Mechanisms of Action of a Dual Cdc7/Cdk9 Kinase Inhibitor against Quiescent and Proliferating CLL Cells

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Abstract
In chronic lymphocytic leukemia (CLL) the proliferation rate and resistance to drug-induced apoptosis are recognized as important factors in the outcome of treatment. In this study, we assess the activity and the mechanism of action of the prototype cell division cycle kinase 7 (Cdc7) inhibitor, PHA-767491, which inhibits the initiation of DNA replication but also has cyclin-dependent kinase 9 (Cdk9) inhibitory activity. We have studied the effects of this dual Cdc7/Cdk9 inhibitor in both quiescent CLL cells and CLL cells that have been induced to proliferate using a cellular coculture system that mimics the lymph node microenvironment. We find that this compound, originally developed as a DNA replication inhibitor, is particularly active in promoting mitochondrial dependent apoptosis in quiescent CLL cells purified from peripheral blood of patients regardless of recognized risk factors. In this setting, apoptosis is preceded by a decrease in the levels of Mcl-1 protein and transcript possibly due to inhibition of Cdk9. Following stimulation by CD154 and interleukin-4, CLL cells become highly chemoresistant, reenter into the cell cycle, reexpress Cdc7 kinase, a key molecular switch for the initiation of DNA replication, replicate their DNA, and undergo cell division. In this context, treatment with PHA-767491 abolished DNA synthesis by inhibiting Cdc7 but is less effective in triggering cell death, although Mcl-1 protein is no longer detectable. Thus, dual Cdc7/Cdk9 inhibition has the potential to target both the quiescent and actively proliferating CLL populations through two distinct mechanisms and may be a new therapeutic strategy in CLL.

Introduction
Chronic lymphocytic leukemia (CLL) is the commonest leukemia in the Western world. Despite advances in treatment, CLL remains an incurable disease. Genetic abnormalities leading to loss of TP53 function and overexpression of antiapoptotic proteins are usually associated with short survival and represent barriers to conventional chemotherapy (1, 2). CLL cells are predominantly found in the peripheral blood, lymphoid organs, and bone marrow. CLL cells in the peripheral blood represent a population of nondividing tumor cells that display high chemosensitivity; in this setting the antiapoptotic protein myeloid cell leukemia sequence 1 (Mcl-1) seems to be a critical survival factor (3–7). Instead CLL cells that reside in secondary lymphoid organs and the bone marrow display high chemoresistance and proliferative capacity (8, 9). A subpopulation of CLL cells expressing the cell cycle markers Ki67 and cyclin D1 can be identified in pseudofollicles or proliferation centers (10). In this environment, the interactions between leukemic and accessory cells, such as monocyte-derived nurse-like cells, CD4+ CD154+ T cells and mesenchymal stromal cells, provide essential signals to maintain CLL survival and growth (11). Stimulated CLL cells express high levels of the antiapoptotic members of the Bcl-2 family (12), which are likely the main determinants of increased chemoresistance (13). Thus, proliferation centers may harbor dividing and resistant leukemic cells, which sustain clonal maintenance, growth, and genetic diversification and may represent a relevant chemotherapeutic target to limit tumor burden and clonal evolution.

A number of different models for pseudofollicles have been established in vitro including culturing CLL cells on CD154-expressing fibroblasts in the presence of interleukin-4 (IL-4; ref. 14). This model system partially recapitulates the phenotypic features seen in CLL cells within the proliferation centers including increased expression...
of the B cell lymphoma X Long (Bcl-X<sub>L</sub>), Bcl-2 homolog A1 (Bcl-A1), and Mcl-1, acquired resistance to spontaneous- and drug-induced apoptosis, and the ability to proliferate (13, 15). Therefore, this model system may be useful in analyzing the proliferative aspect of CLL and testing the efficacy of novel therapeutic agents targeting proliferation.

The cell cycle division 7 (Cdc7) is a protein kinase required for the initiation of DNA replication and cell cycle progression. Cdc7 phosphorylates the minichromosome maintenance 2-7 (MCM2-7) complex (16), activating its intrinsic DNA helicase activity, which is the first step required to establish a competent replication fork for semiconservative DNA synthesis. Phosphorylation of the Mcm2 subunit at Ser40 and Ser53 is observed only during S and G2–M phases of the cell cycle, mirroring Cdc7 activity and is completely dependent on Cdc7 activity (17). These phosphosites on Mcm2 have been shown to be sensitive biomarkers of Cdc7 activity in vivo.

Cdc7 kinase is considered a promising target for cancer therapy (18, 19). Downregulation of Cdc7 by short interfering RNA causes apoptosis in tumor cells independently of TP53, but only arrests cell cycle progression in normal cells (20, 21). This differential killing activity has led to the development of small molecules targeting Cdc7 kinase (18, 19, 22–26). PHA-767491, the prototype of this new class of agents, has cytotoxic activity in a broad range of cancers and displays antitumor activity in various preclinical models (27). An important feature of this compound is its cross reactivity with cyclin-dependent kinase 9 (Cdk9) and its ability to downregulate the expression of the Mcl-1 antiapoptotic protein (27).

In this study, we examined the effects of the dual Cdc7/Cdk9 inhibitor PHA-767491 in both quiescent and proliferating CLL cells.

Materials and Methods

Reagents

Media, serum, penicillin, and streptomycin were from Sigma-Aldrich. Ficoll was from GE Healthcare. IL-4 was from R&D system. PHA-767491 was provided by Neviano Medical Sciences S.r.l. SNS-032 (BMS-387032) and flavopiridol were from Selleck Chemicals LLC and Sigma-Aldrich, respectively. The Cdk9 inhibitor VCC096179 was from Vinchem Chemie Research Ltd. and described as compound 87 in reference (29). The pancaspase inhibitor N-(tert-butyloxycarbonyl)-Asp O-methylated-fluoromethyl ketone (Boc-D(OMe)flk) was from Biovision. 5-Ethynyl-2′-deoxyuridine (EdU) and 6-carboxy-fluorescein-TEG azide were from Budy & Associates. Carboxyfluorescein succinimidyl ester (CFSE) was from Molecular Probes, Invitrogen. Fluorescein isothio-cyanate (FITC, Molecular Probes)-conjugated Annexin V was prepared in house as previously described (29). All other chemicals were from Sigma-Aldrich unless otherwise stated.

CCL cell isolation

This study was approved by the Ethical Review Committee of University College Hospital Galway. Blood samples were obtained after informed consent from patients in accordance with the declaration of Helsinki. Samples were collected in Lithium–Heparin tubes and CLL cells were isolated by Ficoll density-gradient centrifugation. The percentage of double positive CDS<sup>+</sup>/CD19<sup>+</sup> leukemic cells assessed by flow cytometry using CDS-FITC/CD19-PE antibodies (BD Biosciences) was on average 89.9%. Isolated CLL cells were either used immediately for cell culture or frozen in FBS supplemented with 10% dimethyl sulphoxide and stored in liquid nitrogen for subsequent analysis.

Cell culture and drug treatment

Freshly isolated CLL cells were cultured at 2 × 10<sup>6</sup> cells/mL in RPMI medium supplemented with 10% FBS, penicillin (50 units/mL) and streptomycin (50 µg/mL) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. To calculate EC<sub>50</sub>, CLL cells were exposed to 9 different concentrations of PHA-767491 for 12 hours. NIH3T3 fibroblasts expressing human CD154 were kindly provided by Dr. E. Eldering (Department of Pathology, Academic Medical Center, Amsterdam, the Netherlands; ref. 30) and cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS, penicillin (50 units/mL) and streptomycin (50 µg/mL). For in vitro CD40 stimulation, freshly isolated or thawed CLL cells (2 × 10<sup>6</sup> cell/mL) were cultured in RPMI medium on irradiated (30 gray) NIH3T3 fibroblasts expressing human CD154 (ratio 10:1) in the presence of IL-4 (10 ng/mL). Medium and IL-4 were replaced every 3 days.

Immunoblotting

Cells were lysed in buffer containing 50 mmol/L Tris/HCl pH 7.5, 200 mmol/L NaCl, 1% (v/v) Tween 20, 0.2% (v/v) NP-40, 50 mmol/L β-glycerophosphate, 50 mmol/L NaF supplemented with complete protease and phosphatase inhibitors. Protein samples (15 µg) were analyzed by Western blotting with primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Anti-Bcl-A1 antibodies were kindly provided by Prof. J. Borst (Division of Immunology, The Netherlands Cancer Institute, Amsterdam, the Netherlands). Antibodies against Mcl-1, Bax, PARP, and caspase-3 (clone 8G10) were from Cell Signaling Technology. Antibodies anti-Bcl-2 (clone 100, Bcl-X<sub>L</sub> (clone H-5), and Bak (clone G-23) were from Santa Cruz Biotechnology. Anti-RNA Polymerase II (Pol II, clone 8WG16) and phospho Ser2 RNA Pol II (clone H5) were from Covance Research Products. Anti-Cdc7 (clone SPM171) was from Abcam. Anti-X-linked inhibitor of apoptosis (XIAP; clone 2F1) was from Assay Designs. Anti-β-actin (clone AC-15) was from Sigma-Aldrich. Rabbit polyclonal anti-pSer40/41 Mcm2 was previously described (17). Antibodies against Mcm2 were raised against the N terminus of human Mcm2 protein in collaboration with Pocono Rabbit Farm and Laboratory Inc.
DNA replication assay

An assay based on the incorporation of the EdU to detect DNA synthesis in CLL cells was adapted from (31). Briefly, cells (1 x 10⁶) were incubated with 10 µmol/L EdU for 1 hour, collected, washed with PBS, fixed in 2% paraformaldehyde for 5 minutes, washed with PBS and resuspended in 1 mL permeabilization buffer [0.05% w/v saponin in 1% (w/v) BSA/PBS]. For click reaction, 10 mmol/L Na-L-Ascorbate, 100 µmol/L f-carboxy-fluorescein-TEG azide, and 2 mmol/L CuSO₄ were added sequentially. Samples were incubated for 30 minutes at room temperature in the dark, followed by addition of 10 volumes of 1% (w/v) BSA in 0.5% (v/v) Tween 20/PBS and incubated for a further 10 minutes. After 3 washes, samples were resuspended in PBS and analyzed with BD FACSCanto I.

Analysis of CLL proliferation by CFSE staining

Cells (2 x 10⁶) were washed with PBS and resuspended in 1 mL of 0.1% (v/v) PBS/PBS containing 5 µmol/L CFSE. Samples were incubated for 10 minutes at 37°C in the dark, and then 5 volumes of cold media were added and incubated for further 5 minutes on ice. After 2 washes in pre-warmed media, CLL cells were plated onto NIH3T3 CD154 expressing fibroblasts in the presence of IL-4 (10 ng/mL).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism Software. For Fig. 1, unpaired t test or 1-way ANOVA were used to analyze the individual EC₅₀ versus clinical and prognostic markers. For Fig. 2B and C, data were analyzed using paired t test. P values less than 0.05 were considered statistically significant.

Supplementary information describing cells characterization, apoptosis assays, RT-qPCR and immunohistochemistry and immunofluorescences methods are available online.

Results

Peripheral blood CLL cells are sensitive to the Cdc7/Cdk9 inhibitor PHA-767491

Peripheral blood CLL cells do not proliferate (14) but surprisingly, when challenged with PHA-767491 (Fig. 1A and Supplementary Fig. S1), originally developed as a DNA replication inhibitor (27), we observed a concentration and time-dependent induction of apoptosis measured by phosphatidylserine exposure (Supplementary Fig. S2). Apoptosis was induced at 6 hours and increased further at 12 hours with only a marginal increase at 24 hours. On the basis of these initial observations, we extended our studies to a cohort of 27 CLL patients with both favorable and unfavorable prognostic markers, including 2 patients with biallelic inactivation of TP53 (patient #3, #11, Supplementary Table S1) and 1 additional patient with known 17p deletion (patient #23, Supplementary Table S1). PHA-767491 induced apoptosis in all CLL samples tested with an average EC₅₀ of 0.6 µmol/L at 12-hour posttreatment (Fig. 1B, Supplementary Table S1). PHA-767491 was equally effective at inducing apoptosis in CLL cells with either favorable or adverse prognostic factors including IGHV mutational status, CD38 positivity, clinical stage, chromosome abnormalities, and previous treatments (Fig. 1C–G).

PHA-767491 activates the intrinsic apoptotic pathway in resting CLL cells

To investigate the mechanism of PHA-767491-induced apoptosis, we examined the events associated with the intrinsic apoptotic pathway. Exposure of CLL cells to 1 µmol/L PHA-767491 induced Bax activation as early as 4-hour posttreatment with substantial activation detected from 6-hour posttreatment (37% vs. 9% in control cells; Fig. 2A, left panel). Concomitantly, PHA-767491 caused a time-dependent increase in cells with low mitochondrial membrane potential (∆ψm) and in Annexin V-positive cells (Fig. 2A, middle and right panels). By 12-hour posttreatment, nearly 90% of treated cells were Annexin positive and showed depolarized mitochondria.

We next examined the role of caspases during PHA-767491–induced apoptosis using the pan-caspase inhibitor Boc-D-(OMe).fmk. Incubation of CLL cells for 1 hour with 50 µmol/L Boc-D-(OMe).fmk before exposure to PHA-767491 almost completely prevented phosphatidylserine externalization assessed at 6- and 12-hour posttreatment (Fig. 2B). Boc-D-(OMe).fmk also greatly reduced PHA-767491–induced loss of ∆ψm at 6 hours and to a lesser extent at 12-hour posttreatment (Fig. 2C), at which time 60% of CLL cells displayed low ∆ψm but no phosphatidylserine exposure, suggesting that PHA-767491–induced Bax activation occurs upstream of caspase activation. To test this hypothesis, we examined Bax activation 6 hours after PHA-767491 treatment in a CLL sample pretreated for 1 hour with 50 µmol/L Boc-D-(OMe).fmk. Indeed, Boc-D-(OMe).fmk pretreatment did not prevent PHA-767491–induced Bax activation despite complete inhibition of phosphatidylserine externalization (Supplementary Fig. S3A and B), consistent with PHA-767491 directly activating the intrinsic pathway. However, activation of caspases is required to ensure rapid and efficient execution of apoptosis.

PHA-767491 induces downregulation of Mcl-1 protein in a caspase-independent manner

Because PHA-767491–induced apoptosis proceeds via Bax activation, we examined the levels of antiapoptotic Bcl-2 family members, which modulate Bax function (32) and the levels of the antiapoptotic protein XIAP, previously shown to be downregulated by PHA-767491 (27). CLL cells were exposed to 1 µmol/L PHA-767491 and samples were collected at different times posttreatment. Following exposure to PHA-767491, Bax and Bcl-2 protein levels remained constant in all 4 samples tested whereas Bak showed a patient-specific modulation, being upregulated to different degrees in 3 of 4 samples (Fig. 2D and Supplementary Fig. S4). As Bcl-X₇ was poorly expressed (13) and often

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below the detection limit, it was not analyzed. However, we observed that PHA-767491 induced a rapid decrease in Mcl-1 protein levels even at 2 hours in all patients tested, which decreased further over time. The protein levels of XIAP also decreased, albeit to a lesser extent and with slower kinetics. Downregulation of Mcl-1 was followed by processing of procaspase-3, cleavage of PARP, and phosphatidylserine externalization detectable from 4-hour post-treatment. Noxa, the Mcl-1-binding partner, remained constant in 2 of the 3 samples tested while a marked increase was observed in CLL #2 (Fig. 2D and Supplementary Fig. S4).

As Mcl-1 downregulation is the earliest event and seemed to be the only common feature among different patients that are equally sensitive to PHA-767491, we further explored the mechanism of its modulation. In CLL cells
Figure 2. The intrinsic apoptotic pathway is responsible for CLL cell death through caspase-independent Mcl-1 downregulation. A, CLL cells were exposed to 1 μmol/L PHA-767491 for 2, 4, 6, 8, and 12 hours. Samples were collected at each time point and analyzed for Bax activation (left column), ΔΨm depolarization (middle column), and phosphatidylserine exposure (right column) by flow cytometry. Numbers indicate percentage of CLL cells with either active Bax, low ΔΨm, or positive for Annexin V in the treated samples. Data presented are representative of 4 independent experiments. B and C, CLL cells were either untreated, treated with 50 μmol/L Boc-D–(OMe).fmk, treated with 1 μmol/L PHA-767491, or pretreated with 50 μmol/L Boc-D–(OMe).fmk for 1 hour and then treated with 1 μmol/L PHA-767491 for 6 (white bars) or 12 hours (gray bars). Samples were collected at each time point and analyzed for phosphatidylserine exposure (B) and ΔΨm depolarization (C) by flow cytometry. Bars represent the mean ± SEM (n = 8). * and *** denote P < 0.05 and P < 0.001, respectively, when compared with untreated controls. D, CLL cells were exposed to 1 μmol/L PHA-767491 for 2, 4, 6, and 8 hours. Samples were collected at each time point and analyzed for phosphatidylserine exposure by flow cytometry and Western blot using antibodies against the indicated proteins. E, CLL cells from 2 patients were pretreated with either 50 μmol/L Boc-D–(OMe).fmk or 1 μmol/L MG132, followed by treatment with 1 μmol/L PHA-767491 for 6 hours. Samples were collected and analyzed for phosphatidylserine exposure by flow cytometry and Western blot using antibodies against the indicated proteins. Numbers at the bottom of the panels indicate the percentage of Annexin V-positive cells. AV, Annexin V.
that were preincubated with 50 μmol/L Boc-D-(OMe).fmk 1 hour before PHA-767491 treatment, PARP cleavage and phosphatidylserine externalization were prevented, but Mcl-1 levels were not restored (Fig. 2E); preincubation for 1 hour with 1 μmol/L of the proteasome inhibitor MG-132 partially inhibited Mcl-1 downregulation, suggesting that the decrease in Mcl-1 levels is partly due to proteasomal degradation but is caspase independent.

Figure 3. PHA-767491 affects Ser2 CTD RNA polymerase II phosphorylation and Mcl-1 transcription. A, CLL cells from 2 patients were either mock treated or treated with 1 μmol/L PHA-767491 for 2, 4, and 6 hours. Samples were collected and analyzed for phosphatidylserine exposure by flow cytometry and Western blot using antibodies against the indicated proteins. B, CLL cells from 3 patients were exposed to 1 μmol/L PHA-767491 for 2 hours. Samples were collected and Mcl-1 mRNA levels were quantified by RT-qPCR. Bars represent the mean ± 95% CI. C, CLL cells from patient # 2 were treated with the indicated concentrations of PHA-767491 for 12 hours. Samples were collected and analyzed for phosphatidylserine exposure by flow cytometry and Western blot using antibodies against the indicated proteins. Numbers at the bottom of the panels indicate the percentage of Annexin V-positive cells. AV, Annexin V.
Drug treatment causes dephosphorylation of RNA pol II CTD and a rapid decrease in Mcl-1 mRNA levels

Mcl-1 is a short half-life protein that is rapidly down-regulated when its transcription and/or translation is impaired (33). Cdk9 is involved in the regulation of the rate of RNA Pol II-dependent transcription by phosphorylating the carboxy-terminal domain repeat (CTD) at Ser2 (34). The phosphorylation status of Ser2 CTD RNA pol II can be used as a read out of cellular Cdk9 activity (35). Because PHA-767491 inhibits Cdk9 kinase in biochemical assays, we examined whether Cdk9 activity was affected in PHA-767491-treated CLL cells. Indeed, 1 μmol/L PHA-767491 caused a rapid loss of pSer2 CTD RNA Pol II from 2-hour posttreatment that correlates with Mcl-1 downregulation (Fig. 3A). Analysis of Mcl-1 mRNA levels by RT-qPCR at 2-hour posttreatment revealed that drug treatment caused a considerable decrease in Mcl-1 mRNA (Fig. 3B and Supplementary Table S2). Furthermore, in a dose–response experiment, we observed a very tight correlation between loss of pSer2 CTD RNA Pol II phosphorylation, Mcl-1 downregulation, and induction of apoptosis (Fig. 3C). Together these data further support the hypothesis that PHA-767491-induced Mcl-1 downregulation in CLL cells is primarily transcriptional through Cdk9 inhibition.

CLL cells express active Cdc7 kinase in vitro upon stimulation with IL-4 and CD154 and in vivo in the lymph nodes

CLL proliferation has been reported in vivo in discrete compartments such as lymph nodes and to a lesser extent in bone marrow (8, 11). To partially recapitulate the lymph node microenvironment in vitro, we stimulated CLL cells purified from peripheral blood samples with soluble IL-4 in coculture with CD154 expressing NIH3T3 cells (14, 15). In this setting, the kinetics of reentry into the mitotic cell cycle, assessed both as DNA synthesis and loss of CFSE staining, varied across patients starting as early as 2 days after stimulation (Fig. 4A and Supplementary Fig. S5).

Examining protein samples, and as previously reported (13, 15, 30), we observed a rapid induction of both anti-apoptotic proteins Mcl-1 and Bcl-XL (Fig. 4B and Supplementary Fig. S5). In contrast, the levels of the S-phase kinase Cdc7 and the levels of phosphorylation of its substrate Mcm2 at Ser40, which is a considered a specific biomarker for assessing cellular Cdc7 activity (17, 27), increased with a slower kinetic that mirrored the amount of DNA synthesis ongoing in the cell population. The overall levels of Mcm2 before induction and its responsiveness to IL-4 and CD154 stimulation varied across different samples (Fig. 4B and Supplementary Fig. S5).

To assess the relevance of this in vitro model for testing Cdc7 kinase inhibitors, we analyzed the expression of Cdc7, of its substrate Mcm2, and the levels of phosphorylated Ser40 Mcm2 by immunohistochemistry, and we compared their pattern to the one of the well-recognized proliferation marker Ki67 in serial slices of lymph node biopsies from CLL patients. This preliminary analysis revealed that a proportion of cells within the lymph node tissue indeed express Cdc7 and Mcm2, and more importantly, phosphorylated Ser40 Mcm2 (Fig. 5A). The pSer40 Mcm2 expression pattern strongly overlaps with Ki67 staining, indicating that pSer40 Mcm2 staining may also identify the CLL replication centers within the lymph
nodes. To further support this idea, malignant CLL cells were identified with the B-cell CD19 surface marker and assessed for positive Ki67 or pSer40 Mcm2 staining (Fig. 5B, panels I–II). Ki67 and pSer40 Mcm2 staining largely identified the same cells (Fig. 5B, panels III–VI). These results indicate that Cdc7 is functional in a sub-population of lymph node resident CLL cells that are possibly engaged in DNA synthesis.

**DNA synthesis and Cdc7 activity of IL-4 and CD154-stimulated CLL cells can be inhibited by PHA-767491**

We next examined the effects of PHA-767491 on proliferating CLL cells. To this end, 5 days after CD154 and IL-4 stimulation, CLL cells were challenged with increasing doses of PHA-767491. Samples for protein analysis were collected after 12 hours while DNA synthesis, CFSE, and Annexin V positivity were assessed after 24 hours of treatment. We determined that 1 μmol/L PHA-767491 had no effect whereas exposure to 5 μmol/L and 10 μmol/L PHA-767491 caused full inhibition of Cdc7 kinase activity as assessed by loss of Mcm2 phosphorylation (Fig. 6A) and DNA synthesis (Fig. 6B). Interestingly in this setting, PHA-767491 again caused Mcl-1 downregulation that was accompanied also by Noxa and Bel-A1 downregulation, whereas Bak was not affected and only very low levels of cleaved caspase-3 and Annexin V-positive cells were detected (Fig. 6A and C). As expected blockade of DNA synthesis resulted in decreased cell division assessed by CFSE staining (Fig. 6D). Identical results were observed also in CD154 and IL-4-stimulated CLL samples from 2 other patients (Supplementary Fig. S6).

**Discussion**

In this study, we describe the *in vitro* single agent activity of the dual Cdc7/Cdk9 inhibitor PHA-767491 in both quiescent and proliferating CLL cells. The main finding of this work is that PHA-767491, because of its dual activity, has the ability to kill quiescent CLL cells by inhibiting Cdk9 and to restrain CLL proliferation by inhibiting Cdc7 kinase.
Quiescent peripheral blood CLL cells were invariably sensitive to the compound, overcoming poor prognostic parameters, including TP53 dysfunction. Treatment with PHA-767491 induced activation of the intrinsic apoptotic pathway, which correlated with phosphorylation of the CTD tail of RNA polymerase II, and associated with a rapid decrease in Mcl-1 protein. As Mcl-1 mRNA levels drop sharply after a short exposure to the compound, it is very likely that the principal mechanism driving apoptosis in resting CLL cells is indeed the transcriptional inhibition of Mcl-1. This is also in keeping with the mechanisms of action of other kinase inhibitors such as flavopiridol and SNS-032 (Refs. 36, 37; Supplementary Fig. S7).

Proliferating CLL cells normally accumulate in the lymph nodes and to lesser extent in the bone marrow (8, 11). Because of the relevance of the lymph node microenvironment in CLL pathobiology, we analyzed the expression and the activity of Cdc7 kinase, the main molecular target of PHA-767491 in patient-derived material and in an in vitro model that partially recapitulates the lymph node proliferation centers (12–14, 30). In a preliminary analysis, we report that Cdc7 is expressed and active in lymph node biopsies from CLL patients.

In vitro, following CD154 and IL-4 stimulation, we observed that CLL cells purified from peripheral blood reexpress Cdc7, the key regulator of DNA synthesis. Not only is Cdc7 expressed, but it is also fully functional as its key substrate, Mcm2, becomes phosphorylated, thus promoting DNA replication. In this setting, treatment with PHA-767491 was indeed proficient at blocking DNA synthesis, very likely through direct inhibition of Cdc7. In our coculture experiments, however, we noticed that exposure to 5 or 10 μmol/L PHA-767491 was less effective in triggering cell death than 1 μmol/L in resting unstimulated CLL samples, although Mcl-1 was

Figure 6. PHA-767491 inhibits Cdc7 and DNA replication in stimulated CLL cells. CLL cells cultured onto NIH3T3 fibroblasts expressing CD154 in presence of IL-4 for 5 days were exposed to PHA-767491 at the indicated concentrations. Samples were analyzed by Western blot at 12-hour posttreatment using antibodies against the indicated proteins (A) and by flow cytometry at 24-hour posttreatment for DNA synthesis (B) and phosphatidylserine exposure (C). D, proliferation in presence of the indicated concentrations of PHA-767491 was assessed in parallel samples by CFSE staining at 24-hour posttreatment. Bars indicate the peaks corresponding to cells that have undergone cell division in the last 24 hours.
completely depleted in both cases. This possibly reflects a changed balance in the pro- and antiapoptotic factors and the lower dependence of stimulated cells on Mcl-1. Indeed, consistent with previous reports (13, 15), we find that CLL cells stimulated with CD154 and IL-4 express the antiapoptotic proteins Bcl-A1, which is not detectable in circulating, quiescent CLL cells and dramatically increase the expression of Bcl-XI and Mcl-1. Although PHA-767491 was not able to reduce the level of Bcl-A1 in a diffuse B-cell lymphoma cellular system since decreasing Mcl-1 protein levels (38), we find that Bcl-A1 is decreased similarly to Mcl-1 and is partner Noxa whereas Bcl-XI is unaffected. Thus, the expression of Bcl-XI due to CD154 and IL-4 stimulation may promote the survival of CLL cells in response to inhibition of the initiation of DNA replication and Cdk9 activity by PHA-767491. Alternatively a checkpoint mechanism, that has been reported recently to protect normal human primary cells from death caused by Cdc7 inhibition (20, 21), could be proficient in CLL cells used in this study. This hypothesis will require further investigation.

Finally, the ability of PHA-767491 to potently down-regulate Mcl-1 suggests potential synergies with other agents in CLL whose activity is negatively influenced by Mcl-1, including purine nucleoside analogues, alkylating agents, rituximab, (4), and the BH3 mimetic ABT-263 (38, 39).

Disclosure of Potential Conflicts of Interest

A. Montagnoli is an employee of Nerviano Medical Sciences S.r.l.

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