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Abbreviations: ΔΨm, mitochondrial membrane potential; AV, Annexin V; Bak, Bcl-2 homologous antagonist killer; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; Bcl-X_L, B cell lymphoma X Long; Bcl-A1, Bcl-2 homolog A1; Boc-D[OMe].fmk, *N-tert*-butoxycarbonyl-Asp O-methylated -fluoromethyl ketone; CFSE, Carboxyfluorescein succinimidyl ester; CTD , carboxy-terminal domain repeat; CLL, chronic lymphocytic leukemia; Cdc7, Cell division cycle kinase 7; Cdk9, Cyclin dependent kinase 9; EdU, 5-Ethylnyl-2'-deoxyuridine; FISH, interphase fluorescent *in situ* hybridization; IGHV, immunoglobulin heavy-chain variable region; IL-4, interleukin-4; Mcl-1, Myeloid cell leukaemia sequence 1; MCM2-7, minichromosome maintenance 2-7; NLC, nurselike cells; PARP, Poly (ADP-ribose) polymerase; PI, Propidium Iodide; PS, phosphatidylserine; RNA Pol II, RNA polymerase II; TMRE, Tetramethylrhodamine ethyl ester; XIAP, X-linked inhibitor of apoptosis.

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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 co-culture 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 (IL-4), CLL cells become highly chemo-resistant, re-enter into the cell cycle, re-express 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 over-expression of anti-apoptotic 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 non-dividing tumor cells which display high chemo-sensitivity; in this setting the anti-apoptotic protein Myeloid cell leukaemia sequence 1 (Mcl-1) appears to be a critical survival factor (3-7). Instead CLL cells that reside in secondary lymphoid organs and the bone marrow display high chemo-resistance and proliferative capacity (8-9). A sub-population of CLL cells expressing the cell cycle markers Ki67 and cyclin D1 can be identified in pseudo-follicles or proliferation centres (10). In this environment the interactions between leukemic and accessory cells, such as monocyte-derived nurselike cells (NLC), $CD4^+$ $CD154^+$ T cells and mesenchymal stromal cells, provide essential signals to maintain CLL survival and growth (11). Stimulated CLL cells express high levels of the anti-apoptotic members of the Bcl-2 family (12) which are likely the main determinants of increased chemo-resistance (13). Thus, proliferation centres may harbour dividing and resistant leukemic cells, which sustain clonal maintenance, growth, and genetic diversification and may represent a relevant chemo-therapeutic target to limit tumor burden and clonal evolution.

A number of different models for pseudo-follicles have been established *in vitro* including culturing CLL cells on CD154 expressing fibroblasts in the presence of Interleukin-4 (IL-4) (14). This model system partially recapitulates the phenotypic features seen in CLL cells within the proliferation centres including increased expression of the B cell lymphoma X Long (Bcl-X_L), 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 analysing 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 semi-conservative 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 kinase 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 siRNA 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 anti-apoptotic protein (27).

In the present 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 (St Louis, MO). Ficoll was from GE Healthcare (Buckinghamshire, UK). IL-4 was from R & D system (Minneapolis, MM). PHA-767491 was provided by Nerviano Medical Sciences S.r.l. SNS-032 (BMS-387032) and Flavopiridol were from Selleck Chemicals LLC (Houston, TX) and Sigma-Aldrich respectively. The Cdk9 inhibitor VCC096179 was from Vinchem Chemie Research Ltd and described as compound 87 in (28). The pan-caspase inhibitor *N-tert*-butoxycarbonyl-Asp O-methylated -fluoromethyl ketone (Boc-D[OMe].fmk) was from Biovision (Mountain View, CA). 5-Ethylnyl-2'-deoxyuridine (EdU) and 6-Carboxy-fluorescein-TEG azide were from Berry & Associates (Dexter, MI). Carboxyfluorescein succinimidyl ester (CFSE) was from Molecular Probes, Invitrogen (Carlsbad, CA). Fluorescein isothiocyanate (FITC, Molecular Probes)-conjugated Annexin V was prepared in house as previously described (29). All other chemicals were from Sigma-Aldrich unless otherwise stated.

CLL cells 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 $CD5^+/CD19^+$ leukemic cells assessed by flow cytometry using CD5-FITC/CD19-PE antibodies (BD Biosciences, San Jose, CA) was on average 89.9%. Isolated CLL cells were either used immediately for cell culture or frozen in Foetal Bovine Serum (FBS) supplemented with 10 % dimethyl sulphoxide (DMSO) and stored in liquid nitrogen for subsequent analysis.

Cell Culture and Drug Treatment. Freshly isolated CLL cells were cultured at 2 x 10^6 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_2 at 37°C. To calculate EC 50, CLL cells were exposed to nine different concentrations of PHA-767491 for 12 hours. NIH3T3 fibroblasts expressing human CD154 were kindly provided by Dr E. Eldering (30) and cultured in DMEM 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 x 10^6 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 mM Tris/HCl pH 7.5, 200 mM NaCl, 1% (v/v) Tween 20, 0.2% (v/v) NP-40, 50 mM β-Glycerophosphate, 50 mM NaF supplemented with complete protease and phosphatase inhibitors. Protein samples (15 µg) were analysed by western blotting with primary antibodies and horseradish peroxidase (HRP) conjugated secondary antibodies. Anti-Bcl-A1 antibodies were kindly provided by Prof. J. Borst. Antibodies against Mcl-1, Bax, poly (ADP-ribose) polymerase (PARP), and Caspase-3 (clone 8G10) were from Cell Signaling Technology (Danvers, MA). Antibodies anti-Bcl-2 (clone 100), Bcl-X_L (clone H-5) and Bak (clone G-23) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-RNA Polymerase II (Pol II, clone 8WG16) and phospho Ser2 RNA Pol II (clone H5) were from Covance Research Products (Emeryville, CA). Anti-Cdc7 (clone SPM171) was from Abcam (Cambridge, UK). Anti-XIAP (clone 2F1) was from Assay Designs (Ann Arbor, MI). 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. (Canadensis, PA, US).

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 10^6) were incubated with 10 µM EdU for 1 h, collected, washed with PBS, fixed in 2% paraformaldehyde for 5 min, 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 mM Na-L-Ascorbate, 100 µM 6-Carboxy-fluorescein-TEG azide and 2 mM $CuSO_4$ were added sequentially. Samples were incubated for 30 min at RT 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 min. After 3 washes, samples were resuspended in PBS and analysed with BD FACSCanto I.

Analysis of CLL proliferation by CFSE staining. Cells (20 x 10^6/ml) were washed with PBS and resuspended in 1 ml of 0.1% (v/v) FBS/PBS containing 5 µM CFSE. Samples were incubated for 10 min at 37°C in the dark, then 5 volumes of cold media were added and incubated for further 5 min 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 (La Jolla, CA). For Figure 1, unpaired t-test or one-way analysis of variance (ANOVA) were used to analyse the individual EC 50 versus clinical and prognostic markers. For Figure 2B, C, data were analysed using paired t-test. P values less than 0.05 were considered statistically significant.

Supplementary information describing cells characterization, apopotis assays, RT-qPCR and Immunohystochemistry and immunoflourescences 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 (Figure 1A and S1), originally developed as a DNA replication inhibitor (27), we observed a concentration and time dependent induction of apoptosis measured by phosphatidylserine exposure (PS) (Figure S2). Apoptosis was induced at 6 h and increased further at 12 h with only a marginal increase at 24 h. Based on these initial observations, we extended our studies to a cohort of 27 CLL patients with both favourable and unfavourable prognostic markers, including two patients with biallelic inactivation of *TP53* (Patient #3, #11, Table S1) and one additional patient with known 17p deletion (Patient #23, Table S1). PHA-767491 induced apoptosis in all CLL samples tested with an average EC 50 of 0.6 μM at 12 h post-treatment (Figure 1B, Table S1). PHA-767491 was equally effective at inducing apoptosis in CLL cells with either favourable or adverse prognostic factors including *IGHV* mutational status, CD38 positivity, clinical stage, chromosome abnormalities and previous treatments (Figure 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 μM PHA-767491 induced Bax activation as early as 4 h post-treatment with substantial activation detected from 6 h post-treatment (37% versus 9% in control cells; Figure 2A left panel). Concomitantly, PHA-767491 caused a time-dependent increase in cells with low mitochondrial membrane potential (ΔΨm) and in Annexin V (AV) positive cells (Figure 2A middle and right panels). By 12 h post-treatment, nearly 90% of treated cells were AV 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 h with 50 μM Boc-D-(OMe).fmk before exposure to PHA-767491 almost completely prevented PS externalization assessed at 6 and 12 h post-

treatment (Figure 2B). Boc-D-(OMe).fmk also greatly reduced PHA-767491-induced loss of ΔΨm at 6 h and to a lesser extent at 12 h post-treatment (Figure 2C), at which time 60% of CLL cells displayed low ΔΨm but no PS exposure suggesting that PHA-767491-induced Bax activation occurs upstream of caspase activation. To test this hypothesis we examined Bax activation 6 h after PHA-767491 treatment in a CLL sample pre-treated for 1 h with 50 μM Boc-D-(OMe).fmk. Indeed, Boc-D-(OMe).fmk pre-treatment did not prevent PHA-767491-induced Bax activation despite complete inhibition of PS externalization (Figure S3A, S3B), 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

Since PHA-767491-induced apoptosis proceeds via Bax activation, we examined the levels of anti-apoptotic Bcl-2 family members, which modulate Bax function (32) and the levels of the anti-apoptotic protein X-linked inhibitor of apoptosis (XIAP), previously shown to be downregulated by PHA-767491 (27). CLL cells were exposed to 1 μM PHA-767491 and samples were collected at different times post-treatment. Following exposure to PHA-767491, Bax and Bcl-2 protein levels remained constant in all four samples tested while Bak showed a patient specific modulation being upregulated to different degrees in three out of four samples (Figure 2D and S4). As Bcl-X_L is poorly expressed (13) and often below the detection limit it was not analysed. However, we observed that PHA-767491 induced a rapid decrease in Mcl-1 protein levels even at 2 h 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 pro-caspase-3, cleavage of PARP and PS externalization detectable from 4 h post-treatment. Noxa, the Mcl-1 binding partner remained constant in two out of the three samples tested while a marked increase was observed in CLL #2 (Figure 2D and S4).

As Mcl-1 downregulation is the earliest event and appeared 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 that were pre-incubated with 50 μM Boc-D-(OMe).fmk 1 h before PHA-767491 treatment, PARP cleavage and PS externalization were prevented but Mcl-1 levels were not restored (Figure 2E); pre-incubation for 1 h with 1 μM 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.

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 downregulated 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). Since PHA-767491 inhibits Cdk9 kinase activity in biochemical assays we examined whether Cdk9 activity was affected in PHA-767491 treated CLL cells. Indeed, 1 μM PHA-767491 caused a rapid loss of pSer2 CTD RNA Pol II from 2 h post-treatment that correlates with Mcl-1 downregulation (Figure 3A). Analysis of Mcl-1 mRNA levels by RT qPCR at 2 h post-treatment revealed that drug treatment caused a considerable decrease in Mcl-1 mRNA (Figure 3B and 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 (Figure 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 co-culture

with CD154 expressing NIH3T3 cells (14-15). In this setting the kinetics of re-entry into the mitotic cell cycle, assessed both as DNA incorporation and loss of CFSE staining varied across patients starting as early as 2 days after stimulation (Figure 4A and 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-X_L (Figure 4B and 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 kinetics 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 (Figure 4B and S5).

In order to assess the relevance of this *in vitro* model for testing Cdc7 kinase inhibitors, we analysed the expression of Cdc7, of its substrate Mcm2 and the levels of phosphorylated Mcm2 at Ser40 by immunohistochemistry, and we compared their pattern to the one of the well recognised 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 (Figure 5A). The pSer40 Mcm2 expression pattern strongly overlaps with Ki67 staining, indicating that pSer40 Mcm2 staining may also identify the CLL replication centres 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 (Figure 5B, panels I-II). Ki67 and pSer40 Mcm2 stainings largely identified the same cells (Figure 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, five 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 h while DNA synthesis, CFSE and annexin V positivity were assessed after 24 h of treatment. We determined that 1 μM PHA-767491 had no effect while exposure to 5 μM and 10 μM PHA-767491 caused full inhibition of Cdc7 kinase activity as assessed by loss of Mcm2 phosphorylation (Figure 6A) as well as DNA synthesis (Figure 6B). Interestingly in this setting, PHA-767491 again caused Mcl-1 downregulation that was accompanied also by Noxa and Bcl-A1 downregulation while Bak was not affected and only very low levels of cleaved caspase-3 and annexin V positive cells were detected (Figure 6A, 6C). As expected blockade of DNA synthesis resulted in decreased cell division assessed by CFSE staining (Figure 6D). Identical results were observed also in CD154 and IL-4 stimulated CLL samples from two other patients (Figure 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 inhibition of Cdk9 activity measured by 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 (36-37) (Figure 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 analysed 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 centres (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 re-express 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 co-culture experiments, however, we noticed that exposure to 5 or 10 μM PHA-767491 was less effective in triggering cell death than 1 μM in resting unstimulated CLL samples, although Mcl-1 was completely depleted in both cases. This possibly

reflects a changed balance in the pro- and anti-apoptotic 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 anti-apoptotic proteins Bcl-A1, which is not detectable in circulating, quiescent CLL cells and dramatically increase the expression of Bcl-X_L and Mcl-1. While PHA-767491 was not able to reduce the level of Bcl-A1 in a diffuse B-cell lymphoma cellular system despite decreasing Mcl-1 protein levels (38) we find that Bcl-A1 is decreased similarly to Mcl-1 and is partner Noxa while Bcl-X_L is unaffected. Thus, the expression of Bcl-X_L 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 downregulate 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).

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FIGURE LEGENDS

Figure 1. PHA-767491 induces apoptosis in quiescent CLL cells independently of prognostic markers. (A) Molecular structure of PHA-767491. (B) Peripheral blood CLL cells isolated from 27 patients were treated with the indicated concentration of PHA-767491 for 12 h. Samples were collected and apoptosis was assessed by PS exposure using AV/PI assay. Bars represent the mean ± SEM (N=27). The EC 50 calculated for each sample was plotted against *IGHV* status (C), CD38 positivity (D), Binet stage of disease (E), chromosome abnormalities detected by interphase fluorescent in situ hybridization (FISH) analysis (F) and whether patients have received previous treatment or not (G).

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 μM PHA-767491 for 2, 4, 6, 8 and 12 h. Samples were collected at each time point and analysed for Bax activation (left column), ΔΨm depolarization (middle column) and PS exposure (right column) by flow cytometry. Numbers indicate percentage of CLL cells with either active Bax, low ΔΨm or positive for AV in the treated samples. Data presented are representative of 4 independent experiments. (B and C) CLL cells were either untreated, treated with 50 μM Boc-D-(OMe).fmk, treated with 1 μM PHA-767491 or pre-treated with 50 μM Boc-D-(OMe).fmk for 1 h and then treated with 1 μM PHA-767491 for 6 (white bars) or 12 h (gray bars). Samples were collected at each time point and analysed for PS 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 to untreated controls. (D) CLL cells were exposed to 1 μM PHA-767491 for 2, 4, 6 and 8 h. Samples were collected at each time point and analysed for PS exposure by flow cytometry and western blot using antibodies against the indicated proteins. (E) CLL cells from 2 patients were pre-treated with either 50 μM Boc-D-(OMe).fmk or 1 μM MG132 followed by treatment with 1 μM PHA-767491 for 6 h. Samples were collected and

analysed for PS exposure by flow cytometry and western blot using antibodies against the indicated proteins. Numbers at the bottom of the panels indicate the percentage of AV positive cells.

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 μM PHA-767491 for 2, 4 and 6 h. Samples were collected and analysed for PS exposure by flow cytometry and western blot using antibodies against the indicated proteins. (B) CLL cells from 3 patients were exposed to 1 μM PHA-767491 for 2 h. Samples were collected and Mcl-1 mRNA levels were quantified by RT-qPCR. Bars represent the mean ± 95% Confidence Interval (CI). (C) CLL cells from patient # 2 were treated with the indicated concentrations of PHA-767491 for 12 h. Samples were collected and analysed for PS exposure by flow cytometry and western blot using antibodies against the indicated proteins. Numbers at the bottom of the panels indicate the percentage of AV positive cells.

Figure 4. CD154 and IL-4 stimulation leads to DNA replication, cell proliferation and Cdc7 activation. (A) CLL cells were plated onto NIH3T3 fibroblasts expressing CD154 and cultured in the presence of IL-4 for 6 days. Every 24 h, samples were incubated with 10 μM EdU for 1 h before harvesting and analysed for their capability to incorporate the thymidine analogue EdU into their DNA (upper row). Dots in the gated regions and numbers indicate percentage of cells incorporating EdU into their DNA. In the lower row proliferation was assessed in parallel samples by CFSE staining. (B) Protein samples were analysed by western blot using antibodies against the indicated proteins.

Figure 5. Expression of active Cdc7 kinase in CLL cells within the lymph nodes. (A) Lymph node sections from CLL patient #7 were stained for Mcm2, Ki67, Cdc7 and pSer40 Mcm2 and analysed by immunohistochemistry. Pictures were taken at the indicated magnifications using Olympus BX61 microscope with Cell Soft Imaging Software (Olympus UK, Ltd). Data presented are representative of two CLL patients. (B) Lymph node sections from CLL patient #7 were stained for CD19, Ki67,

pSer40 Mcm2 as indicated and analysed by immunofluorescence. DNA was visualised using DAPI. Pictures were taken at 20X (I-II) and 40X (III-VI) using Olympus BX51 Upright Fluorescent Microscope with Improvision Optigrid System and Improvision Volocity Software. Arrows are 32 μm lenghts.

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 analysed by western blot at 12 h post-treatment using antibodies against the indicated proteins (A) and by flow cytometry at 24 h post-treatment for DNA synthesis (B) and PS exposure (C). (D) Proliferation in presence of the indicated concentrations of PHA-767491 was assessed in parallel samples by CFSE staining at 24 h post-treatment. Bars indicate the peaks corresponding to cells that have undergone cell division in the last 24 h.

Figure 1

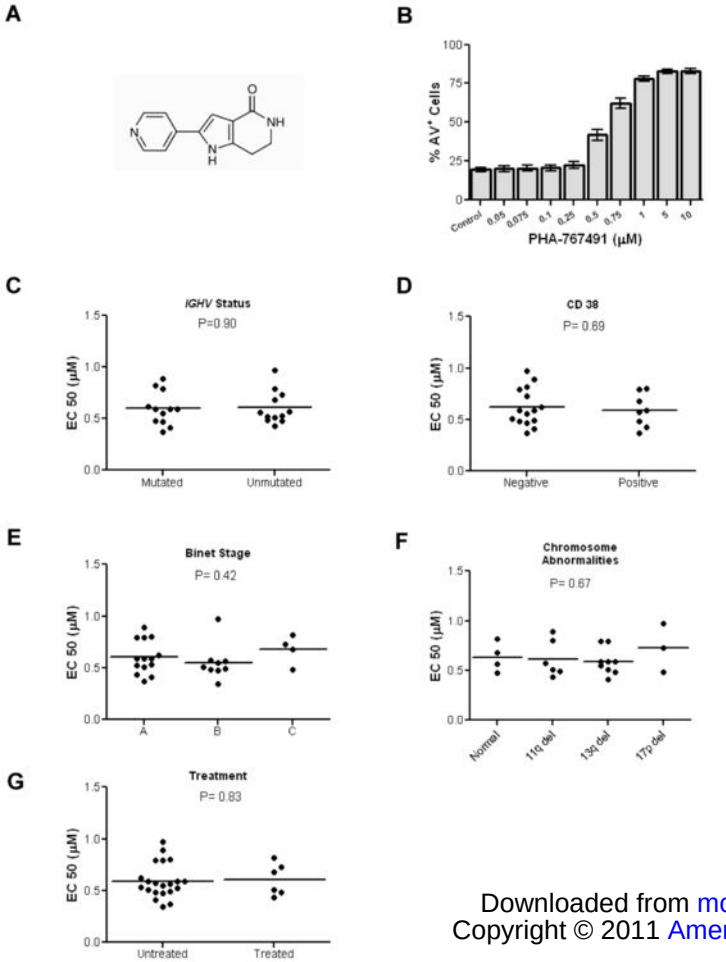
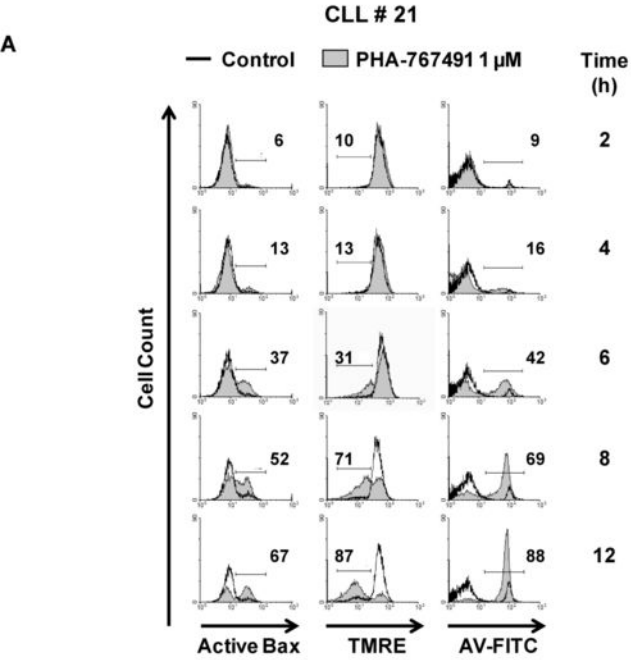
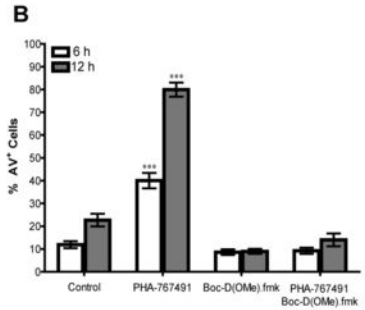
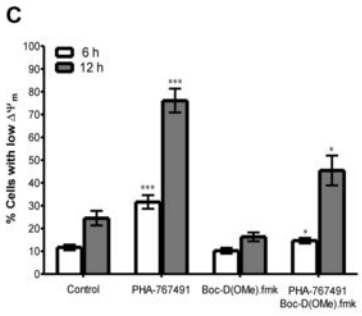
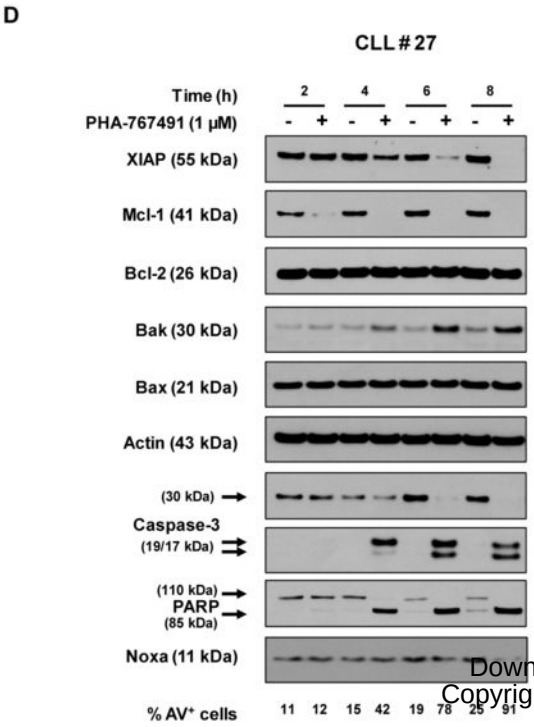


Figure 2









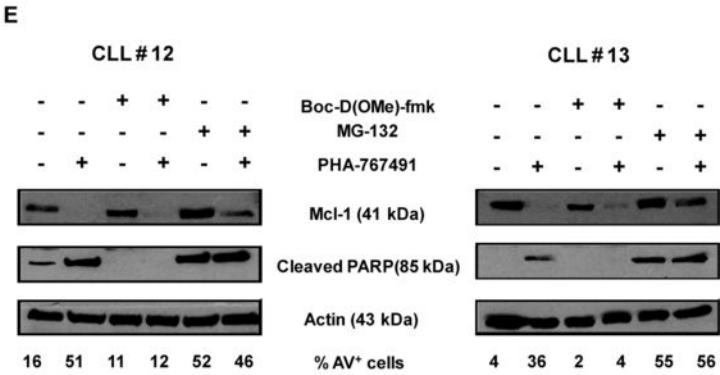
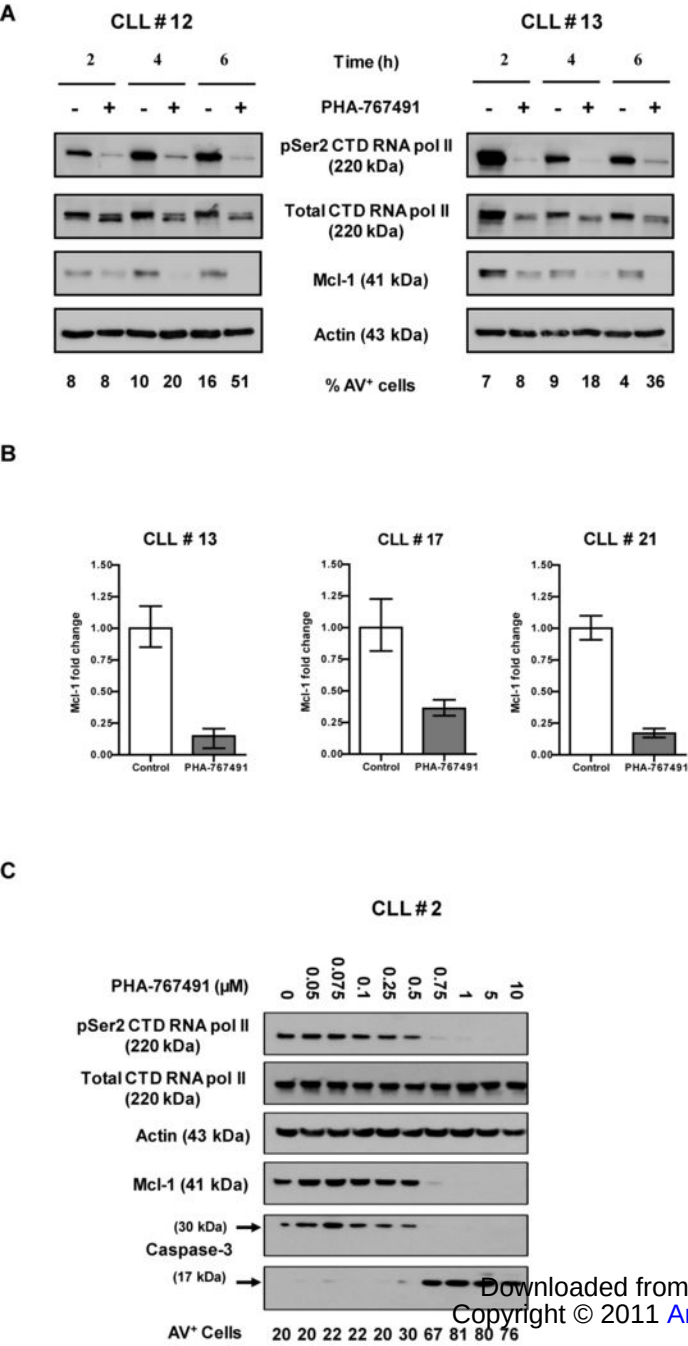


Figure 3



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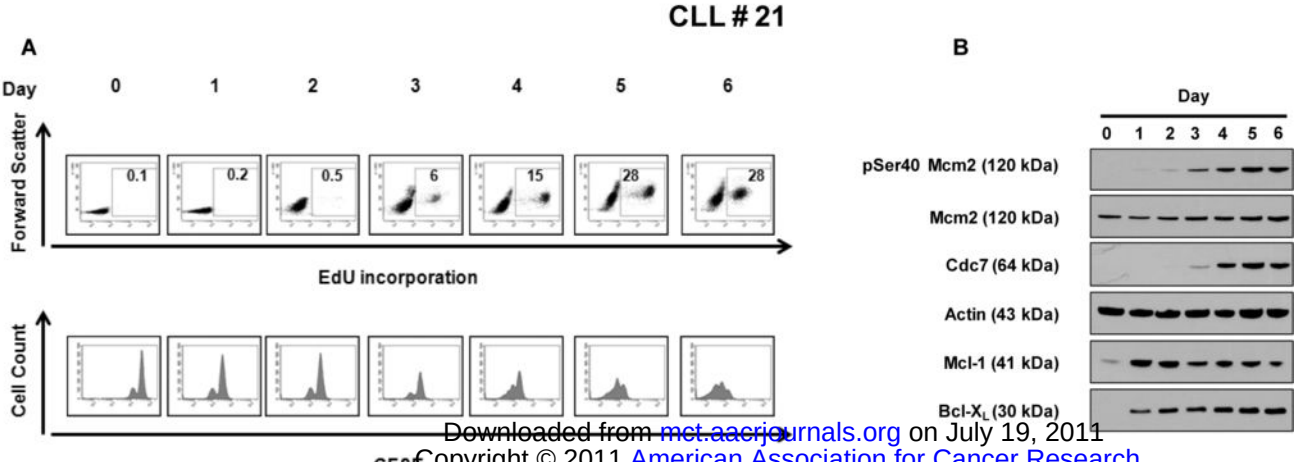
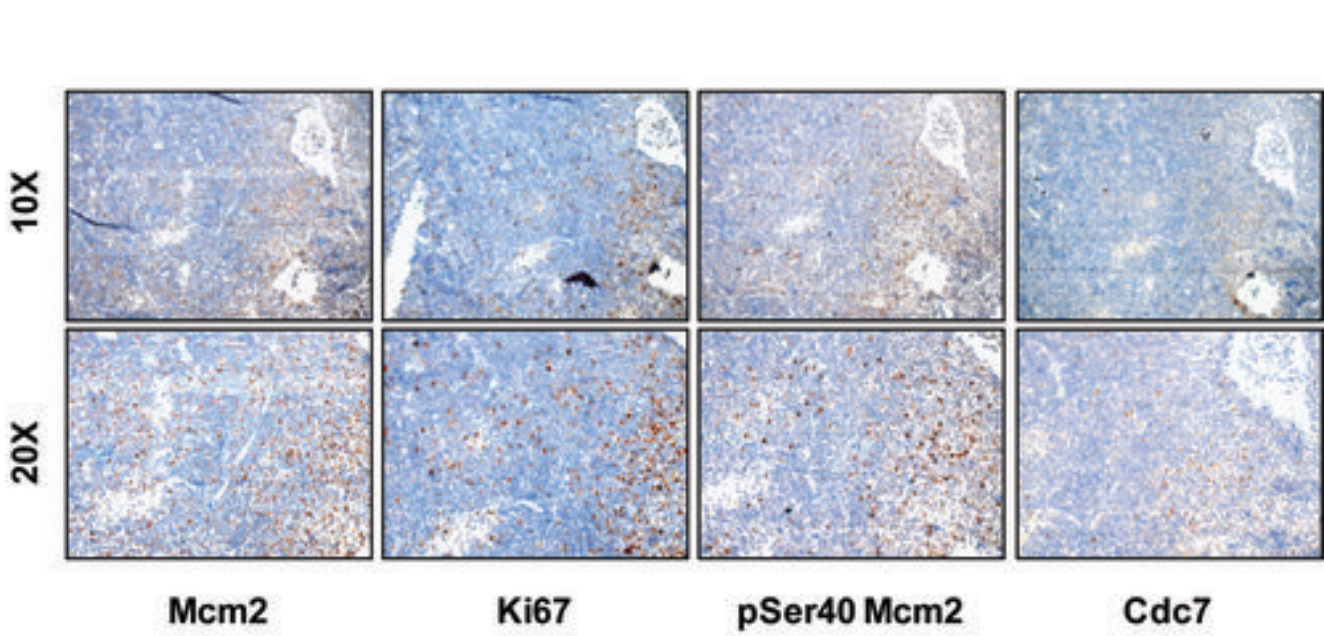


Figure 5

A



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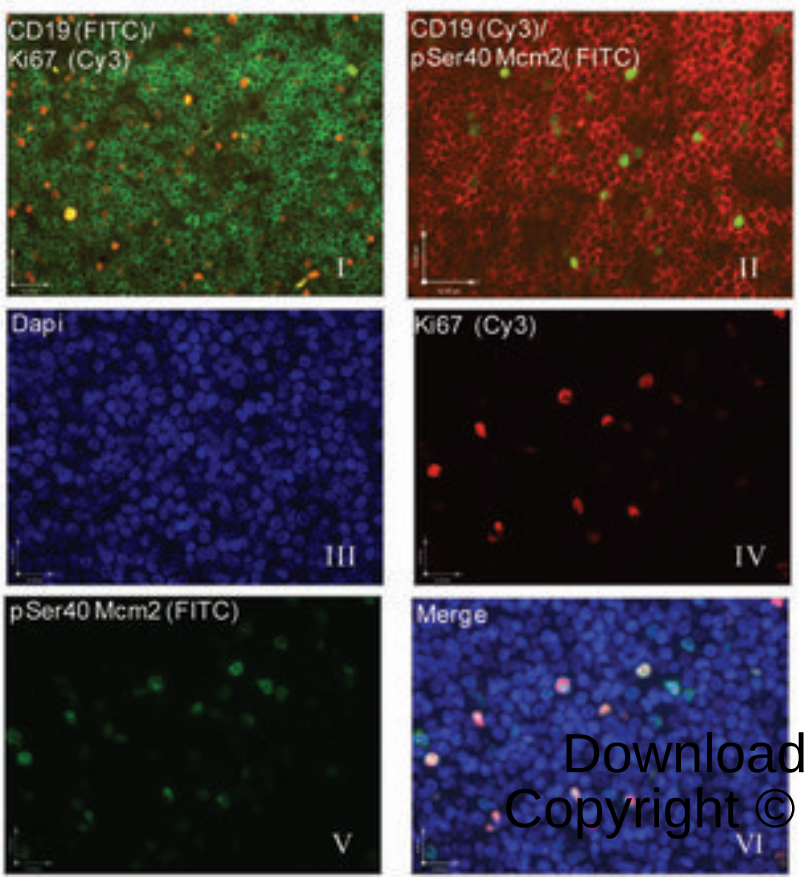

Figure 6

CLL # 21