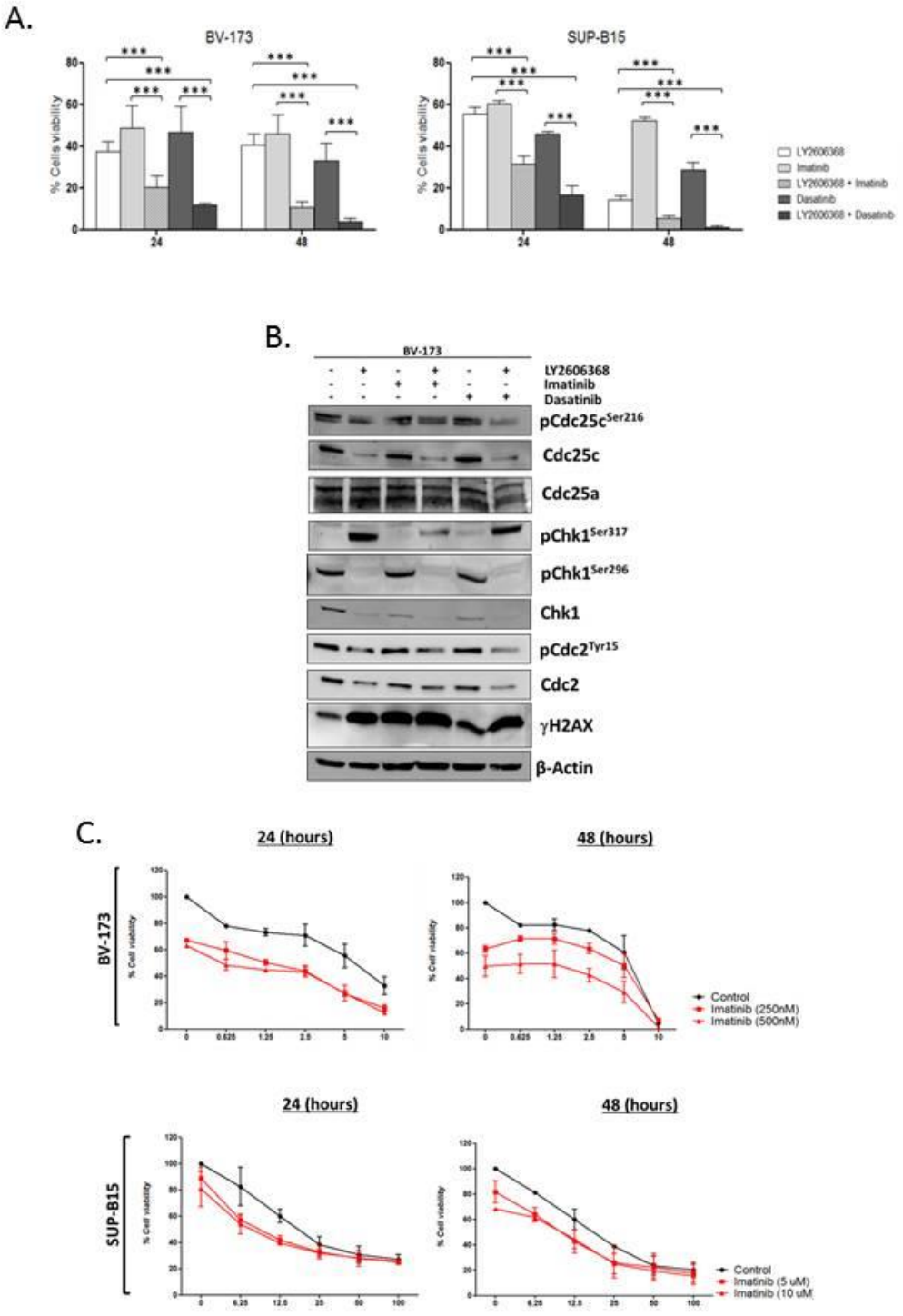


Figure 2: Effect of *LY2606368* in combination with TKIs in Philadelphia-positive cell lines. Cell viability analysis of BV-173 and SUP-B15 cell lines incubated with *LY2606368* and TKIs (imatinib and dasatinib) for 24 and 48 hours. For each cell lines three different experiments have been

performed (A). The blot shows the expression different proteins of the Chk1 pathway after 24 hours of incubation with *LY2606368* in combination with the two TKIs. The cell line BV-173 was incubated with: *LY2606368*: 7n; imatinib: 500nM; dasatinib: 50nM (B). Combination index analysis of BV-173 and SUP-B15 cell lines incubated with increasing concentration of *LY2606368* (BV-173: from 10 to 0.6 nM, dilution rate 1:2; SUP-B15: from 100 to 6.25 nM, dilution rate 1:2) and two increasing concentrations of imatinib (BV-173: 250 and 500 nM; SUP-B15: 5 and 10 μM) for 24 and 48 hours. The black curve (control) represents the effect of the *LY2606368* alone while the two red curves represent the two combinations (full square *LY2606368* + 250 nM (BV-173) or +5 μM (SUP-B15) of imatinib; full triangle LY2606368+ 500 nM (BV-173) or 10□M (SUP-B15) of imatinib). The curves represent the mean of two independent experiments (C). In the figure the samples named Control were cells treated with 0.1 % of DMSO. In the Western blot analysis the homogeneity of the protein loaded (30 μg) was determined by using an internal control (β-actin).

LY2606368 increases the cytotoxicity of clofarabine in Philadelphia-negative cell lines

The efficacy as chemo-sensitizer agent of the compound on Philadelphia-negative B-/T-ALL cell lines was evaluated combining the Chk1/Chk2 inhibitor with the purine nucleoside antimetabolite clofarabine, which is commonly used in clinical trials for the treatment of young and adult ALL patients[140,141]. The effect of clofarabine in term of reduction of the cell viability was evaluated using WST-1 and for each cell lines was calculated the IC_{50} value (data not showed). The effect of the combination was very heterogeneous between the different cell lines (Fig.3A). Interestingly no significant differences in term of effectiveness of the combination was seen between cell lines with p53 mutated, that should have an impaired function of the G1/S checkpoint, and with p53 wild type (Table 1). To deeply investigate the effect of the combination of this two drugs and to evaluate if the cytotoxic effect could be dependent to a particular schedule, different experiments were performed. For this reason NALM-6 and REH cell lines were treated respectively with clofarabine alone for 48 hours (50 nM), *LY2606368* alone for 48 hours (10 nM), pre-incubated with clofarabine for 24 hours and then with *LY2606368* for 24 hours or pre-incubated with *LY2606368* for 24 hours and then with clofarabine for 24 hours. No significant differences were found between the different schedules (Fig. S1C). As with the previous combinations with TKIs, the consequences of the combination on the Chk1 pathway were evaluated using Western blot analysis. REH and NALM-6 cell lines

were incubated for 24 hours with the checkpoint inhibitor in combination with clofarabine using for each drugs the dose nearest to the IC50 after 24 hours (clofarabine: 50 nM; *LY2606368*. respectively 100 and 30 nM). The treatment with clofarabine in single agent did not significantly change the protein level of the main downstream targets of Chk1, except for the amount of γ-H2A.X. However the combination of the two drugs, in both the cell lines, changed the levels of expression of different proteins involved in the Chk1 pathway. In particular the co-treatment increased the DNA damages (γ-H2A.X) and the activation of the apoptosis (Parp-1 and pro-Caspase 3 cleavage) when compared with the cytotoxic effect of the single drugs (Fig. 3B). The ability of *LY2606368* to sensitize leukemic cell lines to the cytotoxicity of clofarabine was evaluated performing different combination index analyses. To this purpose NALM-6 and REH cell lines were incubated with increasing concentration of *LY2606368* (from 0 to 100 nM dilution rate 1:2) and with two different concentrations of clofarabine (sub-toxic concentration: 5 and 10 nM) for 24 and 48 hours. In both the cell lines the reduction of the cell viability was additively stronger in the samples treated with the two compounds than in the samples treated with the two drugs in single agent. For instance in NALM-6 cell line the percentage of living cells in the sample treated with 12.5 nM of *LY2606368* for 48 hours was respectively close to 90%, in the sample treated with 10 nM of clofarabine for 48 hours was close to 54% and in the samples with the combined drugs was less than 25% (Fig.3C).

Figure 3

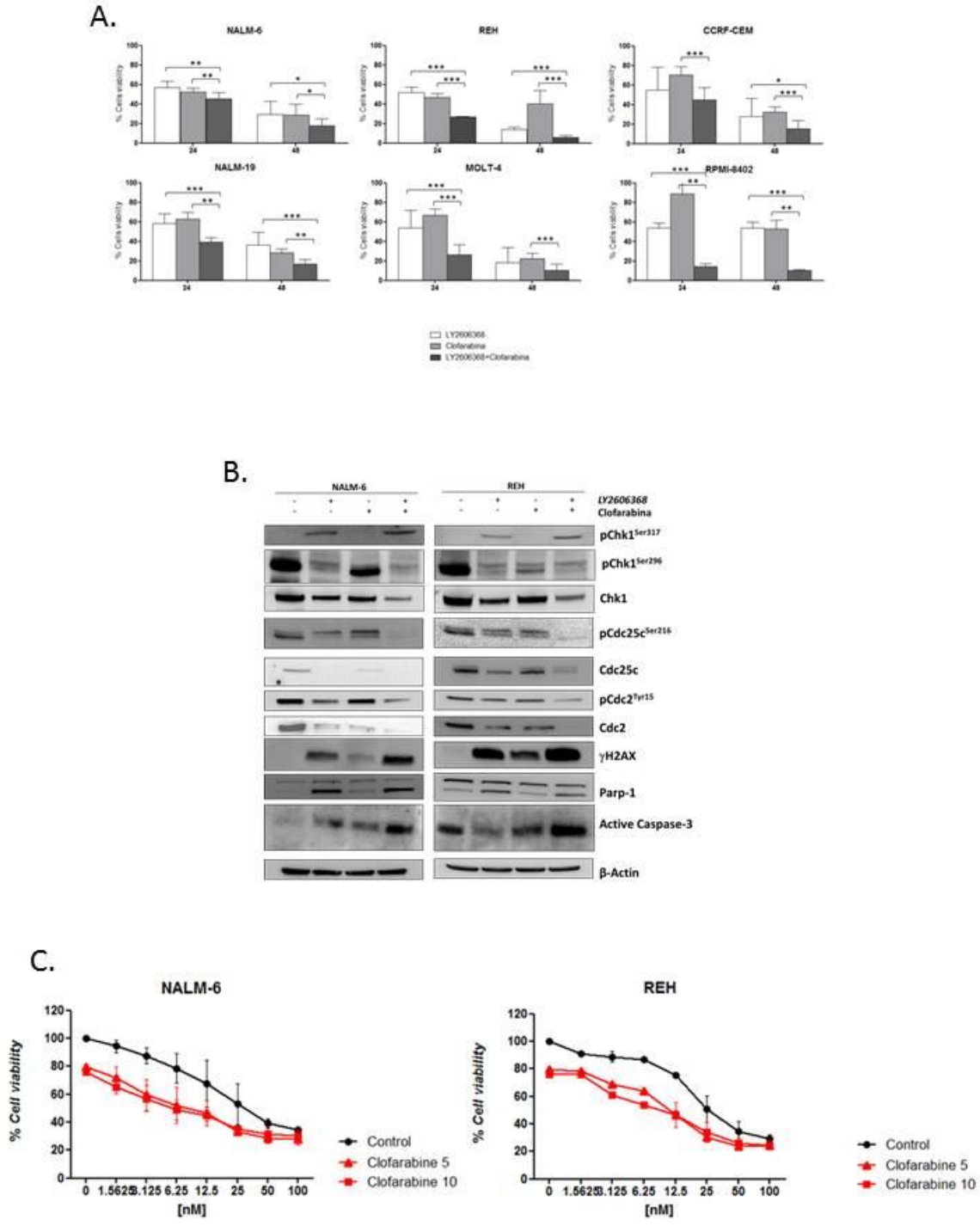


Figure 3: Effect of *LY2606368* in combination with clofarabine in Philadelphia-negative cell lines. Cell viability analysis of Philadelphia-negative ALL cell lines incubated *LY2606368* and clofarabine for 24 and 48 hours. For each cell lines three independent experiments have been

performed (A). The blots show the expression on NALM-6 and REH cell lines of different proteins of the Chk1 pathway after 24 hours of treatment with *LY2606368* in combination with clofarabine. The dosages of each drug have been chosen based on the IC_{50} values after 24 hours of incubation (B). Combination index assay of NALM-6 and REH cell lines treated with increasing concentration of *LY2606368* (from 1.5 to 100 nM) and sub-toxic concentration of clofarabine (5 and 10 nM). The black curve (Control) represents the effect of the *LY2606368* alone while the two red curves represent the effect of the combinations (full triangle *LY2606368* + 5 nM of clofarabine; full square *LY2606368*+10 nM of clofarabine) after 48 hours of incubation with the two drugs. The curves in graph are representative of the mean of two independent experiments (C). In the figure the samples named Control were cells treated with 0.1 % of DMSO. In the Western blot analysis the homogeneity of the protein loaded (30 μg) was determined by using an internal control (β-actin).

LY2606368 targets the Chk1 pathway on leukemic blasts but not on peripheral blood mononuclear cell of healthy donors

Finally the effectiveness of the compound was evaluated on different primary leukemic cells isolated from the bone marrow and the peripheral blood of 9 adult B-ALL patients and on the mononuclear cells isolated from the peripheral blood of 5 healthy donors. To this purpose the primary cells were treated with increasing concentration of *LY2606368* (100, 200 and 500 nM) for 24 hours and then the reduction of the cell viability was evaluated using Trypan blue exclusion dye method. Similarly to the results found on B-/T-ALL cell lines, *LY2606368* progressively reduced the cell viability in a dose-dependent manner in all the primary leukemic cells (Fig. 4A).The sensitivity to the compound was not related with the leukemia sub-types (Fig S1E). However on the mononuclear cells isolated for the healthy donors the treatment did not reduced significantly the cell viability (Fig. 1E). To correlate the reduction of the cell viability with the induction of DNA damages, the primary cells isolated from the bone marrow of 4 adult B-ALL patients were treated with a sub-toxic concentration of the compound (100 nM) and then stained for phospho-Chk1^{ser317}, Chk1 and γH2A.X. The Western blot analysis showed that in all the samples, although with a high heterogeneity, the treatment with *LY2606368* increased both the two markers of DNA damage (phospho-Chk1 ^{ser317}and γH2A.X) (Fig.4B). By contrast, in normal cells, isolated form the peripheral blood of 5 healthy donors, the effect of the compound did not reduced significantly the cell viability and did not modified the amount of

the different downstream targets of Chk1 neither increased the amount of the phosphorylated form of γH2A.X, with the exclusion of one sample (Fig.4E; 4F). The chemo-sensitizer ability of LY2606368 was then evaluated also on the primary leukemic cells. To assess that the primary cells isolated from 3 Philadelphia-positive ALL patients were treated for 24 hours with *LY2606368* (200 nM) in combination with the TKI, imatinib (5 μM). The concomitant treatment showed an additive effect in term of reduction of the cell viability in comparison with the effect of the single treatment (Fig.4C). Finally to correlate the reduction of the cell viability with the induction of DNA damage, a Western blot analysis was performed on the primary cells of one Philadelphia-positive ALL patient. Although the amount of phospho-Chk1ser317 was not significantly higher in the sample treated with the two drug in comparison to the single treatments, the amount of γH2A.X was synergistically increased (Fig. 4D).

Figure 4

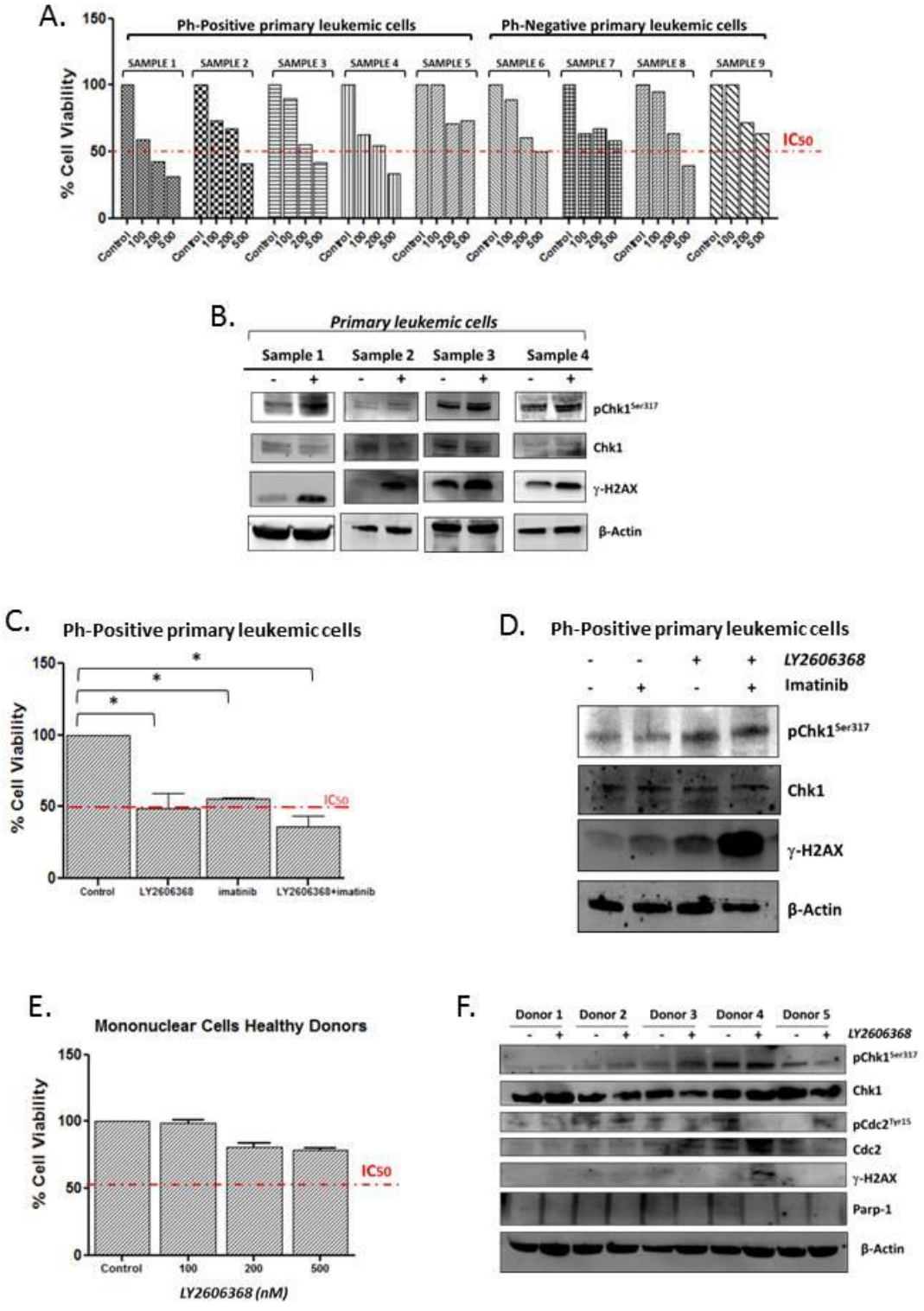


Figure 4: Effect of *LY2606368* on primary leukemic cells isolated from adult ALL patients and on peripheral mononuclear cells isolated from healthy donors. Cell viability analysis on primary leukemia cells isolated from 9 adult ALL patients treated with increasing concentration

of *LY2606368* (100, 200 and 500 nM) for 24 hours.(A). The blot shows the induction of DNA damages on primary leukemic cells isolated from the peripheral blood of 4 newly diagnosed ALL patients treated for 24 hours with *LY2606368* (100nM) (B). Cell viability assay of primary leukemia cells isolated from 3 Philadelphia-positive ALL patients treated with *LY2606368* (100nM) and imatinib (5 μM) for 24 hours. The graph represents the main of response to the combination of the three patients (C).The blot shows the induction of DNA damages on primary leukemia cells isolated from a Philadelphia-positive patient after 24 hours of treatment with *LY2606368* (100nM) in combination with imatinib (5μM)(D). Cell viability assay of mononuclear cells isolated form the peripheral blood of 5 healthy donors, treated with increasing concentration of *LY2606368* (100, 200 and 500 nM)(E). The blot shows the expression of different proteins of the Chk1 pathways after 24 hours of incubation with *LY2606368* (100nM) on the mononuclear cells isolated from the peripheral blood of 5 healthy donors (D). In the Western blot analysis the homogeneity of the protein loaded (30 μg) was determined by using an internal control (β-actin).

Discussion

Various studies have showed the effectiveness of different Chk1/Chk2 inhibitors as monotherapy or in combination with chemotherapy drugs or radiotherapy in different kind of tumors[72,141–146]. The biological hypothesis of these studies is that tumor cells can survive to therapy activating the DDR and delay the cell cycle progression to prevent lethal cell division [60]. Many compounds arrest tumor cells in different phases of the cell cycle. For instance, the purine nucleoside analogue clofarabine, arrests tumor cells in G1/S phase due to the inhibition of DNA synthesis [147]. As already mentioned, one of the most important regulator of the cell cycle is the Checkpoint kinase 1 (Chk1). In BCR/ABL1-positive cells the hyper activation of the ATR-Chk1 pathway after the exposure with different genotoxic agents has been associated with a delay in G2/M progression and thus with a possible mechanism of resistance to treatment[148]. Sarmento and colleagues found that T-ALL cells over-express Chk1 and then demonstrated that the aberrant expression is necessary for the proliferation and the survival of cancer cells[73]. In this study we demonstrated that Chk1 functionality is fundamental for the survival of B-/T-ALL cell lines and primary cells, and that the inhibition of this kinase using, *LY2606368*, sensitized both cell lines and primary cells to the cytotoxicity of different compound normally used for the treatment of adult B-/T-ALL patients. In line with our previous work on the efficacy of the PF-0477736 in single agent, the treatment with *LY2606368* reduced

the cell viability in a time and dose-dependent manner in all the treated cell lines. The sensitivity to the compound was not correlated with the mutational status of p53, with the leukemia sub-type (Table 1) or with the basal expression of Chk1/Chk2 (Data not showed). The reduction of the cell viability was associated with the induction of cell death via apoptosis in all the treated cell lines, confirming the critical role of Chk1 in the proliferation and in the viability of ALL cells. To deeply investigate the consequence of Chk1 inhibition on cell cycle regulation and to clarify mechanisms of cell death, different cell lines were cell cycle and Western blots analyses were performed. The cell cycle analyses highlighted that in B-/T-ALL cell lines the compound reduced the amount of cells G2/M phase and increased the percentage of cells in G1/S and early S phase. The reduction of the G2/M phase together with reduction of the mitotic marker phospho-Histone H3 ($pHH3^{ser10}$) excluded the mechanism of cell death through mitotic catastrophe and corroborated the hypothesis of King C. and colleagues[76] of the *replication catastrophe*. The single agent efficacy of *LY2606368* showed on the cell lines were confirmed on different primary leukemic cells isolated from adult B-ALL patients. Although the compound deeply reduced the cell viability and increased the DNA damages in all the primary cell treated, we could not define any predictive factors of response. The idea of interfering with DNA damage response pathway in human cancer as a means to improve the cytotoxicity of DNA damaging therapies has been demonstrated in many pre-clinical studies. Here we evaluated the effectiveness of the *LY2606368* as a chemo-sensitizer agent for the treatment of ALL. We showed that *LY2606368* sensitized both Philadelphia-positive cell lines and primary leukemic cells to the toxic effect of the two TKIs, imatinib and dasatinib. The combinations (*LY2606368*+TKI) not only significantly reduced the cell viability in comparison to the effect of the single treatments but also increased the amount DNA damages (γH2A.X), confirming the abrogation of the DNA damage response and the consequently accumulation of DNA damages. Similar results were found in Philadephia-negative cell lines, combining *LY2606368* with the 2'-deoxyadenosine analogue, clofarabine. Finally by assessing different schedules in the combination between *LY2606368* and clofarabine, we demonstrate that the sequence on which the compounds were added to the media did not significantly modify the effect of the combination. We believe that the inhibition of Chk1/Chk2 by *LY2606368* could be a promising

strategy to limit adverse events, by reducing the doses needed to reach a sufficient cytotoxic effect, and could increase the effectiveness of imatinib, dasatinib and clofarabine, by inhibiting the survival of cancer cells (Fig.5).

Figure 5

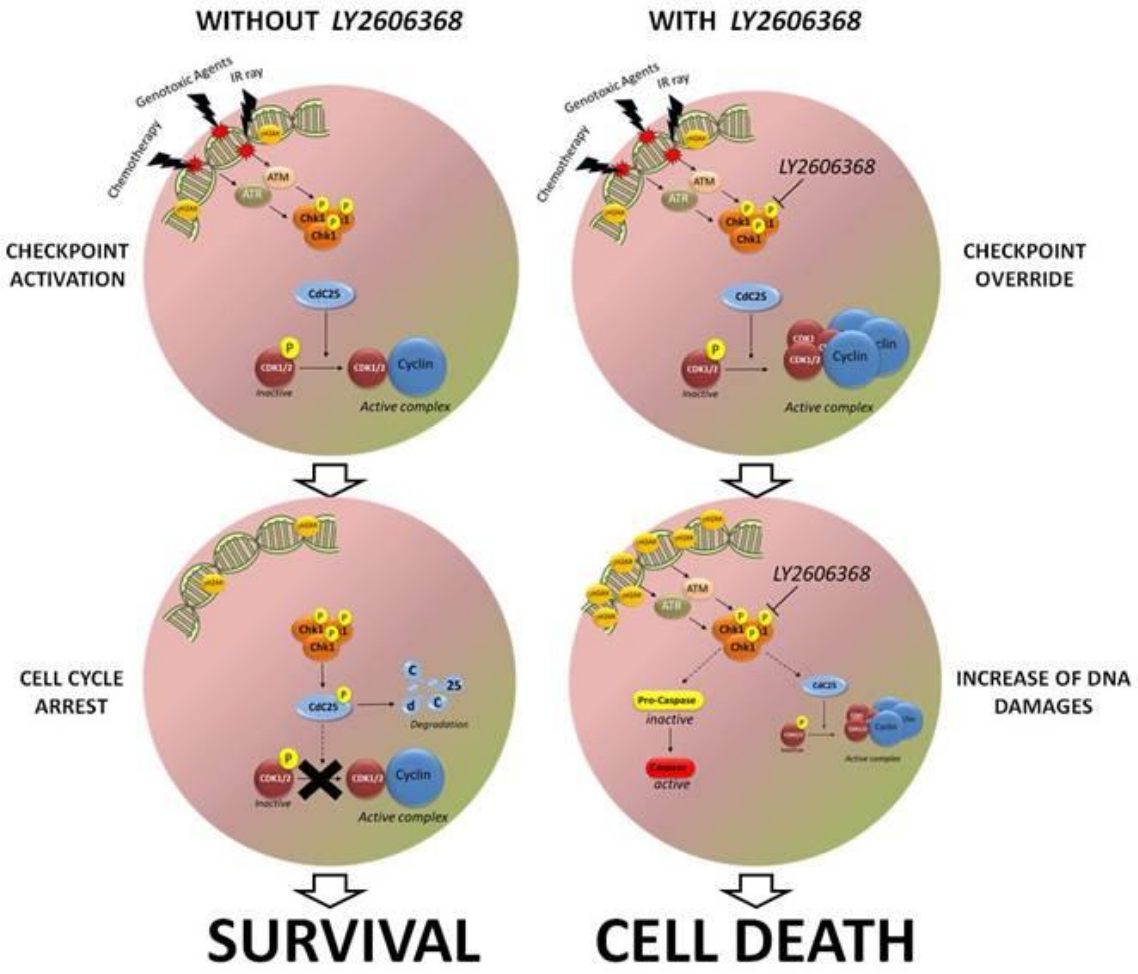


Figure 5: Schematic representation of the effect of *LY2606368* on leukemic blast after the exposure to different genotoxic agent. The left side of the cartoon hypothesizes how leukemic cell could survive to chemotherapy drugs and to other genotoxic agent, activating the cell cycle checkpoint and arresting the cell cycle progression. The right side of the cartoon hypothesizes the mechanism of action of the compound in enhancing cell death and inducing checkpoint override after the exposure to different DNA damaging agents.

Methods

Leukemia cell lines and primary samples

Human B- (BV-173, SUP-B15, REH, NALM-6, NALM-19) and T-ALL (MOLT-4, RPMI-8402, CCRF-CEM) cell lines were obtained from Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Germany). Cells were cultured in RPMI-1640 medium (Invitrogen, Paisley, UK) with 1% *l*-glutamine (Sigma, St. Louis, MO), penicillin and streptomycin (Gibco, Paisley, UK) supplemented with 10%-20% fetal bovine serum (Gibco) in a humidified atmosphere of 5% CO_2 at 37°C. Primary blast cells from 9 newly diagnosed Philadelphia-positive ALL cases were obtained, upon written informed consent, from the bone marrow and the peripheral blood by density gradient centrifugation over Lymphoprep (Nycomed UK, Birmingham). Mononuclear cells were isolated from the peripheral blood of5 healthy donors. Online databases have been interrogated to molecularly characterize leukemia cell lines: International Agency for Research on Cancer (IARC) *TP53* database (http://www-p53.iarc.fr/) and the Catalogue of Somatic Mutations in Cancer, (COSMIC, http:// http://www.sanger.ac.uk/genetics/CGP/cosmic/).

Drugs and Reagents

The Chk1/Chk2 inhibitor *LY2606368* was provided by Lilly Oncology. The purine nucleoside antimetabolite (clofarabine) and the tyrosine kinase inhibitors (imatinib and dasatinib) were bought by Sigma-Aldrich web site.

Cell viability assay

To evaluate the cytotoxic effect of the treatment with *LY2606368*, B- and T-All precursor leukemia cell lines were seeded in 96-well plates at 50,000 cell/100 µl/well with increasing concentrations of drug (1-100 nM) for 24 and 48 and incubated at 37°C. Cell viability was assessed by adding WST-1 reagent (Roche Applied Science, Basel, Switzerland) to the culture medium at 1:10 dilution. Cells were incubated at 37°C and the optical density was measured by

microplate ELISA reader at λ450 after 3 hours. The amount of the formazan formed directly correlates to the number of metabolically active cells. All viability experiments were performed in triplicates and repeated in least two separated experiments.

Primary leukemic cells viability assay

To assess the effect of *LY2606368* on primary leukemic blasts, the cells from 9 newly diagnosed Philadelphia-positive and Philadelphia-negative ALL cases were obtained, upon written informed consent, from the peripheral and bone marrow blood samples by density gradient centrifugation over Lymphoprep (Nycomed UK, Birmingham).Primary cells were seeded on a 6 well plates at 500.000 cells/ml and incubated with increasing concentration of *LY2606368* for 24 hours at 37°C. 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.

Combination index assay

In order to evaluate the effectiveness of *LY2606368* as chemo-sensitizer agents, B-/T-ALL cell lines were seeded in 96-well plastes at 50,000 cell/100µl/well with increasing concentration of the compound (dilution rate 1:2) and increasing concentration of a second drug (dilution rate 1:2), for 24 and 48 hours at 37°C. Cell viability was assessed using WST-1 (Roche Applied Science, Basel, Switzerland). Cells were incubated at 37°C and the optical density was measured by microplate ELISA reader at λ450 after 3 hours. The amount of the formazan formed directly correlates to the number of metabolically active cells. All viability experiments were performed in triplicates and repeated in least two separated experiments.

Annexin V staining of apoptotic cells

To assess the effect of the compound in the induction of apoptosis, three different increasing concentrations of *LY2606368* were used to treat cells lines in order to detect apoptotic, necrotic and cell debris. Cell lines were seeded in 12-well plates at 500,000 cell/1 ml with increasing concentrations of drug (NALM-6, NALM-19, MOLT-4 and CCRF-CEM: 7.5, 15 and 30 nM; BV-173 and RPMI8402: 2, 7.5 and 15 nM; SUP-B15 and REH: 50, 100 and 200 nM) for 24 and 48 hours and incubated at 37°C. Following the treatment, cells were harvested and stained with Annexin

V/Propidium Iodide (PI) according to the manufacturer's instruction (Roche). The percentage of Annexin V-PI positive cells was determined within 1×10^4 cells of the population by flow cytometry (Facs CantoII, BD Biosciences Pharmingen, San Jose, California, USA). The mean percentage of Annexin V-PI positive cells and standard error measurement was calculated from at least two separate experiments.

Western Blot analysis

All cell lines were plated in 6-well plates at 500,000 cell/1 ml with increasing concentrations of drug for different time points and incubated at 37°C. For evaluate the effect of combination of the Chk inhibitor with other drugs (imatinib, dasatinib and clofarabine) were used as dosage the IC_{50} for the single drug after 24 hours of treatment. The drugs in the combination were added simultaneously. At the end of the treatment, the cells were collected and lysate using a specific buffer made of KH2PO4 0,1 M (pH 7,5), Igepal 1% (NP-40), β-glicerofosfato 0,1 mM and complete protease inhibitor cocktail 1X (Roche Diagnostics).For each sample 30-50ug of protein were fractioned on Mini-Protean TGX stain-free precasted gels, blotted to nitrocellulose membranes (Bio-Rad Trans-blot turbo transfer pack) and incubated overnight with the following antibodies: Chk1 (#2345S), phosphorylated Chk1 (Ser317)(#2344S), phosphorylated Chk1(Ser296)(#2349S) and Chk1 (Ser345)(#2348S), Cdc25c (#4688S), phosphorylated Cdc25C (Ser216)(#9528S), Cdc25a (#3652), Cdc2 (#9112S), phosphorylated Cdc2 (Tyr15)(#4539S), phosphorylated H2A.X (Ser139) (γ-H2A.X) (#2577S), Caspase3 (#9662S), Parp (#9542S),phosphorylated Histone 3 (Ser10) from Cell Signaling. Antibody for CDK2 (Cdc1)(sc-163) came from Santa Cruz biotechnology. Antibody to β-actin came from Sigma (St. Louis, MO). Finally all these antibodies were detected using the enhanced chemiluminescence kit ECL (GE) and the compact darkroom ChemiDoc-It (UVP).

Cell Cycle analysis

The cell lines were seeded in a 24 wells plate at the concentration of 500,000 cells/1 ml and treated for 6 and 24 hours at 37°C. After the right incubation time the cells were harvested and washed with cold PBS. After the wash the PBS was discarded and the cell were fixed using ethanol 70% and stored at -20°C for 24 hours. After the fixation period the ethanol was

removed by one wash in PBS and the cells were incubated for 30 minutes at 37°C with the staining mix (sodium citrate pH 2.5 100mM, propidium iodide 2.5mg/ml, RNAse 10mg/ml and dH2O). The cell cycle analysis was conducted using the BD FACS Canto and the quantitative analysis using Modfit LT software (Verity).

Phospho-histone H3/Propidium Iodide co-staining

To evaluate the expression of phospho-Histone H3 (marker of mitosis) different cell lines were treated with *LY2606368* (IC50 after 24 hours). The cell lines were seeded at the concentration of 500,000 cell/ml and treated for 18, 24, 30 and 48 hours at 37°C. After the right period of incubation the cell were harvested, washed twice in ice cold PBS and fixed in -20°C with 70% ETOH for 24 hours. After the right fixation period the cells were washed twice with PBS+0.5%Tween 20 and with PBS+0.1% BSA. The cells were incubated in darkness with FITC-conjugated Phospho-HH3 antibody, dilution rate 1:100 in PBS+0.1%BSA, for 1 hour on ice (Phospho-Histone H3 (ser10) Antibody Alexa Fluor 488 conjugate #9708 Cell Signaling).To remove the exceeded of antibody the samples were washed twice in PBS and the fixed for 30 minutes with -20°C 70%ETOH. After the second fixation the cells were washed twice in cold PBS and then stained with the staining mix((sodium citrate pH 2.5 100mM, propidium iodide 2.5mg/ml, RNAse 10mg/ml and dH2O) for 30 minutes at room temperature. The cell cycle analysis and the detection of the phosphor-HH3 positive cells were conducted using the BD FACS Canto and the quantitative analysis using Modfit LT software (Verity).

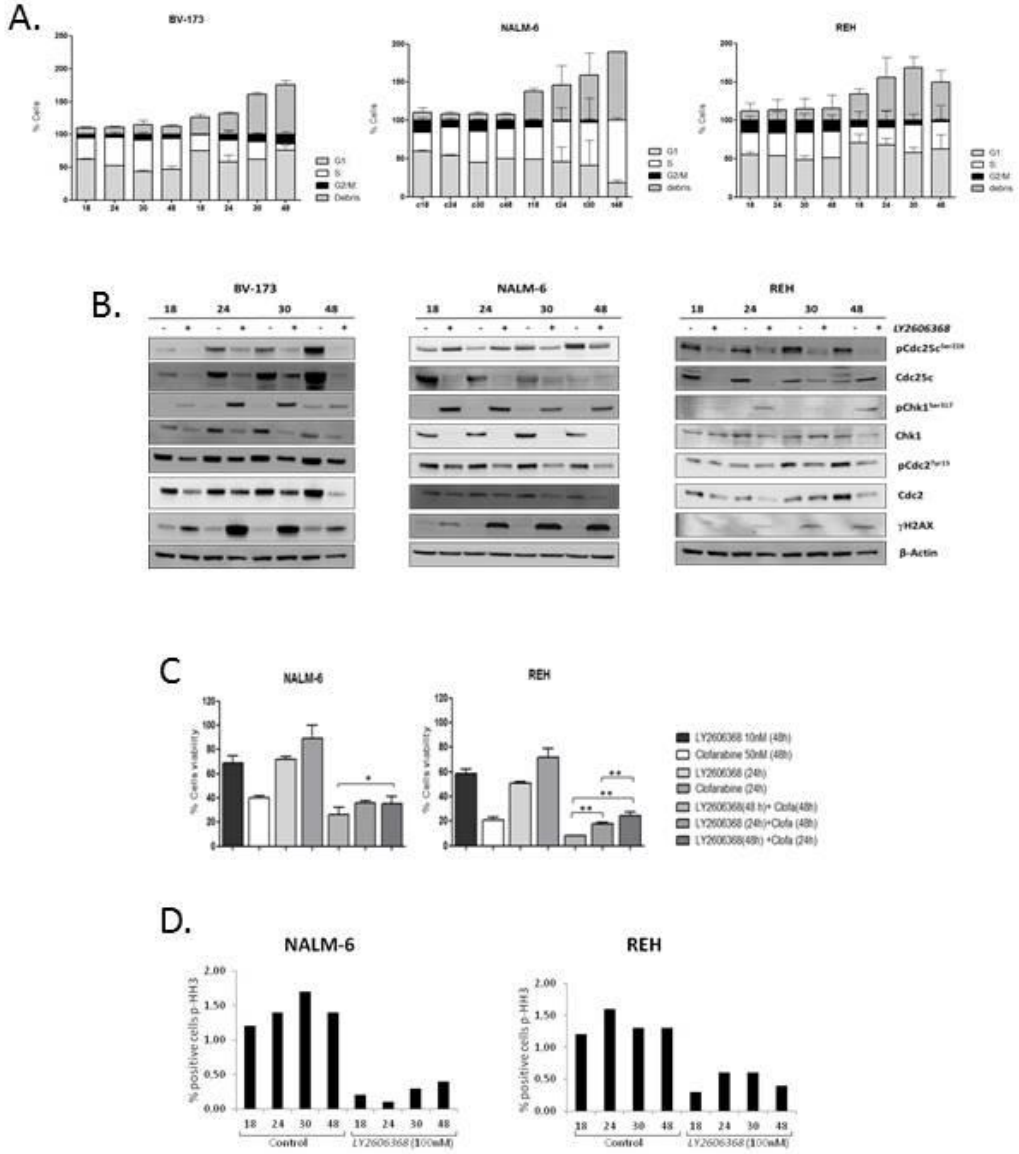
Statistics

All the differences in percentages of reduction of the cell viability were analyzed by unpaired *t*-test ($P \leq 0.05$ was considered as statistically significant).

CELL·LINE¤	LEUKEMIA·SUBTYPE¤	KARYOTYPE*¤	MUTATIONAL·STATUS·OF·P53¤	LY260638·IC_{50}(nM)¤
REH¤	PH-NEG.·B-ALL¤	46(44-47)<2n>X,–X,+16,·del(3)(p22),·t(4;12;21;16)(q32;p13;q22;q24.3)-inv(12)(p13q22),·t(5;12)(q31-q32;p12),·der(16)t(16;21)(q24.3;q22)–·sideline·with·inv(5)der(5)(p15q31),+18–·carries·t(12;21)·and·del(12)·producing·respective·ETV6-RUNX1·(TEL-AML1)·fusion·and·deletion·of·residual·ETC6·(TEL)¤	MUT(R181C0¤	96.7¤
SUP-B15¤	PH-POS.·B-ALL¤	46<2n>XY,·der(1)t(1;1)(p11;q31),·add(3)(q2?7),·der(4)t(1;4)(p11;q35),·t(9;22)(q34;q11),·add(10)(q25),·?del(14)(q23q31),·der(16)t(9;16)(q11;p13)¤	WT¤	61.4¤
NALM-6¤	PH-NEG.·B-ALL¤	46(43-47)<2n>XY,·t(5;12)(q33.2;p13.2)·leading·to·ETV6/PDGFRB¤	WT¤	36.4¤
CCRF-CEM¤	T-ALL¤	90(88-101)<4n>XX,–X,–X,+20,+20,·t(8;9)(p11;p24)x2,·der(9)del(9)(p21-22)del(9)(q11q13-21)x2–·sideline·with·+5,·+21,·add(13)(q3?3),·del(16)(q12)¤	MUT(R175H, R248Q)¤	35.3¤
MOLT-4¤	T-ALL¤	89-99<4n>XXYY,·+4,·+7,·+8,·+20,·+20,·del(6)(q16)x2,·der(7)t(7;7)(p15;q11)x2¤	MUT(R248Q)¤	21.2¤
NALM-19¤	PH-NEG.·B-ALL¤	47(45-48)<2n>XY,+5,·del(9)(p21.2)¤	WT¤	19.1¤
REPMI-8402¤	T-ALL¤	90(79-91)<4n>XXX,–X,+3,+3,–10,–13,–14,+15,–18,–20,·+2mar,·dup(4)(q13q23)x2,·del(6)(q14q22)x2,·t(11;14)(p15;q11)x2,·add(15)(p13)–·sideline·with·der(1)t(1;9)(p35/36;q11),·add(13)(q34)–·carries·t(11;14)·with·LMO1-TRD@·(LMO1-TCRD)·rearrangement·and·cryptic·del(1)(p32)·effecting·STIL-TAL1·(SIL-TAL1)·fusion¤	MUT(R273C)¤	8.07¤
BV-173¤	PH-POS.·B-ALL¤	47(46-48)<2n>X/XY,–9,+22,+mar,·add(1)(q42),·add(8)(p23),·t(9;22)(q34;q11),·der(22)t(9;22)(q34;q11),·der(?)t(9;?)(?p11;?)¤	WT¤	6.33¤
*·Leibniz-Institut·DSMZ-Deutsche·Sammlung·von·Mikroorganismen·und·Zellkulturen·GmbH·(Germany),·www.dsmz.de¤				

Table 1: Leukemia sub-type, karyotype, mutational status of p53 and IC_{50} value (after 24 hours) of the panel of B-/T-ALL cell lines

Figure Supplementary 1



Supplemental figure 1: Cell cycle profile of BV-173, NALM-6 and REH after 18, 24, 30 and 48 hours of incubation with *LY2606368* (IC_{50} value)(A). The blots show the expression of different proteins of the Chk1 pathway on BV-173, NALM-6 and REH cell lines after 18, 24, 30 and 48 hours of incubation with *LY2606368* (IC50 value)(B).Cell viability assay of NALM-6 and REH cell lines treated with *LY2606368* and clofarabine using different schedules (C). Schematic representation of the number of cells positive for phospho-HH3 (ser10) antibody as marker of mitosis. NALM-6 and REH cell lines were incubated with or without *LY2606368* (30 and 100 nM respectively) for 18, 24, 30 and 48 hours. In the graph the amount of positive cells for Phospho-HH3(Ser10) are showed as a percentage of the total amount of cells (D).

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Disclosures

GM has competing interests with Novartis, BMS, Roche, Pfizer, ARIAD, MSD.

Authorship

A.G.L.D.R designed experiments, analyzed data and wrote the manuscript; A.G.L.D.R, II and GM coordinated the research. A.G.L.D.R, EI, AF, VG, and VR performed the laboratory work for this study. CP, SP, CS, MCA and SP contributed to sample collection. A.G.L.D.R, II and GM contributed to data interpretation. All authors read and approved the final manuscript.

Result III

MK-1775

Under submission

The Wee1 inhibitor, MK-1775, sensitizes leukemic cells to different antineoplastic drugs interfering with DNA damage response pathway

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Keywords:

Acute Lymphoblastic Leukemia, Wee1 inhibitor, Chemo-sensitizer agent, G2/M checkpoint

Abstract

Due to inadequate treatments, the survival rate of adult patients with acute lymphoblastic leukemia (ALL) is still very poor. Thus there is a need to improve the efficacy of conventional therapy and to discover novel specific targets for a more personalized therapy. In this study we evaluated the effectiveness of MK-1775, a selective Wee1 inhibitor, as single agent and in combination with different compounds conventionally used for the treatment of B-/T-ALL. The inhibition of Wee1 kinase drastically reduced the cell viability, modified the cell cycle profile and induced apoptosis in B-/T-ALL cell lines and on primary leukemic cells isolated from adult ALL patients. The chemo-sensitizer ability of the compound was then evaluated combining MK-1775 with different compound normally used for the treatment of adult ALL patients. Interestingly, the treatment with MK-1775 strongly sensitized both cell lines and primary

samples to the toxic effect of different compounds and in particular to the tyrosine kinase inhibitor (TKis), bosutinib, to the purine nucleoside antimetabolite, clofarabine, and to the Chk1/Chk2 inhibitor PF-00477736. This manuscript highlights the in vitro/ex-vivo efficacy of MK-1775 for the treatment of ALL.

Introduction

Today different therapeutic approaches are available for the treatment of acute lymphoblastic leukemia (ALL). The identification of novel genetic alterations allowed to better classify ALL patients and to develop innovative therapies. Despite all the enormous progresses that have been made in the treatment of ALL, the cure rate, especially for adult patients, is still very low (less than 35%). The treatment of ALL adult patients, with the exclusion of compounds against particular oncogenes, is mainly associated with the use of chemotherapy [95,136,140,149]. Thus there is a need to increase the efficacy of current treatments in order to amplify the rate of cured patients and reduce the number of relapses. A successful strategy is to deeply understand the mechanisms by which leukemic cells respond and survive to therapies and, consequently, to inhibit them. Nowadays it is known that tumor and normal cells respond to DNA damages activating specific pathways in order to survive and in particular activating the DNA damage response (DDR) pathways. Although different molecular pathways are associated with the DDR, the main three involved in the maintenance of genome integrity and in the cell cycle regulation are the ataxia telangiectasia and Rad3-related protein (ATR)/ Checkpoint kinase 1 (Chk1), the ataxia telangiectasia mutated (ATM) /Checkpoint kinase 2 (Chk2) and the Wee1 pathway[124,150]. These kinases are activated in the presence of DNA damages and prevent cell with altered DNA to move through the cell cycle and complete cell division. Different damaging agents, like IR ray, alkylanting agents, UV ray or oxygen radicals can generate DNA damages and trigger the ATR/Chk1, ATM/Chk2 and Wee1 response [127,33]. Chk1 and Chk2 kinases are mainly involved in the regulation of S and G2 phase and in particular the first one is involved in the stabilization of the replicative forks and the homologous recombination repair. Wee1 is a checkpoint kinase, involved mainly in the regulation of G2/M transition through the inhibitory phosphorylation of both Cyclin-dependent kinase 1 (CDK1, Cdc2) and 2 (CDK2,

Cdc1)respectively [150].In the presence of DNA damages to prevent cell cycle progression both CDK1 and CDK2 are phosphorylated (Tyrosine 15 reside) thus avoid the generation of the CDK/cyclin complex and the progression of cell cycle [151]. The inhibitory phosphorylation of CDK1/CDK2 is directly catalyzed by Wee1 and simultaneously enforced by Chk1 which promote the degradation of both phosphatase CDC25A/C. *WEE1* gene has been found over-expressed in different kind of solid tumor (glioblastoma, osteosarcoma, breast and colon cancer) especially in those with the tumor-suppressor p53 mutated [152–154]. Because of the biological function of Wee1 in cell cycle regulation, different Wee1 inhibitors (PD0166285, PD0407824 and MK-1775) have been developed to improve the efficacy of DNA-damaging agent for the treatment of different cancers [143,155]. In hematological diseases the expression of Wee1 gene has been found higher in tumor samples when compared to the expression of normal peripheral mononuclear cells. Indeed Anderson and colleagues showed that the expression of Wee1 gene was significantly higher in B-/T-ALL leukemic blast isolated from different patients in comparison with the expression of healthy donor[156]. Based on this background in this study we sought to evaluate the effectiveness of the Wee1 inhibitor, MK-1775, as monotherapy and in combination with different drugs, like tyrosine kinase inhibitors (bosutinib authentic or bosutinib isomer), checkpoint kinase (Chk1/Chk2) inhibitors (PF-04477736) and antimetabolites drugs (clofarabine) for the treatment of B-/T-acute lymphoblastic leukemia.

Results

MK-1775 reduces the cell viability and triggers the apoptotic death in B-/T-(ALL) cell lines

To evaluate the efficacy as single agent of the compound, a panel of 8 B-/T-ALL cell lines was treated for 24, 48 and 72 hours with increasing doses of the MK-1775 (from 6 to 5,000 nM, dilution rate 1:3) and the reduction of cell viability was evaluated using the colorimetric assay MTS. MK-1775 reduced the cell viability in dose and time-dependent manner in all cell lines. In particular, the most sensitive cell line after 24 hours of incubation was the Philadelphia-positive cell line, BV-173, with an IC_{50} of 500 nM, while the less sensitive one was the T-ALL cell line, CCRF-CEM, with an IC_{50} of 10 uM (Fig. 1A). In order to explain the high heterogeneity in term of response to the treatment, the basal expression of different proteins involved in the DDR

pathway (Wee1, phospho-Chk1^{ser317}, Chk1, phospho-Chk2^{thr68}, Chk2, phospho-Cdc2^{tyr15}, Cdc2 and γ-H2AX) was evaluated by Western blot. Despite each cell line showed a specific pattern of expression of the different key elements of the DDR, there was no correlation with the sensitivity to MK-1775 (Fig. 1B). Moreover similar to the results of our previous study evaluating the efficacy PF-0477736 on the same panel of cell lines, the sensitivity to the compound did not correlate with the leukemia subtypes, with the karyotype or with the mutational status of the tumor-suppressor p53 [133]. The reduction of the cell viability was then correlated with the activation of cell death via apoptosis. To this purpose different cell lines were incubated for 24 hours with increasing concentration of MK-1775, stained with Annexin V/Propidium Iodide (Pi) and then the amount of apoptotic cells was detected by cytofluorimetry. In all cell lines the inhibition of the Wee1 activated the apoptotic cell death in a dose-dependent manner (Fig. 1E). Then the activation of the apoptotic cascade was correlated with the induction of DNA damages by Western blots. Cells were incubated for 24 hours with MK-1775 (dose nearest to the $IC_{50)}$ and then stained for different antibodies. In the treated samples, the inhibition of Wee1 increased the amount of γH2AX (marker of DNA damages) and of the cleaved isoform of Parp-1 (marker of apoptosis) (Fig. 1C).

The inhibition of Wee1 perturbs the cell cycle profile and increases S and G2/M phases

The biological consequences of Wee1 inhibition were then analyzed in term of perturbation of cell cycle progression. Different cell lines were treated for 24 hours with increasing concentration of MK-1775 and then stained with Pi. The treatment reduced the amount of the cells in G1 phase and progressively increased number of cells in S and in G2/M phase (Fig.1D). The modifications of the cell cycle profile induced by MK-1775 were correlated with the expression of different proteins involved in cell cycle regulation. The treatment reduced the level of expression of both Cdc2 and phospho-Cdc2$^{Tyr\ 15}$, confirming that the catalytic activity of Wee1 was disrupted. Then in order to evaluate the effect of the compound on the checkpoint kinase 1 functionality, the level of expression of phospho-Chk1^{ser317}(marker of ATR-dependent activation of the DDR pathway,), phospho-Chk1^{ser296}(auto-phosphorylation site and marker of

Chk1 functionality) and of the basal form of Chk1 was evaluated in each cell lines. The expression of phospho-Chk1 ^{ser296} was very heterogeneous among the different cell lines while the level of expression of phospho-Chk1^{ser317} was increased, confirming the induction of DNA damage, in all the cell lines. To better understand the mechanism of action of the compound and to evaluate the hypothetic mechanism of death through the mitotic catastrophe, the expressions of the phospho-HH3^{ser10}, markers of mitosis, was evaluated in all the cell lines after the treatment. In different cell lines (SUP-B15, NALM-6, NALM-19, MOLT-4 and RPMI-8402) the incubation with MK-1775 increased the phospho-HH3^{ser10}, confirming the results found in the cell cycle analysis (Fig. 1E; 1D). Finally in order to evaluate the effect of the compound specifically on checkpoint G2/M, BV-173 and CCRF-CEM cell lines were incubated with increasing concentration of MK-1775 (BV-173: 0.4 and 0.8 uM; CCRF-CEM: 5 and 10 uM) for 12 hours and then the level of mRNA expression of different genes involved in the DNA damage response and in the regulation of G2/M checkpoint was analyzed using qPCR (Predesigned 96-well panel for use with SYBR® Green, BioRAD). Different genes involved in cell cycle regulation and in the response to DNA damages were differentially expressed between control and treated samples. In particular in both the cell lines different genes involved in DNA damage response, G2/M transition and in the activation of the apoptosis (ATR, ATM, CDC25C, CDC25B, CDC2, CCNB1, CCNB2, GADD45A and GADD45B) were up-regulated in the treated samples in comparison to the control while genes involved in the negative regulation of the transition G2/M, like PKMYT1, were down-regulated. Interestingly the level of expression of Wee1 was only weakly reduced in both the cell lines (Fig. S1B). Using a specific threshold (2.5), in both the cell lines the expression of PKMYT1 was significantly reduced between treated and untreated cells. The significantly up-regulated genes were GADD45a in BV-173 cell lines and CCNB1/B2 in CCRF-CEM cell line (Fig.1E).

Figure 1

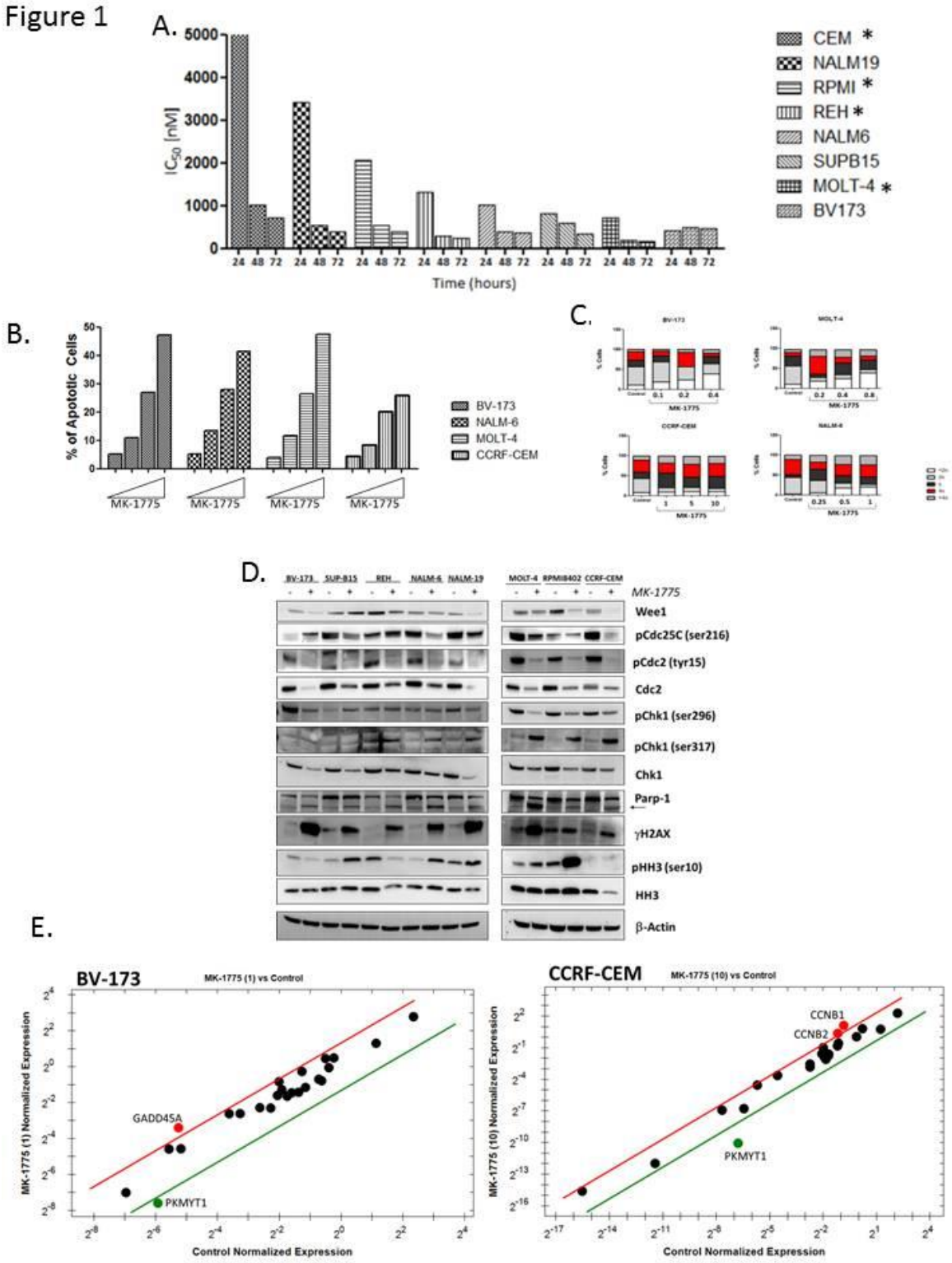

Figure 1: MK-1775 reduces cell viability, increase the apoptosis and deeply change the cell cycle profile in B-/T-ALL cell lines. A) IC50 values of B-/T-ALL cell lines after 24, 48 and 72 hours of incubation with MK-1775. The asterisk (*) in the legend of the graph represent the cell line

with the mutational status of the onco-suppressor p53.**B)** Apoptosis analysis of BV-173, NALM6, MOLT-4 and CCRF-CEM cell lines incubated with increasing concentration of MK-1775 (highest dose close to IC50 value, the other two doses are respectively 1/2 and 1/4 of the IC50) for 24 hours. **C)** Cell cycle profile of BV-173,MOLT-4, CCRF-CEM and NALM-6cell lines incubated with decreasing concentration of MK-1775 (highest dose close to IC_{50} value, the other two doses are respectively 1/2 and 1/4 of the IC_{50}) for 24 hours. **D)** In the blots B-/T-ALL cell lines were incubated for 24 hours with MK-1775 (IC_{50} value). The homogeneity of the protein loaded (40ug) was determined using an internal control (β-actin).**E)** Quantitative analysis of the expression of different elements of the G2/M checkpoint. BV-173 and CCRF-CEM were treated for 12 hours with increasing concentrations of the MK-1775 and then the total amount of RNA was extracted. In the blots the up-regulated genes are represented as red dots while the down-regulated genes between treated samples and controls are represent as blue dots (threshold 2.5) .

MK-1775 reduces the cell viability of primary leukemic cells isolated from adult B-ALL patients

The cytotoxicity of the compound was evaluated on primary leukemic blasts isolated from the bone marrow and peripheral bloods of adult ALL patients (n= 6) at diagnosis, and on normal mononuclear cells isolated from the peripheral blood of healthy donors (n=5). Cells were treated with increasing concentration of the compound (2.5, 5 and 10 uM) for 24 hours and then the reduction of the cell viability was assessed using Trypan blue exclusion dye. In line with the results found on the cell lines, the MK-1775 reduced the cell viability in a dose-dependent manner in all the primary samples treated (Fig.2A), however on the mononuclear cells the compound did not modified the cell viability (Fig. 2B). To evaluate the response of the primary leukemic cells in term of expression of different genes involved in the response to DNA damages, cells were incubated with increasing concentration of MK-1775 (2.5 and 5 uM) for 12 hours and then qPCR were performed. Among all the different genes differentially expressed between control and treated samples, different fundamental genes for the checkpoint G2/M were significantly up-regulated (threshold 2.50): CCNB1, CDC25C, CDK1 (CDC2), WEE1 and GADD45B (Fig. 2C; S1B). The results of the gene expression analysis showed that the inhibition of Wee1 enhanced the expression of different genes involved in the transition from the G2 into M phase. To confirm these data and to evaluate the effect of MK-1775 on mitosis, the primary

leukemic cells isolated from one adult case with Philadelphia-negative B-ALL patient and the mononuclear cells isolated from the peripheral blood of one healthy donor were treated with MK-1775 (2.5, 5, 10 uM) for 24 hours and then stained with the Giemsa and May Grünwald solutions to discern the nuclei from the cytoplasm and to evaluate nuclei morphology. In the primary samples MK-1775 significantly altered the morphology of the nuclei, increasing the number of micro/macro nuclei and of DNA bridges among the nuclei. Interestingly all the alterations were restricted to leukemic samples, indeed in the mononuclear cells there was no difference in the morphology of the nuclei between control and treated cells (Fig. 2D). Conclusively the primary leukemic cells from an adult B-ALL patient were treated with increasing concentration of MK-1775 (2.5, 5 and 10 uM) for 24 hours and then stained for phospho-Chk1^{ser317}, Chk1, phospho-Cdc2^{tyr15}, Cdc2 and γH2AX. Similar to the results found on the different cell lines, the compound deeply reduced the expression of both basal and phosphorylated isoforms of Cdc2 while increased the amount of phospho-Chk1 and γH2AX, confirming at the same time the inhibition of Wee1 functionality and the induction of DNA damages

Figure 2

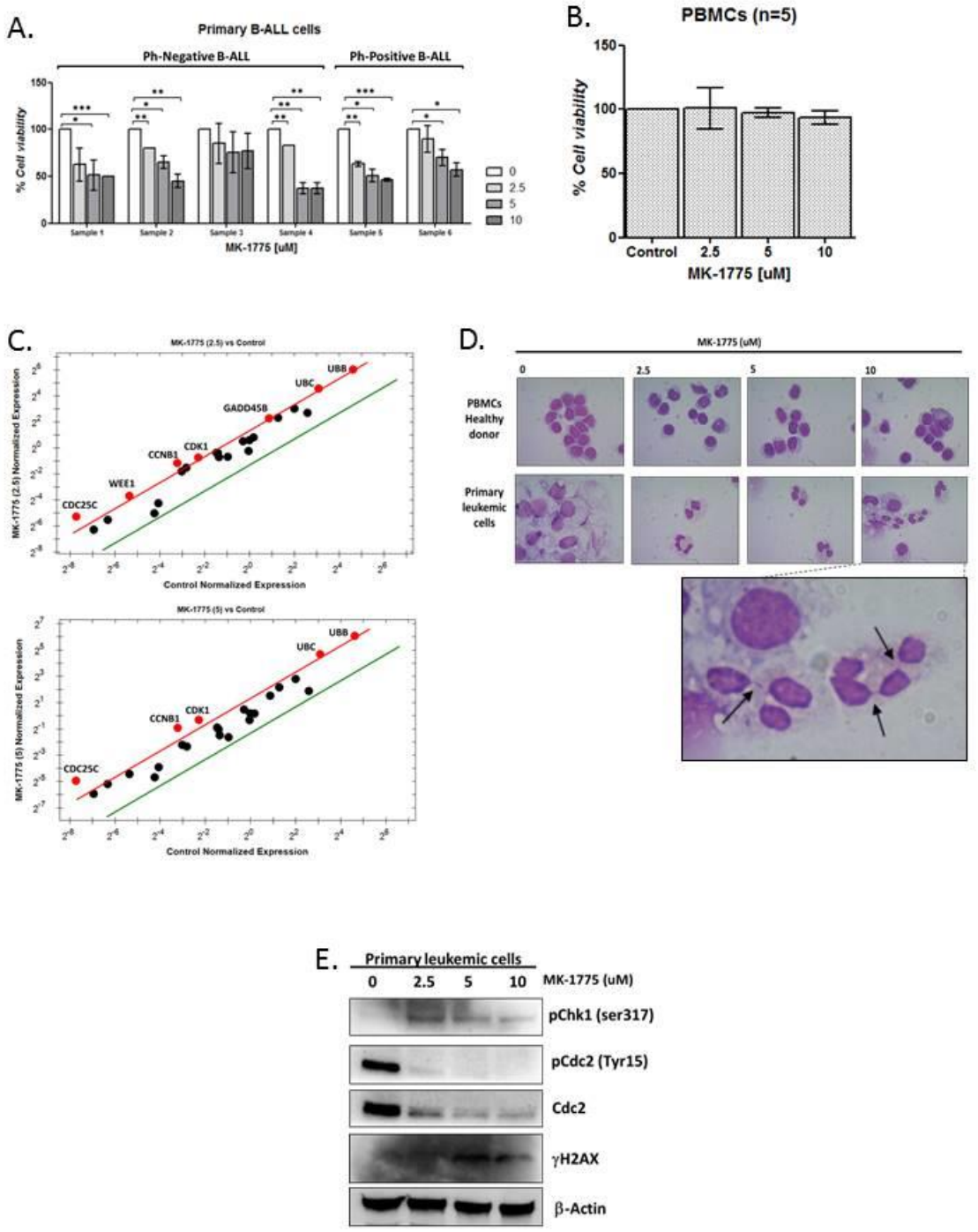

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Figure 2: MK-1775 abrogates the G2/M checkpoint in the primary cells isolated from adult ALL patients. A) Cell viability analysis of primary leukemic cells isolated from 6 adult B-ALL patients incubated for 24 hours with 2.5, 5 and 10 μM of MK-1775. **B)** Cell viability analysis of

mononuclear cells isolated from the peripheral blood of 5 healthy donors incubated for 24 hours with 2.5, 5 and 10 uM of MK-1775. **C)** Quantitative analysis of the expression of different elements of the G2/M checkpoint. Primary leukemic cells were treated for 12 hours with increasing concentrations of the MK-1775 (2.5 and 5 μM) and then the total amount of RNA was extracted. In the blots the up-regulated genes are represent as red dots while the down-regulated genes between treated samples and controls are represent as blue dots. **D)** Light microscopy analysis of primary leukemic cells and mononuclear cells incubated with increasing concentration of MK-1775 (2.5, 5, 10 uM) and then stained with the Giemsa and May Grünwald solutions to discern nuclear structure alterations. The black arrows indicate different DNA bridges, markers of aberrant mitosis. **E)** Western blot analysis of primary leukemic cells treated with MK-1775 (2.5, 5 and 10 uM) for 24 hours. The protein extracts were stained p-Chk1 ser317, Chk1, p-Cdc2 tyr15, Cdc2 and γH2AX ser139. The homogeneity of the protein loaded (70ug) was determined using an internal control (β-actin).

MK-1775 sensitizes leukemia cell lines to the toxic effect of clofarabine.

The efficacy as chemo sensitizer agent of MK-1775 have been showed in different studies, however none of them were focused on the different compounds normally used for the treatment of ALL[84,157]. Thus to evaluate the chemo-sensitizer efficacy of MK-1775 on ALL patient's treatments different combinations have been made using compounds normally used in the clinic. In particular different Philadelphia-negative B-/T-ALL cell lines were treated for 24, 48 and 72 hours with increasing concentration of MK-1775 (from 6 to 5000 nM, dilution rate 1:3) in combination with the purine nucleoside analogue, clofarabine. Based on the sensitivity of each cell lines to clofarabine (data not showed), Nalm6, Nalm19 and REH cell lines were treated with 2.5, 5, 10 nM while CCRF-CEM, MOLT-4 and RPMI-8402 with 5, 10, 20 nM. The combination of the two compounds additively reduced the cell viability in comparison to the effect of the single treatments. The results of the combination index analysis showed that there was no difference in term of sensitivity among the different cell lines and in particular between p53 mutated and p53 wild type cell lines () (Fig. 3A). Based on the results of the combination index analyses, NALM-6, REH, CCRF-CEM and RPMI-8402 cell lines were treated with clofarabine (5nM) and MK-1775 (180 nM) for 24 hours and then stained with Pi. The combination did not significantly alter the cell cycle profiles in comparison with the effects of the single treatments (Fig. 3B). To correlate the effect in term of reduction of the cell viability

with the activation of cell death, two cell lines, MOLT-4 and NALM-6, were treated with MK-1775 (185nM) in combination with clofarabine (MOLT-4 20nM and NALM-6 10 nM) and then the amount of apoptotic cells was detect using cytofluorimetry. In both the cell lines the combination of the two compounds increased significantly the amount of apoptoic cells in comparison to the effect of the single treatments. Despite results of the combination index analysis, in term of induction of apoptosis the p53 mutated cell lines resulted more sensible to the pro-apoptotic effect of the combination (Fig. 3C). Finally the effect of the combination was evaluated in term of reduction of the proliferation. To this purpose REH, NALM-6, MOLT-4 and CCRF-CEM cell lines were incubated with MK-1775 (180nM) and with sub-toxic concentration of clofarabine (respectively: 2.5, 5, 10 and 20 nM) for 6 days and the number of proliferating cells was assessed every 24 hours using Trypan Blue exclusion dye. In all the treated cell lines the combination of the two compounds drastically reduced the number of proliferating cells in comparison to the effect of the single treatments (Fig.3D). The effect of the combination between MK-1775 and clofarabine was then assessed on primary leukemic cells isolated form the peripheral blood and the bone marrow of 8 adult ALL patients. Primary cells were incubated with MK-1775 (5uM) and with increasing concentration of clofarabine (100, 250 and 500 nM) for 24 hours and then the reduction of the cell viability was quantified using Trypan blue exclusion dye. Although with high heterogeneity, in all the primary samples the combination with the two compounds significantly reduced the cell viability in comparison with the effect of the single treatment (Fig. 3E).

Figure 3

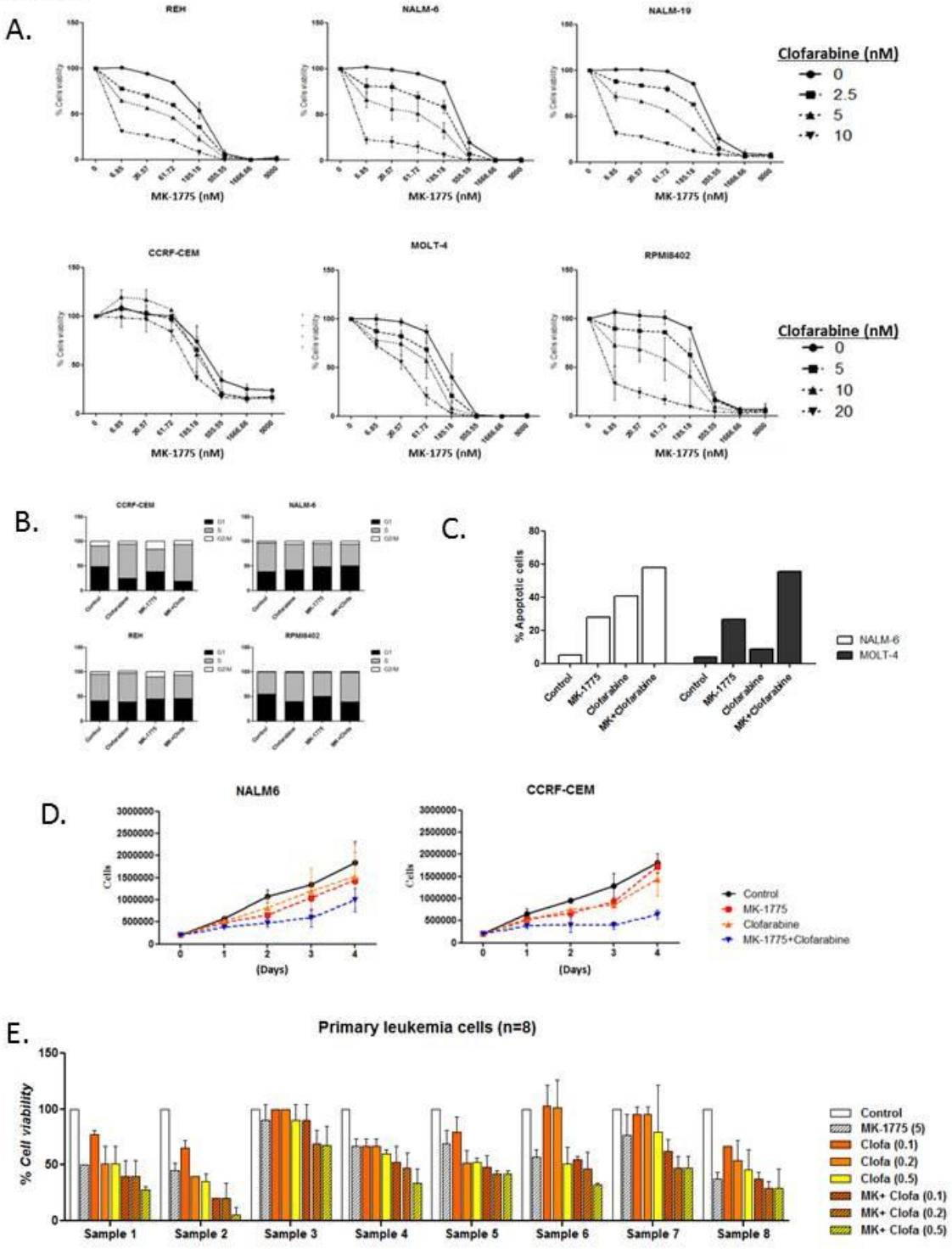


Figure 3: MK-1775 sensitizes Philadelphia-negative cell lines and primary leukemic cells to the cytotoxic effect, clofarabine. **A)** NALM-6, NALM-19, REH, MOLT-4, CCRF-CEM and RPMI-8402 cell lines were treated for 24, 48 and 72 hours with increasing concentration of Mk-1775(from

6 to 5000 nM) with increasing concentration of clofarabine. All the data present are the mean of two independent experiments **B)** Cell cycle analysis of NALM-6, REH, CCRF-CEM and RPMI-8402 cell lines treated with MK-1775 in combination with clofarabine for 24 hours. **C)** Apoptosis analysis of NALM-6 and MOLT-4 cell lines treated for 24 with MK-1775 (185 nM) and clofarabine (NALM-6: 10nM; MOLT-4: 20nM). **D)** Proliferation index assay of NALM-6 and CCRF-CEM cell lines incubated with MK-1775 (185 nM) and sub-toxic concentration of clofarabine (respectively 2.5 and 20 nM) for 6 days. The number of living cells was evaluated using the Trypan Blue exclusion dye analysis. All the data present are the mean of two independent experiments. **E)** Cell viability analysis of primary leukemic cells isolated from adult Philadelphia-negative B-ALL patients (n=8) incubated with MK-1775 (5 uM) and increasing concentration of clofarabine (0.1, 0.2 and 0.5 uM) for 24 hours. The reduction of the cell viability was evaluated using the Trypan Blue exclusion dye analysis.

The inhibition of Wee1/Chk1/Chk2 kinases drastically reduces the cell viability, triggers apoptosis and modifies cell cycle profile in B-/T-ALL cell lines

Due to the central role of both Wee1 and Chk1 kinase in cell cycle regulation the effect of the concomitant inhibition of these two kinases was evaluated in B-/T-ALL cell lines. The sensitivity of each cell lines to the Chk1/Chk2 inhibitors,PF-047736, have been recently published[133]. Thus the most sensitive and the less sensitive cell lines, RPMI-8402 and NALM-6, were chosen to evaluate the effect of the combination with MK-1775. To this purpose cells were treated with increasing concentration of MK-1775 (form 6 to 5000 nM, dilution rate 1:2) and decreasing doses of PF-0477736 (respectively 100, 50,25, 10 and 1000, 500, 250, 100 nM) for 24, 48 and 72 hours. In line with the literature the inhibition of both Chk1 and Wee1 additively reduced the cell viability in both the cell lines[158]. Similar to what found in the co-treatment with Clofarabine, the cytotoxic effect of the combination of the two inhibitors was time and dose-dependent (Fig.4A). To better understand the consequences of the inhibition of both Chk1 and Wee1 on the cell cycle profile, RPMI-8402 and NALM-6 cell lines were treated for 24 hours with MK-1775 (180 nM) and PF-0477736 (respectively 25 and 250 nM) and then stained with Pi. In both cell lines the co-treatment increased the amounts of cells in S phase (Fig.4B). The effect of the inhibition of Chk1 and Wee1 on protein expression was evaluated using Western blot analysis. RPMI-8402 and NALM-6 were treated for 24 hours with MK-1775 and PF-0477736 and

then stained for the expression of phospho-Chk1 (ser345 and ser317), Chk1, phospho-Cdc2 (Tyr15), Cdc2, phospho-HH3 (ser10), γH2AX, pro caspase-3, Parp-1, and β-Actin. In agreement with the results of the viability, the combination of the two compound synergistically increased the expression of γH2AX, confirming the induction of DNA damages, and decreased the expression of phospho-Cdc2, confirming the inhibition of both Wee1 and Chk1 (Fig.4C). The effect of the double treatment was then evaluated in term of inhibition of cell proliferation. RPMI-8402 and NALM-6 cell lines were treated with MK-1775 (180 nM) and PF-0477736 (respectively 25 and 250 nM) for 6 days and then the amount of cells was evaluated every 24 hours. In both the cell lines the inhibition of both Wee1 and Chk1 completely reduce the proliferation rate, while the mono-treatment reduced the cell viability but only in the earliest time points (Fig.4D). The effect of the combination was then evaluated on primary leukemic cells isolated from 2 adult ALL patients. To this purpose primary cells were incubated for 24 hours with MK-1775 (1000 nM) in combination with PF-0477736 (1000nM) and then the reduction of the cell viability was evaluated using Trypan blue exclusion dye. These experiments confirmed the results of the reduction of the cell viability found on the cell lines (Fig.4E).

Figure 4

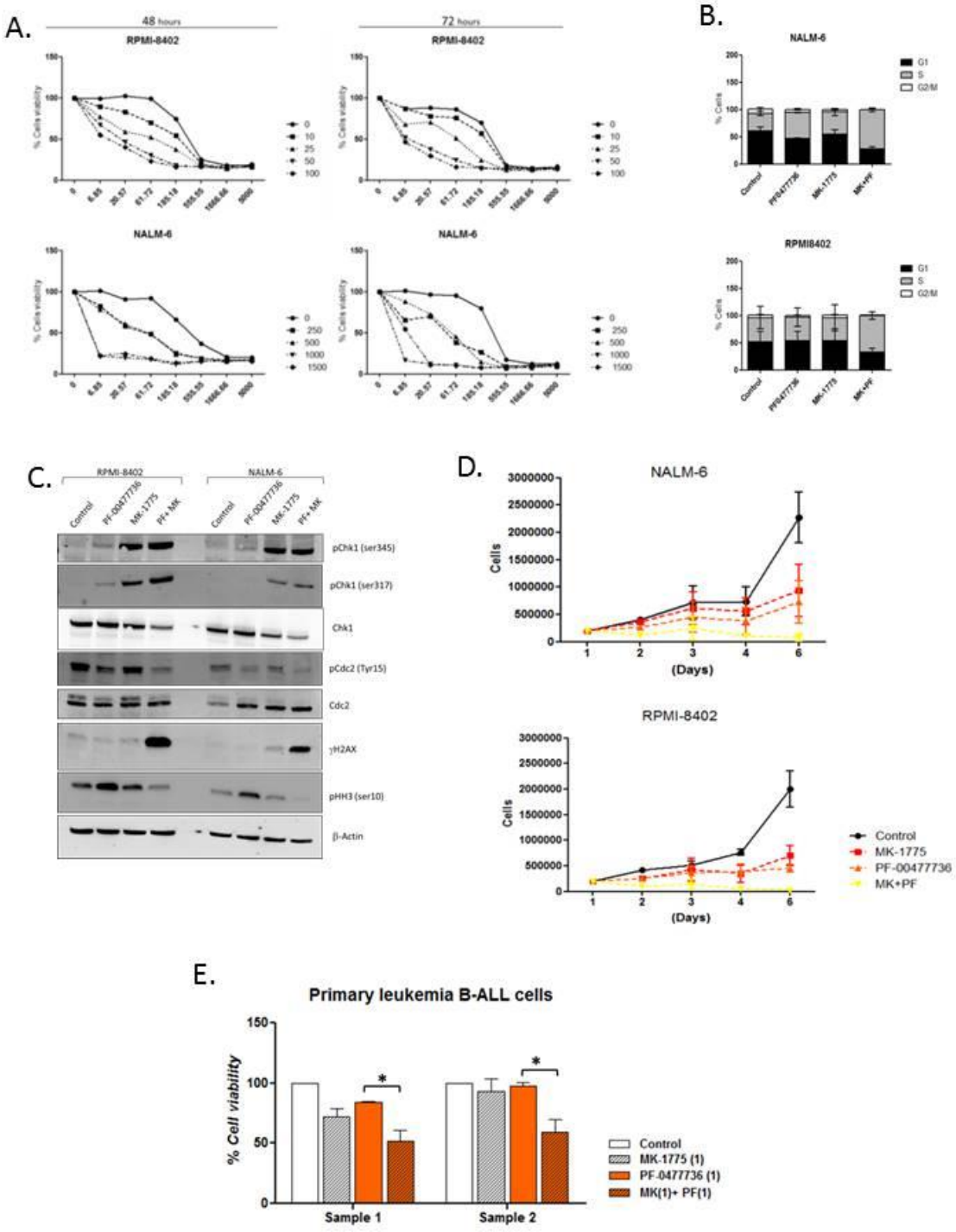


Figure 4: The inhibition of Wee1 and Chk1/Chk2 kinases deeply reduced the cell viability, changed the cell cycle profile and inhibited the proliferation in B-/T-ALL cell lines. **A)** Cell viability assay of RPMI-8402 and NALM-6 cell lines treated for 48 and 72 hours with increasing

concentration of Mk-1775(6nM-5 uM) with increasing concentration of PF-00477736 (RPMI-8402: 10, 25, 50 and 100 nM; NALM-6: 250, 500, 1000 and 1500nM). All the data present are the mean of two independent experiments **B)** Cell cycle analysis RPMI-8402 and NALM-6 cell lines treated with MK-1775 in combination with PF-00477736 for 24 hours. **C)** Western blot analysis of RPMI-8402 and NALM-6 treated with MK-1775 and PF-00477736 for 24 hours. The protein extracts were stained p-Chk1ser317 p-Chk1ser345, Chk1, p-Cdc2tyr15, Cdc2, p-HH3 (ser10) and γH2AX (ser139). The homogeneity of the protein loaded (40ug) was determined using an internal control (β-actin). **D)** Proliferation index assay of RPMI-8402 and NALM-6 cell lines incubated with MK-1775 (185 nM) and sub-toxic concentration of PF-00477736 (respectively 2.5, 5 and 20 nM) for 6 days. The number of living cells was evaluated using the Trypan Blue exclusion dye analysis. All the data present are the mean of two independent experiments. **E)** Cell viability analysis of primary leukemic cells isolated from adult B-ALL patients (n=2) incubated with MK-1775 (1 uM) and with PF-0477736 (1uM) for 24 hours. The reduction of the cell viability was evaluated using the Trypan Blue exclusion dye analysis.

MK-1775 sensitizes leukemic cell lines to the tyrosine kinase inhibitor bosutinb

The chemo-sensitizer activity of MK-1775 was also evaluated combining the Wee1 inhibitor with different tyrosine kinase inhibitors (TKIs), which represents the frontline therapy for the treatment of Philadelphia-positive acute lymphoblastic and chronic myeloid leukemia patients [159–162]. In particular the MK-1775 was combined with two isomers of the TKIs, bosutinib (here hence named as Bos, the authentic isoform, and Bos-I, a specific bosutinib isomer), which differ in term of specificity and of chemical proprieties [143,163]. We have already shown that Bos-I on PANC-1 cells has an off target inhibitory activity on Wee-1 and Chk1 kinases and also that this compound can sensitize tumor cells to gemcitabine [143]. The efficacy of the two isomers, in combination with MK-1775, was evaluated in term of reduction of the cell viability, activation of the apoptosis, and modification of protein expression on ALL cell lines and on primary cells. To assess the cytotoxicity as single agent of the two isomers, cells were incubated for 24, 48 and 72 hours with increasing concentration of Bos/Bos-I (from 6 nM to 5000 nM, dilution rate 1:3) and then the reduction of the cell viability was quantified using MTS reagent. The treatment with Bos reduced the cell viability not only of Philadelphia-positive cell lines (BV-173 and SUP-B15), harboring the BCR-ABL1 fusion transcript, but also of different Philadelphia-negative cells lines, due to the inhibitory effect on Src family kinases. Then efficacy of the Bos-I

was evaluated as single agent on both Philadelphia positive and negative cell lines. In Philadelphia positive cell lines the efficacy of the two isomers was almost the same but in Philadelphia negative cell lines the Bos-I significantly reduced the cell viability in comparison to the effect of the authentic isoform (Fig. 5A). In order to evaluate the efficacy of the two isomers in combination with MK-1775, different combination index assays were performed. The result of the analysis showed that the combination between Bos/Bos-I and MK-1775 additively reduced the cell viability of Philadelphia positive cell lines with no significant differences, in term of efficacy, between the two isomers. On contrary in the Philadelphia-negative cell lines there was a significant difference in term of effectiveness of the two combinations (Fig. 5B). In order to evaluate the effect of the combination on the induction of apoptosis, BV-173, SUP-B15, REH, NALM-6 and MOLT-4 were treated for 24, 48 and 72 hours with MK-1775 (180 nM) and sub-toxic concentration of Bos/Bos-I (BV-173 50nM; SUP-B15 100nM; REH,NALM-6,MOLT-4 1uM). The greater effect in the induction of the apoptosis was seen after 72 hours of incubation with the two drugs. In the two Philadelphia-positive cell lines, BV-173 and SUP-B15, the treatment with MK-1775 and Bos significantly increased the number of apoptotic cells in comparison with the effect of the single treatments and to the effect combination between MK-1775 and Bos-I. On the Philadelphia-negative cell lines the simultaneous treatment with MK-1775 and Bos-I induced a significant increment of necrotic cells in comparison to the effect of effect of the single treatments and to the combination between MK-1775 and Bos (Fig.5C). To assess the effect of the combination of cell proliferation BV-173 and NALM-6 cell lines were incubated with sub-toxic concentration of MK-1775 (185 nM) and Bos/Bos-I (BV-173 50 nM; NALM-6 2uM) for 6 days and the amount of proliferating cells was evaluated every 24 hours. In line with the previous results on BV-173 the strongest effect in the inhibition of the proliferation was seen in the combination between MK-1775 and the authentic isomer of Bosutinib(Bos).Moreover in the combination between MK-1775 and Bos-I, no significant different were seen in the samples treated with MK-1775 alone or in addiction with Bos-I. Interestingly in the experiment with NALM-6 the effects of the two combinations were exactly the opposite of BV-173 cell line (Fig. 5D). Finally the efficacy of the combination between MK-1775 and Bos/Bos-I was evaluated on primary cells isolated form the bone marrow and the

peripheral blood of 2 B-ALL patients. In the first experiment the primary cells isolated form a Philadephia-positive B-ALL patient were incubated with MK-1775 (5uM) and Bos (2uM) for 24 hours and the the reduction of the cell viability was quantified using Trypan Blue. The results showed that the MK-1775 sensitized the primary cells to the effect of TKI, confirming the results on the Philadelphia-positive cell lines (Fig. 5E). Then the effect of MK-1775 in combination with Bos/Bos-I was evaluated on the primary cells isolated from a Philadelphia-negative patient. Cells were incubated with MK-1775 (5uM) and Bos/Bos-I (2 uM) for 24 hours and then the reduction of the cell viability was quantified. The results of the experiment showed that also on Philadelphia-negative primary cells Bos-I, but not the authentic isomer of bosutin, increased the efficacy of MK-1775 in the reduction of the cell viability(Fig.5F).

Figure 5

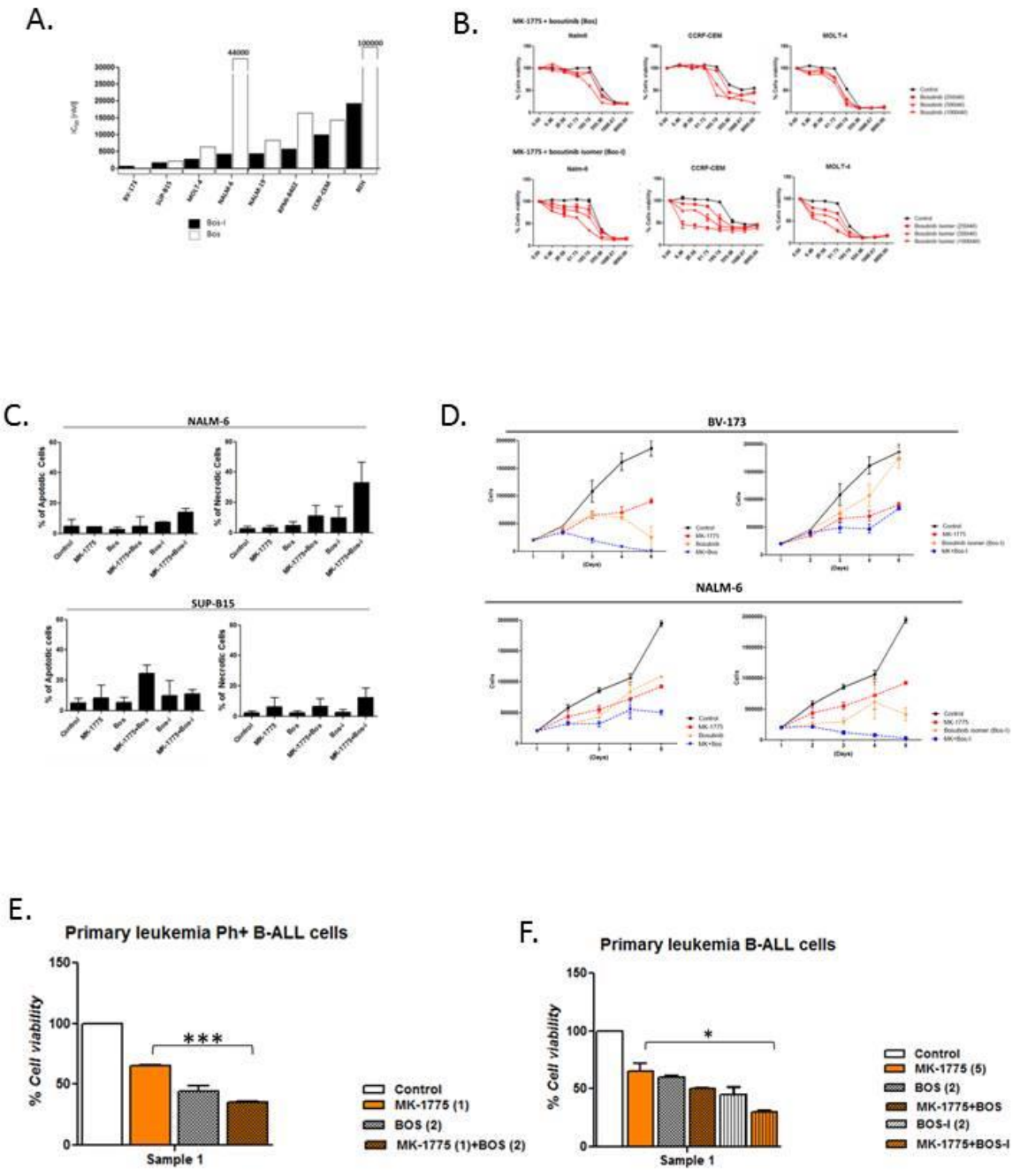


Figure 5: MK-1775 increased the cytotoxicity of both Bos and Bos-I isomers but with differences between Philadelphia-positive and negative cell lines. A) IC50 values of B-/T-ALL cell lines after 24 hours of incubation with Bos (white column) and Bos-I (black column). All the

data presented are the mean of three independent experiments. All the data presented are the mean of two independent experiments. **B)** Cell viability assay of NALM-6, CCRF-CEM and MOLT-4 cell lines treated for 72 hours with increasing concentration of Mk-1775(6nM-5 uM) with increasing concentration of Bos/ Bos-I (250, 500 and 1000nM). All the data present are the mean of two independent experiments. **C)** Apoptosis analysis of SUP-B15 and NALM-6 cell lines incubated with MK-1775 and subtoxic concentration of Bos/Bos-I incubated for 72 hours. All the data present are the mean of two independent experiments. **D)** Proliferation index assay of BV-173 and NALM-6 cell lines incubated with MK-1775 (185 nM) and sub-toxic concentration of Bos/Bos-I (BV-173 50nM, NALM-6 2uM) for 6 days. The number of living cells was evaluated using the Trypan Blue exclusion dye analysis. All the data present are the mean of two independent experiments. **E)** Cell viability analysis of primary leukemic cells isolated from adult Philadelphia-positive B-ALL patient incubated with MK-1775 (5 uM) and with Bos (2uM) for 24 hours. The reduction of the cell viability was evaluated using the Trypan Blue exclusion dye analysis. **F)** Cell viability analysis of primary leukemic cells isolated from adult Philadelphia-negative B-ALL patient incubated with MK-1775 (5 uM) and with Bos/Bos-I (2uM) for 24 hours. The reduction of the cell viability was evaluated using the Trypan Blue exclusion dye analysis.

Discussion

The inhibition of the DNA damage response (DDR) is a promising strategy to sensitize tumor cells to the cytotoxicity of different compound by inhibiting the mechanisms that tumor cells up-regulates to repair DNA damages and to survive The DDR pathway plays a central role in the response to genotoxic drugs by activating the mechanism of DNA repair and by regulating cell cycle progression The efficacy of different inhibitors of the DDR has been well established in vitro and in vivo studies especially in solid tumors. Nowadays only few studies have been done to evaluate the effectiveness of DDR inhibitors in hematological malignacenes[105]. Recently Iacubucci and colleagues have showed the efficacy of PF-00477736, a potent Chk1/Chk2 inhibitor, in single agent on ALL cell lines and primary samples[133]. Here we evaluated the efficacy of MK-1775 a specific Wee1 kinase inhibitor, as single agent and in combination with different compound on B-/T-ALL cell lines and on primary samples. Similar to the results found in the PF-0477736 study the inhibition of a central kinase involved in cell cycle regulation, like Wee1, mined the survival of leukemic cells. The compound deeply reduced the cell viability in all the treated cell lines and on different primary samples. The sensitivity to the compound was not related to the sup-type of leukemia, nether with the mutational status of the tumor-

suppressor p53 nor with the basal expression of the Wee1 protein. The effect of the compound in single agent was not limited to the cell viability, but also inducted cell death via apoptosis and deeply modified the cell cycle profile. The cytotoxic effect of MK-1775 was then confirmed on different primary cells isolated from the bone marrow and the peripheral blood of adult B-ALL patients. Interestingly the toxicity seen on the primary leukemic cells was not found on peripheral mononuclear cells isolated from the peripheral blood of healthy donors. The Western blots analysis highlighted the functional consequences of Wee1 inhibition. The on-target efficacy of the compound was confirmed by the reduction of the phosphorylated form of Cdc2, direct target of Wee1. The treatment with the compound even in single agent increased the DNA damages (H2AX) and triggered the apoptotic death (Parp-1 cleavage) in all the cell lines treated. The increment of DNA damage was also confirmed by the increment of phospho-Chk1 (ser317), confirming that the inhibition of Wee1 increased the intrinsic genetic instability. Interestingly in different cell lines (NALM-19, MOLT-4, CCRF-CEM and RPMI-8402) the treatment with MK-1775 reduced the protein level of phospho-Chk1 (ser296), site of auto-phosphorylation and marker of Chk1 functionality, raising the hypothesis that the compound had an off target effect on Chk1. MK-1775 deeply modified the gene expression (Q-PCR analysis) of different elements of the G2/M checkpoint. The genes more significantly up-regulated were GADD45A, involved in the activation of the apoptosis, and the cyclin B1/B2, fundamental for G2/M transition. Moreover in all the cell lines the treatment down-regulated the expression of PKMYT1, one of the main repressor of G2/M transition. These data confirmed the abrogation of the G2/M checkpoint by MK-1775. Similar results were obtained on the primary leukemic cells isolated from the bone marrow of an adult B-ALL patient. Using the same value of threshold seven genes were significantly up-regulated between treated samples and controls. All the above mentioned genes were involved in the transition through the G2/M checkpoint, confirming also on the primary cells the override of the G2/M checkpoint. The consequence of Wee1 inhibition in term of chemo-sensitization was then evaluated on leukemia cell lines and on primary samples. In order to evaluate if the inhibition of a key element of the DDR could sensitize leukemic cells to different therapies, cells were treated with MK-1775 in combination with different class of antineoplastic agents like tyrosine kinase

inhibitors, purine nucleoside analogue and Chk1/Chk2 inhibitor. The results of these experiments on the cell viability suggested that MK-1775 deeply sensitized the different cell lines to the toxic effect of the different compounds. Moreover similar results were seen in different experiments evaluating the effect to the combinations in the induction of the apoptosis and in the inhibition of the cell proliferation. These results were then confirmed on primary cells isolated form Philadelphia-positive and negative ALL patients. Finally the results of different experiments using the two isomer of the TKI, bosutinib, confirmed an off–target effect of Bos-I on Philadelphia-negative cell lines and primary samples.

Materials and Methods

Drugs and Compounds

MK-1775 was purchased from MedChemexpress (http://www.medchemexpress.com). Clofarabine and PF-0477736 were obtained from Sigma (http://www.sigmaaldrich.com/). Bosutinib isomer (BOS-I) was purchased from LC Labs (http://www.lclabs.com/) while the authentic isoform (BOS) from Tocris (http://www.tocris.com/).

Leukemia cell lines

Human B- (BV-173, SUP-B15, REH, NALM-6, NALM-19) and T-ALL (MOLT-4, RPMI-8402, CCRF-CEM) cell lines were cultured in RPMI-1640 medium (Invitrogen, Paisley, UK) with 1% *l*-glutamine (Sigma, St. Louis, MO), penicillin and streptomycin (Gibco, Paisley, UK) supplemented with 10%-20% fetal bovine serum (Gibco) in a humidified atmosphere of 5% CO_2 at 37°C. All the cell lines were purchased from Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Germany). Cells Online databases have been interrogated to molecularly characterize leukemia cell lines: International Agency for Research on Cancer (IARC) *TP53* database (http://www-p53.iarc.fr/) and the Catalogue of Somatic Mutations in Cancer, (COSMIC, http:// http://www.sanger.ac.uk/genetics/CGP/cosmic/).

Primary leukemic cells

To assess the effect of MK-1775 on primary leukemic cells, samples from 8 B-ALL cases were obtained, upon written informed consent, from the peripheral and bone marrow blood by density gradient centrifugation over Lymphoprep (Nycomed UK, Birmingham) and then treated with the compound in single agent and in combination with different compounds. Primary cells were seeded on a 6 well plates at 500.000 cells/ml and incubated with increasing concentration of MK-1775 (2.5, 5 and 10 uM) for 24 hours at 37°C. For the combination studies cells were incubated with increasing concentration of the MK-1775 and with increasing concentration of clofarabine (100, 250 and 500 nM), PF-0477736 (1uM) or with Bos/Bos-I (1 and 2 uM) for 24 hours at 37°C. In order to evaluate the cytotoxicity of the compound on peripheral mononuclear cells (PBMCs), cells isolated from the peripheral blood of 5 healthy donors, upon written informed consent, were treated with increasing concentration of MK-1775 (2.5, 5 and 10 uM). *In ex vivo* analysis the effect of the compound on the cell viability was detected by counting viable and non-viable cell numbers by the Trypan blue dye exclusion method (Sigma).

Blast morphology and DNA bridge detection assay

In order to evaluate macroscopic modification of cell morphology the primary cells isolated from the bone marrow a B-ALL patient and the mononuclear cells isolated from the peripheral blood of a healthy donor were seeded on a 6 well plates at 500.000 cells/ml and incubated with increasing concentration of MK-1775 (2.5, 5 and 10 uM) for 24 hours at 37°C. Cell were prepared for microscopy analysis spinning down on glass slips, using cytospin centrifuge, and then to discern the nuclei from the cytoplasm and to evaluate nuclei morphology were stained with the Giemsa and May Grünwald solutions. The slides were analyzed using an optical microscope and the pictures were taken using software.

Cell viability assay

For screening the reduction of the cell viability we used the MTS assay (Promega). For the monotherapy studies cells were seeded into 96 well plates $0.5x10^6$cells/ml and incubated at 37 °C with different drugs for 24, 48 and 72 hours. For the combination index assay the two drugs in combination were added simultaneously and the cells were incubated for 24, 48 and 72 hours. Cell viability was then determined by measuring the absorbance at 490nm. All data

points were normalized to untreated cells. All treatments were performed in triplicate and performed a minimum of 3 times.

Cell proliferation assay

To assess the inhibitory effect of the compound on the proliferation, cells were treated with MK-1775 in single agent or in combination with different compounds. Cells were seeded in 6 well plates in a concentration of $0.2x10^6$ cells/ml and incubated at 37 °C with MK-1775 for six days and the number of proliferating cells was evaluated every 24 hours using Trypan blue dye exclusion method (Sigma). All experiments were performed in triplicates and replicated a minimum of 2 times.

Western Blot analysis

To understand the biological and functional consequences of the inhibition of Wee1, Western blot analysis were performed. Cells were seeded in 6-well/plates at $0.5x10^6$ cell/ml with increasing concentrations of MK-1775. After the appropriate time of incubation, proteins were extracted and then stained for different antibodies. In order to evaluate the effect of MK-1775 in combination with other drugs (Bos/Bos-I, PF0477736 and clofarabine) cells were incubated for different time points and with specific concentration of drug. The drugs in the combination were added simultaneously. After the end of the treatment, the cells were collected and lysate using a specific buffer made of KH2PO4 0,1 M (pH 7,5), Igepal 1% (NP-40), β-glicerofosfato 0,1 mM and complete protease inhibitor cocktail 1X (Roche Diagnostics).For each sample 50ug of protein were fractioned on Mini-Protean TGX stain-free precasted gels, blotted to nitrocellulose membranes (Bio-Rad Trans-blot turbo transfer pack) and incubated overnight with the following antibodies: Chk1 (#2345S), phosphorylated Chk1 (Ser317)(#2344S),Chk1 (Ser345)(#2348S) and Chk1 (ser296)(#), Cdc2 (#9112S), phosphorylated Cdc2 (Tyr15)(#4539S), phosphorylated H2A.X (Ser139) (γ-H2A.X) (#2577S), Caspase3 (#9662S), Parp-1 (#9542S),phosphorylated Histone 3 (Ser10) from Cell Signaling. Antibody to β-actin came from Sigma (St. Louis, MO). Finally all these antibodies were detected using the enhanced chemiluminescence kit ECL (GE) and the compact darkroom ChemiDoc-It (UVP).

Cell Cycle analysis

In order to evaluate the effect of Wee1 inhibition in the cell cycle progression, different cell lines were treated with increasing concentrations of MK-1775 and for different time points. In the combination studies the drugs were added simultaneously. The cell lines were seeded in a 24 wells/plate at the concentration of $0.5x10^6$ and treated 24 or 48 hours at 37°C. After the right incubation time the cells were harvested and washed with cold PBS. After the wash the PBS was discarded and the cell were fixed using ethanol 70% and stored at -20°C for 24 hours. After the fixation period the ethanol was removed by one wash in PBS and the cells were incubated for 30 minutes at 37°C with the staining mix (sodium citrate pH 2.5 100mM, propidium iodide 2.5mg/ml, RNAse 10mg/ml and dH2O).The cell cycle analysis was conducted using the BD FACS Canto and the quantitative analysis using Flowjo, Flowing and .

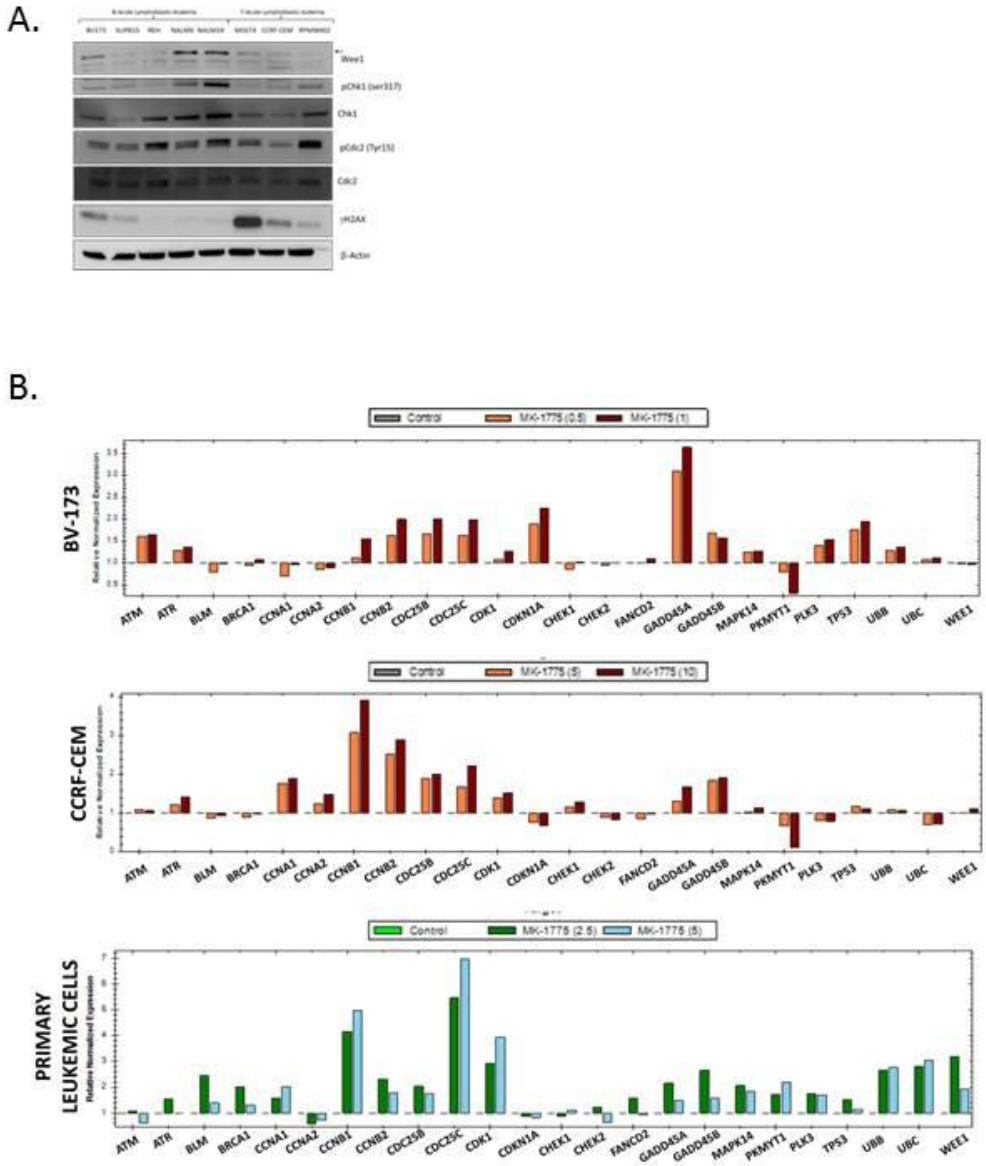
Quantitative PCR

To evaluate the effect of MK-1775 on the expression of different elements of the G2/M checkpoint, different cell lines (BV-173 and CCRF-CEM) and primary cells were treated for 12 hours with increasing concentration of MK-1775. After the right amount of time cells were harvested and the total RNA was extracted using Maxwell simply RNA Blood kit (Promega) and 1 ug of each RNA sample (quantified by ND1000 Spectophotometer) was reverse transcribed using iScript Advanced cDNA Synthesys kit for RT-qPCR (Bio-Rad). For quantification of different elements of G2/M Checkpoint transcript levels, the commercial 96 wells PrimePCR plates (DNA damageDNA-ATM ATR regulation of G2 M checkpoint, Bio-Rad) were employed according to the instructions of the manufacturer: Sso Advanced Universal Sybr Green Supermix (Bio-Rad) and LightCycler 480 System amplification protocol (Roche Diagnostics, Mannheim, Germany). For the normalization of the relative amount of mRNA, three differnent housekeeping genes were amplified (GAPDH, TBP and HPRT1). Data analysis was performed with PrimePCR analysis software (Bio-Rad).

Statistical analysis

All the differences in percentages of reduction of the cell viability were analyzed by unpaired *t*-test (P≤0.05 was considered as statistically significant).

Supplementary 1



Supplementary Figure 1: A) Basal expression of Wee1, p-Chk1ser317, Chk1, p-Cdc2tyr15, Cdc2, γH2AX in all the B-/T-ALL cell lines treated in this study. **B)** Graphic representation of the relative normalized expression of different genes involved in the ATR/ATM response to DNA

damages and to the regulation of the G2/M checkpoint. In the graph the expression of each gene in the treated samples is normalized to the expression of the controls.

Discussion

The present thesis aimed to evaluate the efficacy of the inhibition of the checkpoint kinase pathway as a novel strategy for the treatment of adult ALL patients. The results of these studies, that will be discussed in details below, showed the general idea that Chk1, Chk2 and Wee1 kinases are crucial for the leukemic cells not only to survive but also to sustain the intrinsic genetic instability that characterize this kind of hematological malignance. In line with the study of Sarmento and colleagues[73], in which the Chk1 kinase has been found over-expressed and hyper-activated in T-ALL patients, we showed that the same kinase is highly expressed in our B-ALL patients cohort in comparison with its expression in mononuclear cells isolated form the peripheral blood of healthy donors. Moreover using immunohistochemistry we demonstrate that both the Chk1 and Chk2 pathways are hyper-activated in the tumor samples in comparison to normal tissue. On these bases we speculate that the inhibition of these kinases could mine the genetic stability of ALL cells, pushing the leukemic cells to progressively accumulate DNA damages up to a point of no return. For this reason we firstly evaluate the efficacy in single agent of the Chk1/Chk2 inhibitor, PF-0477736 on different cell lines and on primary cells isolated from adult B-ALL patients. The results of these experiments confirmed that the activities of these kinases are crucial for cell proliferation and cell survival. We demonstrate that the inhibition of both Chk1 and Chk2 promote the reduction of the cell viability, the activation of apoptosis and the modification of the expression of different elements of the G2/M pathway (both proteins and mRNA level). The consequences of Chk1/Chk2 inhibition firstly evaluated on different cells lines and on primary leukemic cells where then confirmed *in vivo* using mouse models. Despite the great response, in both primary and leukemic cell lines using PF-0477736 in single agent, we could not find any predictive markers of response. The sensitivity to the compound was not related with the different sub-type of leukemia, with the basal expression of the target kinases, with the mutational status of p53 (marker of G1/S checkpoint functionality) and with the karyotypes. Similar results were obtained treating in single agent different cell lines and primary cells with the Chk1/Chk2 inhibitor, LY2606368, and with the Wee-1 inhibitor, MK-1775. The consequences of Chk1/Chk2

and Wee1 inhibition on the cell cycle progression were then analyzed treating different cell lines respectively with LY2606368 and MK-1775 in single agent. The treatment with LY2606368 progressively arrested the cells in S phase and, simultaneously, increased cell debris, supporting the hypothetical mechanism of death through “replication catastrophe”[76]. However in the inhibition of Wee1 increased the amount of cells in late S phase and in G2/M phase highlighting the potential mechanism of death through “mitotic catastrophe”. These data were supported by gene expression studies in which in both primary cells and leukemic cell lines the treatment with MK-1775 enhanced the expression of protein like, Cdc25C and the Cdc2-Cyclin B complex while repressed the expression of inhibitor the G2/M transition like Myt1. The consequence of the inhibition of the G2/M checkpoint after the treatment with MK-1775 was also analyzed in term of alterations of nuclear morphology. To this purpose primary cell were incubated with MK-1775 and the analyzed in light microscopy. The treated samples showed alterations in the morphology of the nucleus. Several DNA bridges, marker of mitotic alterations, were seen in all the treated samples but not in the controls. These alterations could not be detected on mononuclear cells isolated from healthy donors. To assess if the inhibition of the checkpoint kinase pathway could sensitize leukemic cells to the toxicity of different compound normally used in the clinic, different combination studies were performed. The Chk1/Chk2 inhibitor, LY2606368, and the Wee1 inhibitor, MK-1775, were combined with different Tyrosine Kinase inhibitors (imatinb, dasatinib and bosutinib), front line therapy for Philadelphia-positive B-ALL patients, and with the purine nucleoside analogue, clofarabine, used for the treatment of Philadelphia-negative B-ALL patients. The two checkpoint inhibitors deeply sensitized leukemic cell lines to the cytotoxicity of different compounds. The efficacy of the combinations was not only evaluated in term of reduction of the cell viability but also in term of induction of apoptosis (confirmed both by Western blots and Annexin V/Pi stainig) and induction of DNA damages (Western blots). The results found using different cell lines were then confirmed on primary cells isolated from the bone marrow and the peripheral blood of adult Philadelphia-positive and negative B-ALL patients. Interestingly the combination between MK-1775 and clofarabine seems to be more efficacies in the p53 mutated cell lines than in the p53 wild type but this hypothesis was not evaluated on primary cells. Finally different class of checkpoint

kinase inhibitors were combined together in order to evaluate their interaction. On this purpose the Chk1/Chk2 inhibitor, PF-0477736, was combined together with the Wee1 inhibitor, MK-1775. This combination should drastically mine the integration of the genetic stability. Interestingly using sub-toxic concentration of both compounds, the combination strongly reduced the cell viability and synergistically increased the DNA damages in all the cell lines treated. Moreover the co-treatment deeply modified the cell cycle profile, arresting the cell in S phase and inhibited the proliferation in all the treated cell lines.

In our opinion the preclinical data presented in this thesis are the basis for a future evaluation of this class of compound in clinical trials in the treatment of adult Acute Lymphoblastic Leukemia (ALL) patients. Although the mechanisms of action as well as the potential side effects of the checkpoint kinase inhibition must be better elucidate, we believe that the inhibition of Chk1/Chk2/Wee1 kinases could be a promising strategy to optimize the actual treatments and, consequently, to increase the cure rate of the patients. Finally future analyses must be performed to evaluate the potential synergistic effect of the combinations between checkpoint kinase inhibitors and other compound today used for the treatment of ALL patients.

Bibliography

1. Hunger SP, Mullighan CG. Rede fi ning ALL classi fi cation : toward detecting high-risk ALL and implementing precision medicine. Blood. 2015;125(26):3977–88.
2. Mrózek K, Harper DP, Aplan PD. Cytogenetics and Molecular Genetics of Acute Lymphoblastic Leukemia. Hematology/Oncology Clinics of North America. 2009. p. 991–1010.
3. Hunger SP, Mullighan CG. Redefining ALL classification: toward detecting high-risk ALL and implementing precision medicine. Blood [Internet]. 2015;125(26):3977–87. Available from: http://www.bloodjournal.org/content/125/26/3977.abstract
4. Kuiper RP, Schoenmakers EFPM, van Reijmersdal S V, Hehir-Kwa JY, van Kessel AG, van Leeuwen FN, et al. High-resolution genomic profiling of childhood ALL reveals novel recurrent genetic lesions affecting pathways involved in lymphocyte differentiation and cell cycle progression. Leuk Off J Leuk Soc Am Leuk Res Fund, UK. 2007;21(6):1258–66.
5. Mullighan CG, Goorha S, Radtke I, Miller CB, Coustan-Smith E, Dalton JD, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature. 2007;446(7137):758–64.
6. Mullighan CG, Su X, Zhang J, Radtke I, Phillips LAA, Miller CB, et al. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. N Engl J Med [Internet]. 2009;360(5):470–80. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19129520\nhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2674612
7. Mullighan CG. The genomic landscape of acute lymphoblastic leukemia in children and young adults. ASH Educ Progr B [Internet]. 2014;2014(1):174–80. Available from: http://asheducationbook.hematologylibrary.org/content/2014/1/174\nhttp://asheducationbook.hematologylibrary.org/content/2014/1/174.full.pdf\nhttp://www.ncbi.nlm.nih.gov/pubmed/25696852\nhttps://signin.hematology.org/Login.aspx?vi=9&vt=dca01674822b02dfe939
8. Hoelzer D. Personalized medicine in adult acute lymphoblastic leukemia. Haematologica [Internet]. 2015;100(7):855–8. Available from: http://www.haematologica.org/content/100/7/855.abstract
9. Larson S, Stock W. Progress in the treatment of adults with acute lymphoblastic leukemia. Curr Opin Hematol. 2008;15(4):400–7.
10. Fedorov VD, Upadhyay VA, Fathi AT. The Approach to Acute Lymphoblastic Leukemia in Older Patients: Conventional Treatments and Emerging Therapies. Curr Hematol Malig Rep [Internet]. 2016; Available from: http://link.springer.com/10.1007/s11899-016-0316-3

11. Hoelzer D, Ludwig WD, Thiel E, Gassmann W, Löffler H, Fonatsch C, et al. Improved outcome in adult B-cell acute lymphoblastic leukemia. Blood [Internet]. 1996;87(2):495–508. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8555471

12. Kantarjian HM, O'Brien S, Smith TL, Cortes J, Giles FJ, Beran M, et al. Results of treatment with hyper-CVAD, a dose-intensive regimen, in adult acute lymphocytic leukemia. J Clin Oncol. 2000;18(3):547–61.

13. Dombret H, Gabert J, Boiron J-M, Rigal-Huguet F, Blaise D, Thomas X, et al. Outcome of treatment in adults with Philadelphia chromosome–positive acute lymphoblastic leukemia—results of the prospective multicenter LALA-94 trial. Blood [Internet]. 2002;100(7):2357–66. Available from: http://www.bloodjournal.org/content/100/7/2357.abstract

14. Kantarjian H, Thomas D, Jorgensen J, Jabbour E, Kebriaei P, Rytting M, et al. Inotuzumab ozogamicin, an anti-CD22-calecheamicin conjugate, for refractory and relapsed acute lymphocytic leukaemia: A phase 2 study. Lancet Oncol. 2012;13(4):403–11.

15. Bargou R, Leo E, Zugmaier G, Klinger M, Goebeler M, Knop S, et al. Tumor regression in cancer patients by very low doses of a T cell-engaging antibody. Science. 2008;321(5891):974–7.

16. Portell CA, Wenzell CM, Advani AS. Clinical and pharmacologic aspects of blinatumomab in the treatment of B-cell acute lymphoblastic leukemia. Clinical Pharmacology: Advances and Applications. 2013. p. 5–11.

17. Wolach O, Stone RM. Blinatumomab for the treatment of Philadelphia chromosome-negative, precursor B-cell acute lymphoblastic leukemia. Clin Cancer Res. 2015;21(19):4262–9.

18. Goekbuget N, Dombret H, Bonifacio M, Reichle A, Graux C, Havelange V, et al. BLAST: A Confirmatory, Single-Arm, Phase 2 Study of Blinatumomab, a Bispecific T-Cell Engager (BiTE®) Antibody Construct, in Patients with Minimal Residual Disease B-Precursor Acute Lymphoblastic Leukemia (ALL). Blood [Internet]. 2014;124(21):Abstract 379. Available from: https://ash.confex.com/ash/2014/webprogram/Paper67851.html

19. Davila ML, Riviere I, Wang X, Bartido S, Park J, Curran K, et al. Efficacy and toxicity management of 19-28z {CAR} {T} cell therapy in {B} cell acute lymphoblastic leukemia. Sci Transl Med. 2014;6(224):224ra25.

20. Brentjens RJ, Davila ML, Riviere I, Park J, Wang X, Cowell LG, et al. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. Sci Transl Med [Internet]. 2013;5(177):177ra38. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3742551&tool=pmcentrez&rendertype=abstract

21. Sadelain M. CAR therapy: The CD19 paradigm. Journal of Clinical Investigation. 2015. p. 3392–400.

22. Liang Y, Lin S-Y, Brunicardi FC, Goss J, Li K. DNA damage response pathways in tumor suppression and cancer treatment. World J Surg [Internet]. 2009;33(4):661–6. Available

from: http://www.ncbi.nlm.nih.gov/pubmed/19034564

23. Jackson SP, Bartek J. The DNA-damage response in human biology and disease. Nature [Internet]. 2009;461(7267):1071–8. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2906700&tool=pmcentrez&rendertype=abstract

24. Velic D, Couturier A, Ferreira M, Rodrigue A, Poirier G, Fleury F, et al. DNA Damage Signalling and Repair Inhibitors: The Long-Sought-After Achilles' Heel of Cancer. Biomolecules [Internet]. 2015;5(4):3204–59. Available from: http://www.mdpi.com/2218-273X/5/4/3204

25. Shiloh Y, Ziv Y. The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. Nat Rev Mol Cell Biol [Internet]. 2013;14(4):197–210. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23486281

26. Falck J, Coates J, Jackson SP. Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. Nature [Internet]. 2005;434(7033):605–11. Available from: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=15758953&retmode=ref&cmd=prlinks\npapers3://publication/doi/10.1038/nature03442

27. Williams RS, Moncalian G, Williams JS, Yamada Y, Limbo O, Shin DS, et al. Mre11 Dimers Coordinate DNA End Bridging and Nuclease Processing in Double-Strand-Break Repair. Cell. 2008;135(1):97–109.

28. De Jager M, Van Noort J, Van Gent DC, Dekker C, Kanaar R, Wyman C. Human Rad50/Mre11 is a flexible complex that can tether DNA ends. Mol Cell. 2001;8(5):1129–35.

29. Lee J-H, Paull TT. ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. Sci (New York, NY) [Internet]. 2005;308(5721):551–4. Available from: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=15790808&retmode=ref&cmd=prlinks\npapers3://publication/doi/10.1126/science.1108297

30. Byun TS, Pacek M, Yee MC, Walter JC, Cimprich KA. Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. Genes Dev. 2005;19(9):1040–52.

31. Weber AM, Ryan AJ. ATM and ATR as therapeutic targets in cancer. Pharmacology and Therapeutics. 2015. p. 124–38.

32. Meek DW. Tumour suppression by p53: a role for the DNA damage response? [Internet]. Nature reviews. Cancer. 2009. p. 714–23. Available from: http://dx.doi.org/10.1038/nrc2716

33. Cuadrado M, Martinez-Pastor B, Murga M, Toledo LI, Gutierrez-Martinez P, Lopez E, et al. ATM regulates ATR chromatin loading in response to DNA double-strand breaks. J Exp Med. 2006;203(2):297–303.

34. Satyanarayana A, Kaldis P. Mammalian cell-cycle regulation: several Cdks, numerous cyclins and diverse compensatory mechanisms. Oncogene [Internet]. 2009;28(33):2925–

39. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19561645

35. Lim S, Kaldis P. Cdks, cyclins and CKIs: roles beyond cell cycle regulation. Development [Internet]. 2013;140(15):3079–93. Available from: http://dev.biologists.org/content/140/15/3079.abstract

36. Khosravi R, Maya R, Gottlieb T, Oren M, Shiloh Y, Shkedy D. Rapid ATM-dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA damage. Proc Natl Acad Sci. 1999;96(26):14973–7.

37. Origanti S, Cai S, Munir AZ, White LS, Piwnica-Worms H. Synthetic lethality of Chk1 inhibition combined with p53 and/or p21 loss during a DNA damage response in normal and tumor cells. Oncogene. 2012.

38. Bartek J, Lukas J. Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell. 2003. p. 421–9.

39. Chipuk JE, Green DR. Dissecting p53-dependent apoptosis. Cell Death Differ [Internet]. 2006;13(6):994–1002. Available from: http://www.nature.com/doifinder/10.1038/sj.cdd.4401908

40. Donzelli M, Draetta GF. Regulating mammalian checkpoints through Cdc25 inactivation. EMBO Rep [Internet]. 2003;4(7):671–7. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1326326&tool=pmcentrez&rendertype=abstract

41. Schmitt E, Boutros R, Froment C, Monsarrat B, Ducommun B, Dozier C. CHK1 phosphorylates CDC25B during the cell cycle in the absence of DNA damage. J Cell Sci [Internet]. 2006;119(Pt 20):4269–75. Available from: http://jcs.biologists.org/content/119/20/4269.long\npapers3://publication/doi/10.1242/jcs.03200

42. Forrest a, Gabrielli B. Cdc25B activity is regulated by 14-3-3. Oncogene. 2001;20(32):4393–401.

43. Do K, Doroshow JH, Kummar S. Wee1 kinase as a target for cancer therapy. Cell Cycle. 2013. p. 3159–64.

44. Watanabe N, Arai H, Iwasaki J-I, Shiina M, Ogata K, Hunter T, et al. Cyclin-dependent kinase (CDK) phosphorylation destabilizes somatic Wee1 via multiple pathways. Proc Natl Acad Sci U S A. 2005;102(33):11663–8.

45. Perry JA, Kornbluth S. Cdc25 and Wee1: analogous opposites? Cell Div [Internet]. 2007;2:12. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1868713&tool=pmcentrez&rendertype=abstract

46. Guardavaccaro D, Pagano M. Stabilizers and Destabilizers Controlling Cell Cycle Oscillators. Molecular Cell. 2006. p. 1–4.

47. Pawlowska E, Blasiak J. DNA repair—a double-edged sword in the genomic stability of cancer cells—the case of chronic myeloid leukemia. Int J Mol Sci. 2015;16(11):27535–49.

48. Sørensen CS, Hansen LT, Dziegielewski J, Syljuåsen RG, Lundin C, Bartek J, et al. The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair. Nat Cell Biol. 2005;7(2):195–201.

49. Zhang J, Willers H, Feng Z, Ghosh JC, Kim S, Weaver DT, et al. Chk2 phosphorylation of BRCA1 regulates DNA double-strand break repair. Mol Cell Biol [Internet]. 2004;24(2):708–18. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=343805&tool=pmcentrez&rendertype=abstract

50. Davis AJ, Chen DJ. DNA double strand break repair via non-homologous end-joining. Transl Cancer Res [Internet]. 2013;2(3):130–43. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3758668&tool=pmcentrez&rendertype=abstract

51. Lieber MR. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annu Rev Biochem [Internet]. 2010;79:181–211. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3079308&tool=pmcentrez&rendertype=abstract

52. Valerie K, Povirk LF. Regulation and mechanisms of mammalian double-strand break repair. Oncogene. 2003;22(37):5792–812.

53. Muraki K, Han L, Miller D, Murnane JP. The Role of ATM in the Deficiency in Nonhomologous End-Joining near Telomeres in a Human Cancer Cell Line. PLoS Genet. 2013;9(3).

54. Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer. 2009;9(3):153–66.

55. Bartkova J, Hamerlik P, Stockhausen M-T, Ehrmann J, Hlobilkova a, Laursen H, et al. Replication stress and oxidative damage contribute to aberrant constitutive activation of DNA damage signalling in human gliomas. Oncogene [Internet]. 2010;29(36):5095–102. Available from: http://dx.doi.org/10.1038/onc.2010.249

56. Halazonetis TD, Gorgoulis VG, Bartek J. An oncogene-induced DNA damage model for cancer development. Science. 2008;319(5868):1352–5.

57. Collins I, Garrett MD. Targeting the cell division cycle in cancer: CDK and cell cycle checkpoint kinase inhibitors. Curr Opin Pharmacol [Internet]. 2005;5(4):366–73. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15964238 \npapers://497fa013-e17c-4362-b4c6-8f807eac5791/Paper/p1010

58. Landau HJ, McNeely SC, Nair JS, Comenzo RL, Asai T, Friedman H, et al. The Checkpoint Kinase Inhibitor AZD7762 Potentiates Chemotherapy-Induced Apoptosis of p53-Mutated Multiple Myeloma Cells. Mol Cancer Ther. 2012;11:1781–8.

59. Ma CX, Cai S, Li S, Ryan CE, Guo Z, Schaiff WT, et al. Targeting Chk1 in p53-deficient

triple-negative breast cancer is therapeutically beneficial in human-in-mouse tumor models. J Clin Invest. 2012;122(4):1541–52.

60. Dent P, Tang Y, Yacoub A, Dai Y, Fisher PB, Grant S. CHK1 inhibitors in combination chemotherapy: thinking beyond the cell cycle. Mol Interv. 2011;11(2):133–40.

61. Curtin NJ. DNA repair dysregulation from cancer driver to therapeutic target. Nat Rev Cancer [Internet]. 2012;12(12):801–17. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23175119

62. a.I. D, M.T. A, J. S, J.W. G, D. M, G. S, et al. Phase I dose-escalation trial of checkpoint kinase 1 inhibitor MK-8776 as monotherapy and in combination with gemcitabine in patients with advanced solid tumors. J Clin Oncol [Internet]. 2015;33(9):1060–6. Available from: http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L603620168\nhttp://dx.doi.org/10.1200/JCO.2014.57.5027

63. Prudhomme M. Novel checkpoint 1 inhibitors. Recent Pat Anticancer Drug Discov. 2006;1(1):55–68.

64. Welch S, Hirte HW, Carey MS, Hotte SJ, Tsao MS, Brown S, et al. UCN-01 in combination with topotecan in patients with advanced recurrent ovarian cancer: A study of the Princess Margaret Hospital Phase II consortium. Gynecol Oncol. 2007;106(2):305–10.

65. Ma CX, Ellis MJC, Petroni GR, Guo Z, Cai SR, Ryan CE, et al. A phase II study of UCN-01 in combination with irinotecan in patients with metastatic triple negative breast cancer. Breast Cancer Res Treat. 2013;137(2):483–92.

66. Engelke CG, Parsels LA, Qian Y, Zhang Q, Karnak D, Robertson JR, et al. Sensitization of pancreatic cancer to chemoradiation by the Chk1 inhibitor MK8776. Clin Cancer Res. 2013;19(16):4412–21.

67. Grabauskiene S, Bergeron EJ, Chen G, Chang AC, Lin J, Thomas DG, et al. CHK1 levels correlate with sensitization to pemetrexed by CHK1 inhibitors in non-small cell lung cancer cells. Lung Cancer. 2013;82(3):477–84.

68. Dai Y, Chen S, Kmieciak M, Zhou L, Lin H, Pei X-Y, et al. The novel Chk1 inhibitor MK-8776 sensitizes human leukemia cells to HDAC inhibitors by targeting the intra-S checkpoint and DNA replication and repair. Mol Cancer Ther [Internet]. 2013;12(6):878–89. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3681875&tool=pmcentrez&rendertype=abstract

69. Morgan MA, Parsels LA, Zhao L, Parsels JD, Davis MA, Hassan MC, et al. Mechanism of radiosensitization by the Chk1/2 inhibitor AZD7762 involves abrogation of the G2 checkpoint and inhibition of homologous recombinational DNA repair. Cancer Res. 2010;70(12):4972–81.

70. Landau HJ, McNeely SC, Nair JS, Comenzo RL, Asai T, Friedman H, et al. The Checkpoint Kinase Inhibitor AZD7762 Potentiates Chemotherapy-Induced Apoptosis of p53-Mutated Multiple Myeloma Cells. Molecular Cancer Therapeutics. 2012. p. 1781–8.

71. Didier C, Demur C, Grimal F, Jullien D, Manenti S, Ducommun B. Evaluation of checkpoint kinase targeting therapy in acute myeloid leukemia with complex karyotype. Cancer Biol Ther. 2012;13(5):307–13.

72. Kim MK, James J, Annunziata CM. Topotecan synergizes with CHEK1 (CHK1) inhibitor to induce apoptosis in ovarian cancer cells. BMC Cancer [Internet]. 2015;15(1):1–10. Available from: http://www.biomedcentral.com/1471-2407/15/196

73. Sarmento LM, Póvoa V, Nascimento R, Real G, Antunes I, Martins LR, et al. CHK1 overexpression in T-cell acute lymphoblastic leukemia is essential for proliferation and survival by preventing excessive replication stress. Oncogene [Internet]. 2014;0. Available from: http://dx.doi.org/10.1038/onc.2014.248

74. Calvo E, Chen VJ, Marshall M, Ohnmacht U, Hynes SM, Kumm E, et al. Preclinical analyses and phase I evaluation of LY2603618 administered in combination with pemetrexed and cisplatin in patients with advanced cancer. Investigational New Drugs. 2014. p. 955–68.

75. King C, Diaz H, Barnard D, Barda D, Clawson D, Blosser W, et al. Characterization and preclinical development of LY2603618: A selective and potent Chk1 inhibitor. Invest New Drugs. 2014;32(2):213–26.

76. King C, Diaz HB, McNeely S, Barnard D, Dempsey J, Blosser W, et al. LY2606368 causes replication catastrophe and anti-tumor effects through CHK1-dependent mechanisms. Mol Cancer Ther [Internet]. 2015; Available from: http://www.ncbi.nlm.nih.gov/pubmed/26141948

77. Panek RL, Lu GH, Klutchko SR, Batley BL, Dahring TK, Hamby JM, et al. In vitro pharmacological characterization of PD 166285, a new nanomolar potent and broadly active protein tyrosine kinase inhibitor. J Pharmacol Exp Ther. 1997;283(3):1433–44.

78. Wang Y, Li J, Booher RN, Kraker A, Lawrence T, Leopold WR, et al. Radiosensitization of p53 mutant cells by PD0166285, a novel G2 checkpoint abrogator. Cancer Res. 2001;61(22):8211–7.

79. Hirai H, Iwasawa Y, Okada M, Arai T, Nishibata T, Kobayashi M, et al. Small-molecule inhibition of Wee1 kinase by MK-1775 selectively sensitizes p53-deficient tumor cells to DNA-damaging agents. Mol Cancer Ther [Internet]. 2009;8(11):2992–3000. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19887545

80. Hirai H, Arai T, Okada M, Nishibata T, Kobayashi M, Sakai N, et al. MK-1775, a small molecule Wee1 inhibitor, enhances antitumor efficacy of various DNA-damaging agents, including 5-fluorouracil. Cancer Biol Ther. 2010;9(7):514–22.

81. Bridges KA, Hirai H, Buser CA, Brooks C, Liu H, Buchholz TA, et al. MK-1775, a novel wee1 kinase inhibitor,radiosensitizes p53-defective human tumor cells. Clin Cancer Res. 2011;17(17):5638–48.

82. Kreahling JM, Gemmer JY, Reed D, Letson D, Bui M, Altiok S. MK1775, a selective Wee1 inhibitor, shows single-agent antitumor activity against sarcoma cells. Mol Cancer Ther [Internet]. 2012;11(1):174–82. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22084170

83. Van Linden A a, Baturin D, Ford JB, Fosmire SP, Gardner L, Korch C, et al. Inhibition of Wee1 sensitizes cancer cells to antimetabolite chemotherapeutics in vitro and in vivo, independent of p53 functionality. Mol Cancer Ther [Internet]. 2013;12(8):2675–84. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24121103

84. Qi W, Xie C, Li C, Caldwell J, Edwards H, Taub JW, et al. CHK1 plays a critical role in the anti-leukemic activity of the wee1 inhibitor MK-1775 in acute myeloid leukemia cells. J Hematol Oncol [Internet]. 2014;7(1):53. Available from: http://www.jhoonline.org/content/7/1/53

85. Zhou L, Zhang Y, Chen S, Kmieciak M, Leng Y, Lin H, et al. A regimen combining the Wee1 inhibitor AZD1775 with HDAC inhibitors targets human acute myeloid leukemia cells harboring various genetic mutations. Leukemia [Internet]. 2014;(August):1–12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25283841

86. Qi W, Zhang W, Edwards H, Chu R, Madlambayan GJ, Taub JW, et al. Synergistic anti-leukemic interactions between panobinostat and MK-1775 in acute myeloid leukemia ex vivo. Cancer Biol Ther [Internet]. 2015; Available from: http://www.ncbi.nlm.nih.gov/pubmed/26529495

87. Chaudhuri L, Vincelette ND, Koh BD, Naylor RM, Flatten KS, Peterson KL, et al. CHK1 and WEE1 inhibition combine synergistically to enhance therapeutic efficacy in acute myeloid leukemia ex vivo. Haematologica. 2014;99(4):688–96.

88. Guertin AD, Martin MM, Roberts B, Hurd M, Qu X, Miselis NR, et al. Unique functions of CHK1 and WEE1 underlie synergistic anti-tumor activity upon pharmacologic inhibition. Cancer Cell Int [Internet]. 2012;12(1):45. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3517755&tool=pmcentrez&rendertype=abstract

89. Russell MR, Levin K, Rader J, Belcastro L, Li Y, Martinez D, et al. Combination therapy targeting the Chk1 and Wee1 kinases shows therapeutic efficacy in neuroblastoma. Cancer Res. 2013;73(2):776–84.

90. Do K, Wilsker D, Ji J, Zlott J, Freshwater T, Kinders RJ, et al. Phase I Study of Single-Agent AZD1775 (MK-1775), a Wee1 Kinase Inhibitor, in Patients With Refractory Solid Tumors. J Clin Oncol [Internet]. 2015;33(30):3409–15. Available from: http://jco.ascopubs.org/content/early/2015/05/08/JCO.2014.60.4009.long

91. Conter V, Aricò M, Basso G, Biondi A, Barisone E, Messina C, et al. Long-term results of the Italian Association of Pediatric Hematology and Oncology (AIEOP) Studies 82, 87, 88, 91 and 95 for childhood acute lymphoblastic leukemia. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K. 2010.

92. Pui C-H, Robison LL, Look AT. Acute lymphoblastic leukaemia. Lancet. 2008;371(9617):1030–43.

93. Moorman A V. The clinical relevance of chromosomal and genomic abnormalities in B-cell precursor acute lymphoblastic leukaemia. Blood Rev. 2012;26(3):123–35.

94. Bailey LC, Lange BJ, Rheingold SR, Bunin NJ. Bone-marrow relapse in paediatric acute

lymphoblastic leukaemia. The Lancet Oncology. 2008. p. 873–83.

95. Fielding AK. Current Therapeutic Strategies in Adult Acute Lymphoblastic Leukemia. Hematology/Oncology Clinics of North America. 2011. p. 1255–79.

96. Blasina A, Hallin J, Chen E, Arango ME, Kraynov E, Register J, et al. Breaching the DNA damage checkpoint via PF-00477736, a novel small-molecule inhibitor of checkpoint kinase 1. Mol Cancer Ther. 2008;7(8):2394–404.

97. Kortmansky J, Shah MA, Kaubisch A, Weyerbacher A, Yi S, Tong W, et al. Phase I trial of the cyclin-dependent kinase inhibitor and protein kinase C inhibitor 7-hydroxystaurosporine in combination with fluorouracil in patients with advanced solid tumors. J Clin Oncol. 2005;23(9):1875–84.

98. Zabludoff SD, Deng C, Grondine MR, Sheehy AM, Ashwell S, Caleb BL, et al. AZD7762, a novel checkpoint kinase inhibitor, drives checkpoint abrogation and potentiates DNA-targeted therapies. Mol Cancer Ther. 2008;7(9):2955–66.

99. Sha S-K, Sato T, Kobayashi H, Ishigaki M, Yamamoto S, Sato H, et al. Cell cycle phenotype-based optimization of G2-abrogating peptides yields CBP501 with a unique mechanism of action at the G2 checkpoint. Mol Cancer Ther. 2007;6(1):147–53.

100. Zhang C, Yan Z, Painter CL, Zhang Q, Chen E, Arango ME, et al. PF-00477736 mediates checkpoint kinase 1 signaling pathway and potentiates docetaxel-induced efficacy in xenografts. Clin Cancer Res. 2009;15(14):4630–40.

101. Syljuåsen RG, Sørensen CS, Nylandsted J, Lukas C, Lukas J, Bartek J. Inhibition of Chk1 by CEP-3891 accelerates mitotic nuclear fragmentation in response to ionizing radiation. Cancer Res. 2004;64(24):9035–40.

102. Syljuåsen RG, Sørensen CS, Hansen LT, Fugger K, Lundin C, Johansson F, et al. Inhibition of human Chk1 causes increased initiation of DNA replication, phosphorylation of ATR targets, and DNA breakage. Mol Cell Biol. 2005;25(9):3553–62.

103. Parsels LA, Morgan MA, Tanska DM, Parsels JD, Palmer BD, Booth RJ, et al. Gemcitabine sensitization by checkpoint kinase 1 inhibition correlates with inhibition of a Rad51 DNA damage response in pancreatic cancer cells. Mol Cancer Ther. 2009;8(1):45–54.

104. Karp JE, Thomas BM, Greer JM, Sorge C, Gore SD, Pratz KW, et al. Phase I and pharmacologic trial of cytosine arabinoside with the selective checkpoint 1 inhibitor Sch 900776 in refractory acute leukemias. Clin Cancer Res. 2012;18(24):6723–31.

105. Maugeri-Saccà M, Bartucci M, De Maria R. Checkpoint kinase 1 inhibitors for potentiating systemic anticancer therapy. Cancer Treatment Reviews. 2013. p. 525–33.

106. Mailand N, Falck J, Lukas C, Syljuâsen RG, Welcker M, Bartek J, et al. Rapid destruction of human Cdc25A in response to DNA damage. Science. 2000;288(5470):1425–9.

107. Kuntz K, O'Connell MJ. The G2 DNA damage checkpoint: Could this ancient regulator be the achilles heel of cancer? Cancer Biology and Therapy. 2009.

108. Sørensen CS, Syljuåsen RG, Falck J, Schroeder T, Rönnstrand L, Khanna KK, et al. Chk1 regulates the S phase checkpoint by coupling the physiological turnover and ionizing

radiation-induced accelerated proteolysis of Cdc25A. Cancer Cell. 2003;3(3):247–58.

109. Chen M-S, Ryan CE, Piwnica-Worms H. Chk1 kinase negatively regulates mitotic function of Cdc25A phosphatase through 14-3-3 binding. Mol Cell Biol. 2003;23(21):7488–97.

110. Stolz A, Ertych N, Bastians H. Tumor suppressor CHK2: Regulator of DNA damage response and mediator of chromosomal stability. Clin Cancer Res. 2011;17(3):401–5.

111. Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, et al. ONCOMINE: A Cancer Microarray Database and Integrated Data-Mining Platform1. Neoplasia. 2004;6(1):1–6.

112. Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: An early marker of chemotherapy-induced apoptosis. Cancer Res. 1993;53(17):3976–85.

113. Peng CY, Graves PR, Thoma RS, Wu Z, Shaw AS, Piwnica-Worms H. Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science. 1997;277(5331):1501–5.

114. Xu Y, Price BD. Chromatin dynamics and the repair of DNA double strand breaks. Cell Cycle. 2011;10(2):261–7.

115. Matsuura K, Wakasugi M, Yamashita K, Matsunaga T. Cleavage-mediated activation of Chk1 during apoptosis. J Biol Chem. 2008;283(37):25485–91.

116. Wisdom R, Johnson RS, Moore C. c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms. EMBO J. 1999;18(1):188–97.

117. Went P, Agostinelli C, Gallamini A, Piccaluga PP, Ascani S, Sabattini E, et al. Marker expression in peripheral T-cell lymphoma: a proposed clinical-pathologic prognostic score. J Clin Oncol. 2006;24(16):2472–9.

118. Vignetti M, Fazi P, Cimino G, Martinelli G, Di Raimondo F, Ferrara F, et al. Imatinib plus steroids induces complete remissions and prolonged survival in elderly Philadelphia chromosome-positive patients with acute lymphoblastic leukemia without additional chemotherapy: Results of the Gruppo Italiano Malattie Ematologiche dell'Adu. Blood. 2007;109(9):3676–8.

119. Foa R, Vitale A, Vignetti M, Meloni G, Guarini A, Propris MS De, et al. Dasatinib as first-line treatment for adult patients with Philadelphia chromosome – positive acute lymphoblastic leukemia. Therapy. 2011;118(25):6521–8.

120. Clarke CAL, Clarke PR. DNA-dependent phosphorylation of Chk1 and Claspin in a human cell-free system. Biochem J. 2005;388(Pt 2):705–12.

121. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. Nature. 2004;432(7015):316–23.

122. Bartek J, Lukas J. Pathways governing G1/S transition and their response to DNA damage. FEBS Lett. 2001;490(3):117–22.

123. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. 1999;13(12):1501–12.

124. Finn K, Lowndes NF, Grenon M. Eukaryotic DNA damage checkpoint activation in response to double-strand breaks. Cell Mol Life Sci [Internet]. 2012;69(9):1447–73. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22083606

125. Zhao H, Piwnica-Worms H. ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. Mol Cell Biol. 2001;21(13):4129–39.

126. Zhang Y, Hunter T. Roles of Chk1 in cell biology and cancer therapy. Int J Cancer [Internet]. 2014;134(5):1013–23. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23613359

127. Smith J, Mun Tho L, Xu N, Gillespie DA. The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. Advances in Cancer Research. 2010. 73-112 p.

128. Patil M, Pabla N, Dong Z. Checkpoint kinase 1 in DNA damage response and cell cycle regulation. Cell Mol Life Sci [Internet]. 2013;70(21):4009–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23508805

129. Garrett MD, Collins I. Anticancer therapy with checkpoint inhibitors: What, where and when? Trends Pharmacol Sci. 2011;32(5):308–16.

130. McNeely S, Beckmann R, Bence Lin AK. CHEK again: Revisiting the development of CHK1 inhibitors for cancer therapy. Pharmacol Ther. 2014;142(1):1–10.

131. Dillon MT, Good JS, Harrington KJ. Selective Targeting of the G2/M Cell Cycle Checkpoint to Improve the Therapeutic Index of Radiotherapy. Clin Oncol. 2014;26(5):257–65.

132. Derenzini E, Agostinelli C, Imbrogno E, Iacobucci I, Brighenti E, Righi S, et al. Constitutive activation of the DNA damage response pathway as a novel therapeutic target in diffuse large B-cell lymphoma. 2015;6(9).

133. Iacobucci I, Di Rorà AGL, Falzacappa MVV, Agostinelli C, Derenzini E, Ferrari A, et al. In vitro and in vivo single-agent efficacy of checkpoint kinase inhibition in acute lymphoblastic leukemia. J Hematol Oncol [Internet]. Journal of Hematology & Oncology; 2015;8(1):125. Available from: http://www.jhoonline.org/content/8/1/125

134. Thompson R, Eastman A. The cancer therapeutic potential of Chk1 inhibitors: How mechanistic studies impact on clinical trial design. Br J Clin Pharmacol. 2013;76(3):358–69.

135. Reader JC, Matthews TP, Klair S, Cheung KMJ, Scanlon J, Proisy N, et al. Structure-guided evolution of potent and selective CHK1 inhibitors through scaffold morphing. J Med Chem. 2011;54(24):8328–42.

136. Narayanan S, Shami PJ. Treatment of acute lymphoblastic leukemia in adults. Critical Reviews in Oncology/Hematology. 2012. p. 94–102.

137. Information C, Information O. Tyrosine kinase inhibitors in Ph + acute lymphoblastic leukaemia : facts and perspectives. Ann Hematol. 2016;(800).

138. Majda K, Lubecka K, Kaufman-Szymczyk A, Fabianowska-Majewska K. Clofarabine (2-chloro-2???-fluoro-2???-deoxyarabinosyladenine) - Biochemical aspects of anticancer activity. Acta Pol Pharm - Drug Res. 2011;68(4):459–66.

139. Barba P, Sampol A, Calbacho M, Gonzalez J, Serrano J, Mart??nez-S??nchez P, et al. Clofarabine-based chemotherapy for relapsed/refractory adult acute lymphoblastic leukemia and lymphoblastic lymphoma. The Spanish experience. American Journal of Hematology. 2012. p. 631–4.

140. Lech-Maranda E, Korycka A, Robak T. Clofarabine as a novel nucleoside analogue approved to treat patients with haematological malignancies: mechanism of action and clinical activity. Mini Rev Med Chem. 2009;9(7):805–12.

141. Xiao Y, Ramiscal J, Kowanetz K, Del Nagro C, Malek S, Evangelista M, et al. Identification of Preferred Chemotherapeutics for Combining with a CHK1 Inhibitor. Mol Cancer Ther [Internet]. 2013;12(11):2285–95. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24038068

142. Nguyen T, Hawkins E, Kolluri A, Kmieciak M, Park H, Lin H, et al. Synergism between bosutinib (SKI-606) and the Chk1 inhibitor (PF-00477736) in highly imatinib-resistant BCR/ABL+ leukemia cells. Leuk Res [Internet]. Elsevier Ltd; 2015;39(1):65–71. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0145212614003312

143. Beeharry N, Banina E, Hittle J, Skobeleva N, Khazak V, Deacon S, et al. Re-purposing clinical kinase inhibitors to enhance chemosensitivity by overriding checkpoints. Cell Cycle. 2014;13(14):2172–91.

144. Wang FZ, Fei HR, Cui YJ, Sun YK, Li ZM, Wang XY, et al. The checkpoint 1 kinase inhibitor LY2603618 induces cell cycle arrest, DNA damage response and autophagy in cancer cells. Apoptosis. 2014;19(9):1389–98.

145. Bryant C, Scriven K, Massey AJ. Inhibition of the checkpoint kinase Chk1 induces DNA damage and cell death in human Leukemia and Lymphoma cells. Mol Cancer [Internet]. 2014;13(1):147. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4082411&tool=pmcentrez&rendertype=abstract

146. Bryant C, Stokes S, Massey a. J. Abstract 4458: Chk1 inhibition as a novel therapeutic strategy for treating triple negative breast and ovarian cancers. Cancer Res. 2011;71(8 Supplement):4458–4458.

147. Seedhouse C, Grundy M, Shang S, Ronan J, Pimblett H, Russell N, et al. Impaired S-phase arrest in acute myeloid leukemia cells with a FLT3 internal tandem duplication treated with clofarabine. Clin Cancer Res. 2009;15(23):7291–8.

148. Nieborowska-Skorska M, Stoklosa T, Datta M, Czechowska A, Rink L, Slupianek A, et al. ATR-Chk1 axis protects BCR/ABL leukemia cells from the lethal effect of DNA double-strand breaks. Cell Cycle. 2006;5(9):994–1000.

149. Onciu M. Acute Lymphoblastic Leukemia. Hematology/Oncology Clinics of North America. 2009. p. 655–74.

150. Sørensen CS, Syljuåsen RG. Safeguarding genome integrity: The checkpoint kinases ATR, CHK1 and WEE1 restrain CDK activity during normal DNA replication. Nucleic Acids Research. 2012. p. 477–86.

151. Hochegger H, Takeda S, Hunt T. Cyclin-dependent kinases and cell-cycle transitions: does one fit all? Nat Rev Mol Cell Biol. 2008;9(11):910–6.

152. Mir SE, De Witt Hamer PC, Krawczyk PM, Balaj L, Claes A, Niers JM, et al. In Silico analysis of kinase expression identifies WEE1 as a gatekeeper against mitotic catastrophe in glioblastoma. Cancer Cell. 2010;18(3):244–57.

153. Wang H, Huang M, Zhang DY, Zhang F. Global profiling of signaling networks: study of breast cancer stem cells and potential regulation. Oncologist. 2011;16(7):966–79.

154. PosthumaDeBoer J, Würdinger T, Graat HCA, van Beusechem VW, Helder MN, van Royen BJ, et al. WEE1 inhibition sensitizes osteosarcoma to radiotherapy. BMC Cancer. 2011;11:156.

155. Vriend LEM, De Witt Hamer PC, Van Noorden CJF, Würdinger T. WEE1 inhibition and genomic instability in cancer. Biochimica et Biophysica Acta - Reviews on Cancer. 2013. p. 227–35.

156. Andersson a, Ritz C, Lindgren D, Edén P, Lassen C, Heldrup J, et al. Microarray-based classification of a consecutive series of 121 childhood acute leukemias: prediction of leukemic and genetic subtype as well as of minimal residual disease status. Leukemia [Internet]. 2007;21(6):1198–203. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17410184

157. Chilà R, Basana A, Lupi M, Guffanti F, Gaudio E, Rinaldi A, et al. Combined inhibition of Chk1 and Wee1 as a new therapeutic strategy for mantle cell lymphoma. Oncotarget [Internet]. 2014;6(5). Available from: www.impactjournals.com/oncotarget\nwww.impactjournals.com/oncotarget/

158. Guertin AD, Martin MM, Roberts B, Hurd M, Qu X, Miselis NR, et al. Unique functions of CHK1 and WEE1 underlie synergistic anti-tumor activity upon pharmacologic inhibition. Cancer Cell Int [Internet]. 2012;12(1):45. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3517755&tool=pmcentrez&rendertype=abstract

159. Puttini M, Coluccia AML, Boschelli F, Cleris L, Marchesi E, Donella-Deana A, et al. In vitro and in vivo activity of SKI-606, a novel Src-Abl inhibitor, against imatinib-resistant Bcr-Abl+ neoplastic cells. Cancer Res. 2006;66(23):11314–22.

160. Kantarjian HM, Cortes JE, Kim DW, Khoury HJ, Brümmendorf TH, Porkka K, et al. Bosutinib safety and management of toxicity in leukemia patients with resistance or intolerance to imatinib and other tyrosine kinase inhibitors. Blood. 2014;123(9):1309–18.

161. Chiaretti S, Foà R. Management of adult Ph-positive acute lymphoblastic leukemia. Hematology Am Soc Hematol Educ Program [Internet]. 2015;2015(1):406–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26637750

162. Liu-Dumlao T, Kantarjian H, Thomas DA, O'Brien S, Ravandi F. Philadelphia-positive acute lymphoblastic leukemia: Current treatment options. Curr Oncol Rep. 2012;14(5):387–94.

163. Levinson NM, Boxer SG. Structural and spectroscopic analysis of the kinase inhibitor bosutinib and an isomer of bosutinib binding to the Abl tyrosine kinase domain. PLoS

One. 2012;7(4).