

**Pro-survival signal inhibition by CDK inhibitor dinaciclib  
in Chronic Lymphocytic Leukaemia**

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**Short title:** Effects of Dinaciclib on pro-survival signals in CLL.

## SUMMARY

Dinaciclib is a cyclin-dependent kinase inhibitor with clinical potential in different cancers, including Chronic Lymphocytic Leukemia (CLL). In order to better understand its cytotoxic action, we characterized its effects on signalling pathways important for the survival of CLL cells. We found that dinaciclib induced apoptosis through the activation of caspases 8 and 9, which was independent of the presence of cytokines to mimic the environment of proliferation centres or *IGVH* mutation status. Moreover, treatment with dinaciclib led to the inhibition of oncogenic pathways normally activated in stimulated CLL cells, such as STAT3, NF- $\kappa$ B and p38. Also, PI3K/AKT and RAF/MEK/ERK pathways showed a transient and early activation, followed by a later, permanent inhibition. Dinaciclib was also able to block the expression of anti-apoptotic proteins of the BCL-2 family such as MCL-1 and BCL-xL. Finally, we show that low concentrations of dinaciclib enhanced cell sensitivity to ibrutinib and BCL-2 inhibitor ABT-199, two drugs with known effects on CLL. Taken together, our data show that dinaciclib targets multiple pro-survival signalling pathways in CLL, which provides a mechanistic explanation for its potent induction of apoptosis. They also support a therapeutic application of CDK inhibitors in CLL in combination with other relevant targeted therapies.

**Keywords:** dinaciclib, CDK inhibitors, CLL, apoptosis, combination therapy.

## INTRODUCTION

Chronic Lymphocytic Leukemia (CLL) is a B-cell malignancy characterized by a progressive accumulation of functionally incompetent B lymphocytes in the peripheral blood, lymph nodes and bone marrow (Chiorazzi, *et al* 2005, Mittal, *et al* 2014). Recent therapeutic strategies significantly improved survival of patients, although it remains incurable. Thus, efforts to develop effective treatments are still necessary (Dyer, *et al* 2013). Although CLL cells in peripheral blood are mostly non-dividing and easily targeted by many inhibitors, a small number of cells residing in bone marrow and lymphoid nodes have higher chemoresistance and proliferative capacity (Meads, *et al* 2008). A variety of cytokines, including T lymphocyte-expressed CD40 ligand (CD40L or CD154), BAFF and IL-4, are produced within this microenvironment niche and, together with stromal cells, nurse like cells and T cells, they are thought to be the major cause of resistance CLL therapy (ten Hacken and Burger 2014). To mimic this microenvironment *in vitro*, a number of models have been established, including stimulation with soluble CD154/IL-4, and co-cultures with CD154-expressing fibroblasts together with interleukin-4 (IL-4) (Willimott, *et al* 2007a). Under these conditions, the expression of anti-apoptotic members of the Bcl-2 family, in particular Bcl-xL and Mcl-1, are greatly increased, which contributes to the resistance to spontaneous- and drug-induced apoptosis in CLL (Vogler, *et al* 2009, Willimott, *et al* 2007a, Willimott, *et al* 2007b).

B-cell receptor (BCR) signalling can be induced by ligand-independent stimulation and/or ligation of auto-antigens present in the microenvironment. Once engaged, it activates pro-survival signals such as the RAF/MEK/ERK, PI3K/AKT, p38, NF- $\kappa$ B and STAT3 pathways, which play a pathogenic role in CLL (Cuní, *et al* 2004, Hazan-Halevy, *et al* 2010, Kawauchi, *et al* 2002, Ogasawara, *et al* 2003, Pickering, *et al* 2007, Ringshausen, *et al* 2002,

Rozovski, *et al* 2014, Sainz-Perez, *et al* 2006). Nearly half of CLL cases have high levels of constitutively phosphorylated ERK1/2 (Muzio, *et al* 2008) and in freshly isolated CLL cells, phosphorylated p38 is predominately activated (Sainz-Perez, *et al* 2006). PI3K is also frequently activated in CLL cells and its inhibition induces apoptosis in some models (Cuní, *et al* 2004). PI3K/AKT activates NF- $\kappa$ B and the loss of AKT and NF- $\kappa$ B is also associated with apoptosis in these cells (Cuní, *et al* 2004). NF- $\kappa$ B RelA/p65 activation correlates chemoresistance and poorer clinical outcome in CLL (Cuní, *et al* 2004, Furman, *et al* 2000, Hewamana, *et al* 2008). Moreover, NF- $\kappa$ B activation can be regulated by STAT3 (Liu, *et al* 2011), which is constitutively phosphorylated at serine 727 in CLL cells (Hazan-Halevy, *et al* 2010, Rozovski, *et al* 2014).

Disruption of activated pro-survival signals such as these has become a field of major interest in the development of antineoplastic drugs, and many small molecular kinase inhibitors that selectively target them have already been developed (Dyer, *et al* 2013). Finding treatments that target multiple signalling pathways simultaneously in diseases such as CLL is important to avoid recurrences and eventually provide a curative strategy (Dyer, *et al* 2013).

Cyclin-dependent kinases (CDKs) are essential in the regulation of cell division, cell-cycle progression and gene transcription, and therefore deregulated in many cancers (Malumbres and Barbacid 2009). Their inhibition represents a promising therapeutic approach in blood malignancies (Blachly, *et al* 2016). Although initial clinical studies with CDK inhibitor alvocid showed high toxicity due to hyperacute tumour lysis, there have been successful trials with subsequent inhibitors (Blachly, *et al* 2016). Dinaciclib (MK-7965, formerly SCH727965), a novel, selective CDK1-4/7/9 inhibitor with improved therapeutic index, was found to induce apoptosis in CLL cells (Johnson, *et al* 2012). It has already been tested in initial trials for solid tumours (Nemunaitis, *et al* 2013), myeloma (Kumar, *et al* 2015)

and refractory CLL (Flynn, *et al* 2015). Encouraging Phase I and II results provide a rationale for the use of dinaciclib alone or in combination with immunotherapies in CLL and lymphoma (Blachly, *et al* 2016). However, the molecular events that contribute to dinaciclib-induced apoptosis of malignant B cells are not fully understood.

Here, we studied the effects of dinaciclib on the pro-survival signals present in CLL to better understand the mechanisms involved defining its therapeutic potential. We show that dinaciclib simultaneously targets several pro-survival signalling pathways and suppresses expression of anti-apoptotic proteins to induce a caspase-dependent cell death. We also present evidence that supports the hypothesis that dinaciclib could be of therapeutic use in CLL, alone or in combination with other targeted drugs.

## **METHODS**

**Cell culture and reagents.** MEC-1, Eskol and JVM3 cell lines were cultured in RPMI-1640 with 10% Fetal Bovine Serum (FBS) serum and 2 mmol/L L-glutamine, 50 U/mL penicillin, 50 mg/mL streptomycin, which was called complete medium, at 37°C in incubator containing 5% CO<sub>2</sub>. Peripheral blood samples were obtained from CLL patients attending clinics at the Leicester Royal Infirmary (Leicester, UK) following informed consent and approval from the local Research Ethics Committee and in accordance with the Declaration of Helsinki. All patients, diagnosed according to IWCLL-NCI 2008 guidelines (Hallek, *et al* 2008), were treatment free for at least 6 months and had a cell count  $>50 \times 10^9/l$ . Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by density centrifugation. Heparinized whole blood was diluted 1:1 with PBS and gently layered onto 15ml Ficoll (Histopaque 1077, Sigma-Aldrich, Poole, UK) prior to centrifugation at 400g for 30 minutes.

The mononuclear cell layer was removed from the interphase, washed and resuspended in RPMI-1640 supplemented with 10 ng/mL IL-4 and CD154 (CD40L) to maintain cells survival *in vitro*. Alternatively, mouse fibroblast L cells (NTL) and NTL cells stably expressing CD154 (NTL/CD154) were used in co-culture experiments to support CLL cell survival/proliferation. NTL/CD154 cells were irradiated with 30 Gy and then co-cultured with CLL patient cells at 1:25 ratio. Inhibitors dinaciclib (SCH727965), ibrutinib (PCI-32765), Entospletinib (GS-9973), Ver155008, BX-912, RAF265, Sorafenib, Vemurafenib (PXL4032), Trametinib (GSK1120212), MG-132, Fedratinib and ABT-199 used in this study were obtained from Selleckchem. Z-Vad-Fmk was bought from Sigma-Aldrich Co. Ltd.

**MTS assay.** The CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) was used to estimate cell viability and proliferation, following manufacturer's instructions. Briefly, 100µl 0.25×10<sup>6</sup>/ml cells from cell lines or 2×10<sup>6</sup>/ml CLL cells were seeded in 96-well plates. Inhibitors were added at different concentration for 48 hours before 20 µl MTS was added to each well. After incubation for 1-3 hours at 37°C, the absorbance at 490 nm was recorded on a TECAN infinite F50 reader (Labtech International). Cell viability was calculated based on the reducing potential of the viable cells using a colorimetric reaction. Assays were performed in triplicate and repeated at least 2 independent times and results were plotted and analysed using GraphPad Prism 6 (GraphPad Software Inc.).

**Cell-cycle analysis.** Cells were harvested, fixed with precooled 70% ethanol at -20°C overnight, and then stained with PI (Sigma-Aldrich) diluted in PBS containing RNase A at 37°C for 30 minutes in the dark. Cell-cycle distribution was then determined using a FACS Canto II cytometer (BD Biosciences).

**Cell death analysis.** Cells were harvested and stained with Annexin V (Sigma-Aldrich) for 15 minutes at 4°C. Percentages of apoptotic cells (positive for Annexin V) were determined by flow cytometry using a FACS Canto II cytometer (BD Biosciences) following manufacturer's instructions. Alternatively, cell death was measured staining the cells with PI for 30 minutes at 4°C. Percentage of PI-positive cells (dead) were determined by flow cytometry.

**Western blotting.** Total protein was prepared in RIPA lysis buffer (10mM Tris pH 7.4, 150mM NaCl, 1% TritonX-100, 0.1% sodium deoxycholate, 0.1% sodium dodecylsulfate (SDS), 5mM EDTA) supplemented with protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail 2 (Sigma). Proteins were separated with SDS-PAGE and incubated with specific antibodies. Protein bands were visualized and quantified with an Odyssey system (Pierce). The antibodies used were: ERK, phospho-ERK (Thr202/Tyr204), AKT, phospho-AKT (Ser473), IκBα, phospho-IκBα (Ser32/36), STAT3, phospho-STAT3 (Tyr705), p38, phospho-p38 (Thr180/Tyr182), PARP, caspase-8 and caspase-9 (Cell Signaling Technology); BCL-2 (Dako); β-actin (Millipore); BCL-xL/S, Mcl-1 and NF-κB(p65) (Santa Cruz Biotechnology). Fluorescent-conjugated secondary anti-rabbit or anti-mouse antibodies were purchased from Enzo life sciences.

## **RESULTS**

**CLL cells are highly sensitive to dinaciclib.** In order to better understand the sensitivity of CLL cells to CDK inhibitor dinaciclib, we compared its effects on the viability of MEC-1, an EVB-positive B-cell line derived from a patient with CLL that is often used as a model for

this disease (Stacchini, *et al* 1999), to that of different targeted inhibitors of pro-survival pathways often activated in leukaemia. We selected other drugs that have been studied in the context of leukaemia, such as MG-132 (a proteasome inhibitor (Guo and Peng 2013)), ibrutinib (a BTK inhibitor (Byrd, *et al* 2013, Danilov 2013)), vemurafenib (a <sup>V600E</sup>BRAF inhibitor (Samuel, *et al* 2014)), ver155008 (a HSP70 inhibitor (Reikvam, *et al* 2013)), BX-912 (a PDK1 inhibitor targeting PI3K/Akt pathway (Feldman, *et al* 2005)) and fedratinib (a Jak/STAT pathway inhibitor (Pardanani, *et al* 2007)). As shown in Figure 1A, dinaciclib demonstrated more than 100-fold greater inhibitory effects on the metabolic activity of MEC-1 than any of the other drugs tested, confirming its potential as a therapeutic approach for CLL and consistent with recent results that showed the strong cytotoxic effects of CDK inhibitors in CLL (Johnson, *et al* 2012, Paiva, *et al* 2015, Sylvan, *et al* 2016). We determined that the effects of dinaciclib on cell viability were the consequence of a dose-dependent increase in cell death but not cell cycle arrest, as tested by PI staining (Figure 1B). Specifically, we found that this was due to the induction of apoptosis (Figure 1C). Cell death induction was progressive and reached a maximum after 48 hours (Figure 1D). Of note, these figures show that a fraction of cells were resistant to even the highest concentration of dinaciclib tested, for reasons that will need to be elucidated. A similar response to these drugs was also observed in other malignant B-cell lines such as Eskol and JVM3 (Supplementary Figure 1A).

We next confirmed these results in primary CLL cells (Table S1) cultured in the presence of CD154 and IL-4 to mimic the protective microenvironment of proliferation centres. As shown in Figure 1E, dinaciclib was also able to reduce cell viability at low concentrations under these conditions, showing a higher effect than the other inhibitors. This was also dose-dependent (Figure 1F), with an IC<sub>50</sub> below 5nM, and not determined by the *IGHV* mutation status of the patients (Figure 1G). Similar results were also observed in the



primary CLL patient cells co-cultured with mouse fibroblast L (NTL) cells stably expressing CD154 and supplemented with IL-4, an alternative stimulation system (Supplementary Figure 1B). Taken together, these data support the hypothesis that dinaciclib is highly toxic for malignant B cells at submicromolar concentrations, with effects stronger than any of the inhibitors of pro-survival signals tested, and that this is due to the induction of apoptosis. Also, we show that dinaciclib can strongly affect cell viability even in different culture conditions that mimic protective microenvironments, which underscores its potential to target resistant CLL cells.

**Dinaciclib-induced toxicity in CLL is dependent on caspase activation.** We further explored the apoptotic effects of dinaciclib on CLL cells by analysing the activation of the caspase pathway. Our data showed that dinaciclib can activate caspase 8 and caspase 9, and induce PARP cleavage in CLL patient cells (Figure 2A), which indicates induction of apoptosis with involvement of both the intrinsic (mediated by caspase 9) and extrinsic (mediated by caspase 8) pathways (Parrish, *et al* 2013). We next treated CLL cells with the pan-caspase inhibitor Z-Vad-Fmk before exposing them to dinaciclib. As shown in Figure 2B, the presence of the caspase inhibitor partially reduced the PARP cleavage and apoptosis induced by dinaciclib, suggesting that the cytotoxicity of dinaciclib in CLL is at least in part dependent on the activation of the caspase cascade.

**Dinaciclib inhibits pro-survival signals in CLL cells.** To further investigate the molecular events that determine the effect of dinaciclib on CLL cell survival, we measured the activation status of multiple pro-survival oncogenic signalling pathways that have been reported to be upregulated in B cell malignancies, such as STAT3, NF- $\kappa$ B, p38, PI3K/AKT and RAF/MEK/ERK (Cuní, *et al* 2004, Hazan-Halevy, *et al* 2010, Kawauchi, *et al* 2002,

Ogasawara, *et al* 2003, Pickering, *et al* 2007, Ringshausen, *et al* 2002, Rozovski, *et al* 2014, Sainz-Perez, *et al* 2006). The phosphorylation level of STAT3 (Tyr705), I $\kappa$ B $\alpha$  (Ser32/36), p38 (Thr180/Tyr182), AKT (Ser473) and ERK (Thr202/Tyr204) were assessed as markers of activation of these pathways in MEC-1. As shown in Figure 3A (0.1  $\mu$ M dinaciclib) and Supplementary Figure 2A (0.01  $\mu$ M dinaciclib), in treated cells there was a marked decrease in the phosphorylation of STAT3 and I $\kappa$ B $\alpha$ , together with a more discreet decrease in the phosphorylation of p38. On the other hand, AKT and ERK phosphorylation increased temporarily, and started to decline after 8 hours. Inhibition of these signals was maintained after that (Figure 3B). Of note, these changes in signalling pathways occurred before PARP cleavage and the induction of apoptosis, which started after 12 hours of exposure to dinaciclib (see Figure 3A and Supplementary Figure 2A), suggesting they are an early event in the cellular response to the drug.

Next, we confirmed these results in primary CLL cells. We found that the phosphorylation of STAT3, I $\kappa$ B $\alpha$  and p38 decreased after 12 hours exposure to dinaciclib in cells cultured in the presence of CD154/IL4 (Figure 3C). Total levels of I $\kappa$ B $\alpha$  were reduced as well. Moreover, we observed that ERK, AKT (Figure 3D) and NF- $\kappa$ B (Figure 3E) signalling were also inhibited by dinaciclib in primary cells. Of note, although a similar response was observed in the ESKOL, JVM3 showed reduction in the phosphorylation of AKT but not in that of ERK, perhaps due to an over-activation of this pathway driven by the BRAF K601N mutation present in these cells (Supplementary Figure 2B).

We also observed that the levels of MCL-1, an anti-apoptotic protein of the BCL-2 family, quickly decreased in MEC-1 after treatment with dinaciclib in a time and concentration dependent manner, which correlated to PARP cleavage and induction of apoptosis (see Figure 3A and Supplementary Figure 2A). This is consistent with recent reports showing that MCL-1 expression was decreased by dinaciclib in various cancer cells

(Chen, *et al* 2015, Fu, *et al* 2011, Gregory, *et al* 2015, Varadarajan, *et al* 2015). As shown in Figure 3F, we confirmed that dinaciclib inhibited both MCL-1 and BCL-xL in stimulated primary cells and MEC-1. However, its effects on BCL-2 were patient-dependent (Figure 3G). Since levels of BCL-2-related proteins such as MCL-1, BCL-xL and BCL-2 have been linked to the resistance of malignant B cells to apoptosis (Kitada, *et al* 1998, Pepper, *et al* 2008), this inhibition could contribute to the cytotoxicity of dinaciclib. Taken together, our data shows that dinaciclib has an inhibitory effect on several oncogenic signalling pathways usually activated in malignant B-cells, which provides a mechanism to explain its potent effects in these cells.

**Positive effects of dinaciclib in combination therapies.** Despite their strong apoptotic effects on malignant B cells, the efficacy of CDK inhibitors as monotherapy has not been as successful as expected (Bose, *et al* 2013). Dinaciclib has only a 54% overall response rate in relapsed/refractory CLL patients and a tumour lysis syndrome is sometimes induced (Flynn, *et al* 2015). This suggests that combination with other targeted therapies could improve its clinical impact. To explore this possibility, we assessed the combination of dinaciclib with low concentrations of other specific inhibitors relevant to B cell malignancies, such as ABT-199 (Bcl-2 inhibitor), ibrutinib (BTK inhibitor) and entospletinib (SYK inhibitor). As shown in Figure 4A, ibrutinib and ABT-199 had their effects on MEC-1 survival increased by being combined with nanomolar concentrations of dinaciclib, while there was no variation with entospletinib. The greatest effect was seen with ABT-199, for which concentrations that on their own were only capable of triggering low levels of cell death (~20%) induced over 80% when combined with 0.01  $\mu$ M dinaciclib. This is consistent with previous reports showing that CDK inhibitors sensitize CLL cells to BH3 mimetics like ABT-737 (Paiva, *et al* 2015). For ibrutinib, the increase in cell death was from ~50 to ~90%. A similar response was

observed in stimulated primary cells (Figure 4B). The increase in apoptosis induced in this case by ibrutinib was from ~60 to ~90%, and by ABT-199 from ~80 to ~90%. These results suggest that nanomolar concentrations of dinaciclib could sensitize CLL cells to ibrutinib or Bcl2 inhibitors, potentially enhancing their effects and reducing chemoresistance.

## DISCUSSION

Despite having been developed many years ago, CDK inhibitors have only recently been considered for antineoplastic therapies. First-generation CDK inhibitors, such as flavopiridol (PHA-793887) and roscovitine (CYC202), had a narrow therapeutic window and low selectivity (Blachly and Byrd 2013, Blachly, *et al* 2016). Dinaciclib was designed maximizing the therapeutic index and minimizing the toxicity associated with this type drug. It specifically shows a strong and selective inhibition of CDK1, CDK2, CDK5 and CDK9 and was shown to induce death in various cancer cells (Parry, *et al* 2010, Paruch, *et al* 2010). Dinaciclib has already been evaluated in clinical trials and results show that has encouraging activity in relapsed/refractory CLL (Fabre, *et al* 2014, Flynn, *et al* 2015).

In this study, we explored its effects on pro-survival signalling pathways in CLL cells. Our data confirmed that malignant B cells have a high sensitivity to dinaciclib, even in the presence of stimulatory cytokines that recapitulate the protective features of the microenvironment in proliferation centres. Reaching these cells is necessary for a curative effect in CLL, since they are less sensitive to therapies than circulating cells and may thus be the cause of resistant disease (Dyer, *et al* 2013). Importantly, the concentration of dinaciclib needed to exert an effect on B cell survival is several orders of magnitude below that of other small molecule inhibitors we tested. All this greatly supports the clinical relevance of

dinaciclib as a treatment for CLL. However, our results also show a percentage of refractory cells (10-25%), even at concentrations above therapeutic limits (see Figures 1A-C), which suggests that dinaciclib may not be a monotherapy of choice.

It has been previously reported that CDK inhibitors promote apoptosis in different cancer cells via suppression of Rb phosphorylation (Fu, *et al* 2011, Parry, *et al* 2010) or in a JNK/p38-dependent manner (Paiva, *et al* 2015). Our data showed that this requires the activation of caspase 8 and 9, suggesting an involvement of both the extrinsic and intrinsic pathways. The use of patients in our experiments with p53 mutations or deletions (see Table S1) indicates that dinaciclib-induced apoptosis can also be p53-independent.

We show that treating CLL cells with dinaciclib has wide ranging effects and interferes with at least five major oncogenic pathways often hyperactivated in these cells: RAF/MEK/ERK, p38, PI3K/AKT, NF- $\kappa$ B and STAT3. Given the fact that these are well known pro-survival signals, it is reasonable to assume that their inhibition would greatly contribute to the induction of cell death. Moreover, these are early effects, observed before the activation of the apoptotic machinery and thus would play a role in the downstream events. It is likely that the strong effect of dinaciclib on CLL survival is in part mediated by the combined inhibition of two or more of these oncogenic pathways.

According to our results, this molecular response of CLL cells to dinaciclib follows two different phases. The early effects include an immediate inhibition of STAT3, NF- $\kappa$ B and p38 signalling. In parallel, the ERK and AKT pathways may initially show a paradoxical activation. However, in a late phase, which starts after 8-10 hours, as apoptosis begins, both signals are also suppressed. This suggests that the first three pathways may be directly inhibited by dinaciclib, while the latter two may probably be an indirect or secondary response. Of note, dinaciclib could effectively induce cell death in JVM3 despite the fact that it did not inhibit ERK phosphorylation in them, suggesting that either this effect may be dispensable in

triggering apoptosis or inhibiting other pathways simultaneously is sufficient. The importance of signalling pathways in cell responses to dinaciclib is likely to vary in different CLL patients. For instance, not all patients have a high level ERK activation (Muzio, *et al* 2008) and AKT Serine 473 phosphorylation, a marker of its activation, was very low in unstimulated CLL cells (Ringshausen, *et al* 2002). These data supports the view that the signalling networks in CLL cells are very diverse and complex and that drugs that can target more than one of these signals are likely to be effective at treating a greater number of patients.

It remains to be determined which of these pathways needs to be inhibited for dinaciclib to induce cells apoptosis and which inhibition may be dispensable. Our data suggests that induction of apoptosis is linked to an early inhibition of STAT3, NF- $\kappa$ B and p38 signalling, which is then followed by blocking of the PI3K/AKT and RAF/MEK/ERK pathways. NF- $\kappa$ B has been recently shown to be essential for CDK inhibitors to induce apoptosis in CLL cells (Cosimo, *et al* 2013). This is consistent with the fact that cytokines expressed in the microenvironment of proliferation centres induce NF- $\kappa$ B transcriptional activity to up-regulate chemokines, cell cycle regulators and anti-apoptotic proteins that further increase the resistance of CLL cells to apoptosis. Similarly, STAT3 regulates the expression of anti-apoptotic, pro-proliferative and immune response genes, therefore playing a role in the interaction between cancer cells and their microenvironment (Grivennikov and Karin 2010). Although this supports their importance in the cellular response to dinaciclib, further experiments will needed to confirm it.

Dinaciclib also has an inhibitory effect on anti-apoptotic proteins, which is likely to reinforce its induction of CLL cell death. Indeed, we observed that MCL-1 and BCL-xL levels decreased upon dinaciclib treatment. Similar results were observed with other CDK inhibitors, such as Seliciclib/R-roscovitine/CYC202 (Hallaert, *et al* 2007). However, the

levels of BCL-2 showed a variable response in primary cells. Specific inhibition of BCL-2 greatly enhanced the effects of dinaciclib (see Figure 4), which suggests that the anti-apoptotic effects of BCL-2 could provide cellular resistance to dinaciclib in certain patients. The downregulation of these and other proteins by dinaciclib, including AKT, I $\kappa$ B $\alpha$  and NF- $\kappa$ B, could be due to its ability to regulate transcription (Alvi, *et al* 2005, Natoni, *et al* 2011), particularly, the inhibition on CDK9, which targets RNA polymerase II activity (Oelgeschläger 2002). Thus, repression of transcription has been considered as a mechanism that explains how CDK inhibitors kill cancer cells (Alvi, *et al* 2005, Natoni, *et al* 2011).

Dinaciclib is being tested in clinical trials, alone or in combination with drugs like rituximab, especially in the context of relapsed or refractory CLL (Blachly, *et al* 2016). Our results suggest that dinaciclib is unlikely to be a useful monotherapy, due to its toxicity and the existence of a percentage of refractory cells, but it could be combined with other specific small molecular inhibitors, like ibrutinib or ABT-199, and be highly effective at nanomolar concentrations. Ibrutinib is an irreversible inhibitor of Bruton's tyrosine kinase (BTK) that has shown robust clinical activity in CLL (Byrd, *et al* 2013, Herman, *et al* 2011) and has been approved by US Food and Drug Administration (FDA) for the treatment of CLL. Resistance to its monotherapy appears in CLL patients due to the mutation of cysteine to serine at position 481 of BTK (Woyach, *et al* 2014). According to our data, combination with low concentrations of dinaciclib could potentiate the effects of ibrutinib and reduce chemoresistance. Similarly, ABT-199, a third-generation Bcl-2 inhibitor, has shown clinical potential in a wide variety of hematologic malignancies, including CLL (Pan, *et al* 2014, Scarfò and Ghia 2013, Souers, *et al* 2013). However, it selectively targets BCL-2 but not MCL-1. This may confer resistance to this drug, since MCL-1 can compensate for the loss of BCL-2 function (Moulding, *et al* 2000). Consistent with this, down-regulation of MCL-1 increases BCL-2 inhibitor ABT-737 lethality in human leukemia cells (Chen, *et al* 2007). We

showed that the capacity of ABT-199 to induce apoptosis in CLL cells was improved by combining it with dinaciclib, perhaps mediated by its effects on MCL-1. This supports the hypothesis that dinaciclib could sensitize CLL cells to BCL-2 inhibitors and enhance its therapeutic effects.

In summary, our data proposes a mechanistic explanation for the effects of dinaciclib on CLL cell survival and support the idea of using CDK inhibitors in combination with other specific inhibitors could improve current treatments of CLL.

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## **AUTHOR CONTRIBUTION**

YC and SM designed the experiments, analysed the data and prepared the manuscript, with contributions from MJSD, SJ and SG. YC performed the experiments, with help from SG, CC, JS, GS and SJ. All authors reviewed the manuscript.

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## FIGURE LEGENDS

**Figure 1. MEC-1 cells are highly sensitive to dinaciclib.** (A) MEC-1 cell viability measured by MTS assay. Cells were treated with various small molecule inhibitors (0.001 to 10 $\mu$ M) or DMSO (0 $\mu$ M) for 48 hours. Experiments were performed in triplicate and repeated at least 2 times. Graphics show mean values and error bars represent standard deviation, as in all the other panels of this figure. (B) Percentage of cells in each phase of the cell cycle, as measured by PI staining of MEC-1 cells treated with dinaciclib for 72 hours. +: positive control (0.1% Triton X). Experiments were performed in triplicate and repeated at least 2 independent times. (C) Induction of apoptosis in MEC-1, as measured by FACS analysis of Annexin V-stained cells. Cells were treated with inhibitors for 72 hours. Graphics show

percentage of apoptotic cells (Annexin positive). **(D)** Time-course analysis the induction of apoptosis in MEC-1 cells treated with different concentrations of dinaciclib, as measured by PI staining. Experiments were performed in triplicate and data were expressed as mean  $\pm$  standard deviation in all the panels of this figure. **(E)** CLL patient cell viability measured by an MTS assay. Cells were cultured for 24 hours in the presence of 10 ng/mL IL-4 and CD154 and then treated with 0.01 and 0.1  $\mu$ M of different inhibitors for 48 hours. 3 to 10 samples were tested for each inhibitor, and experiments were performed in triplicate. Graphics show mean values and error bars represent standard deviation. **(B)** Primary cells cultured as above, treated with dinaciclib at various concentrations for 48 hours. Patient used (see Supplementary Table 2): P2, P5, P8, P9, P10, P14, P16, P20, P26, P27, P28, P31 and P32. **(C)** Viability of primary patient cells treated with different concentrations of dinaciclib for 48 hours, as measured by MTS. P9 (*IGVH* mutated) and P32 (unmutated) were used as representative. Experiments were performed in triplicate and repeated at least 2 independent times. Data are expressed as mean  $\pm$  standard deviation.

**Figure 2. Cell death induced by Dinaciclib in primary cells is dependent on caspase cleavage.** **(A)** Western blot showing caspase-9, caspase-8 and PARP cleavage in CLL primary cells (P1 and P3). Cells were cultured as above and treated by dinaciclib for 16 hours.  $\beta$ -actin served as a loading control. Bottom graph shows quantitation of these two blots, expressed as the ratio of cleaved to uncleaved proteins, normalized to  $\beta$ -actin. **(B)** Patient cells (P22) were cultured as above and treated with 25  $\mu$ M caspase inhibitor Z-Vad-Fmk for 1 hour before being treated with different concentrations of dinaciclib for 16 hours. Numbers show percentage of apoptotic cells in the same experiment, as measured by Annexin staining.

**Figure 3. Dinaciclib inhibits pro-survival signals in CLL cells.** (A) Western blot performed on lysates of MEC-1 cells treated with dinaciclib at 0.1 $\mu$ M concentration and collected at 2, 4, 6, 8 and 10 hours after treatment. Levels of activation of signalling pathways was measured by the phosphorylation of Erk (Thr202/Tyr204), Akt (Ser473), I $\kappa$ B $\alpha$  (Ser32/36), STAT3 (Tyr705) and p38 (Thr180/Tyr182). Mcl-1 and PARP levels were also measured. Right-panel graph shows quantitation the Western blot bands normalized to  $\beta$ -actin and expressed as relative to control (0 hour) treatment. (B) Representative Western blot of MEC-1 cells treated with dinaciclib for 12 hours. Levels of total and phosphorylate Erk and Akt were measured, as well as PARP cleavage. (C) Representative Western blot of patient CLL cells (P21 and P12), cultured as described and treated with dinaciclib for 12 hours. Levels of total and phosphorylated STAT3, I $\kappa$ B $\alpha$  and p38 were measured. (D) Same as above, measuring the levels of P-ERK, ERK, P-AKT and AKT, as well as PARP cleavage. (E) Same as above, showing levels of NF- $\kappa$ B (p65 and p50). (F) Same as above, showing levels of MCL-1 and Bcl-xL in CLL patient cells and MEC-1 treated with dinaciclib. (G) Same as above, showing levels of Bcl-2.

**Figure 4. Dinaciclib enhances sensitivity of CLL cells to ibrutinib and ABT-199.** (A) Percentage of apoptotic MEC-1 cell in cells treated with different concentrations of dinaciclib for 48 hours, alone or in combination with 0.1  $\mu$ M ABT-199, 10  $\mu$ M ibrutinib or 10  $\mu$ M entospletinib, as measured by PI staining. Graphs show average of three independent experiments. (B) Same for combinations of dinaciclib with 0.1  $\mu$ M ABT-199 and 10  $\mu$ M ibrutinib on CLL patient cells (P22) cultured for 24 hours in the presence of 10 ng/mL IL-4 and CD154. Graphs show average of two independent experiments.

**Supplementary Figure 1.** (A) Same as Figure 1A, using Eskol and JVM3 cells. (B) Viability of unmutated and mutated CLL cells co-cultured with NTL/CD154/IL-4 for 48 hours, before being treated with 0.1 or 0.01 $\mu$ M of different drugs for 48 hours, as measured by MTS assay. Patient cells used: P11, P4, P5, P7, P8, P15, P20, P23, P28, P29, P31, P32.

**Supplementary Figure 2.** (A) Same as Figure 3A, using 0.01 $\mu$ M dinaciclib. (B) Same as Figure 3B, using ESKOL and JVM3.

**Table S1.** Information on the CLL patient samples used in the figures.

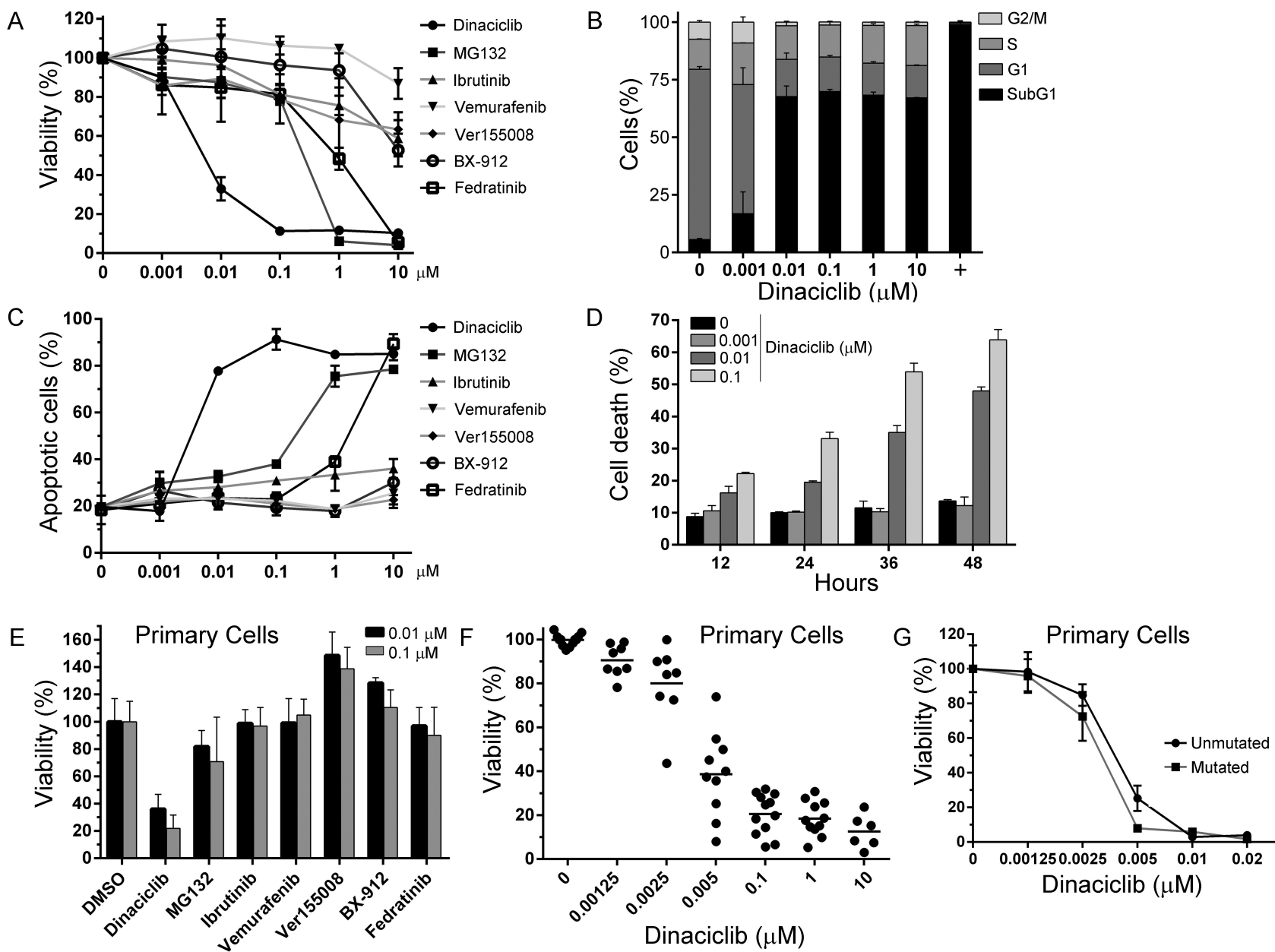
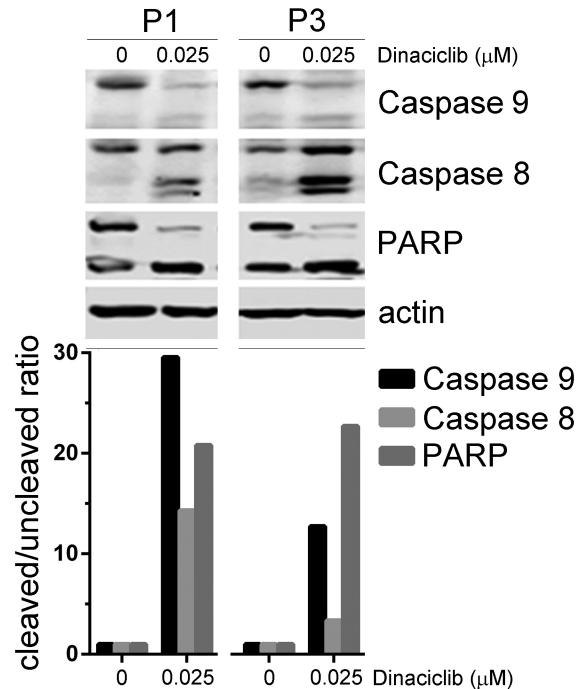


Figure 1

A



B

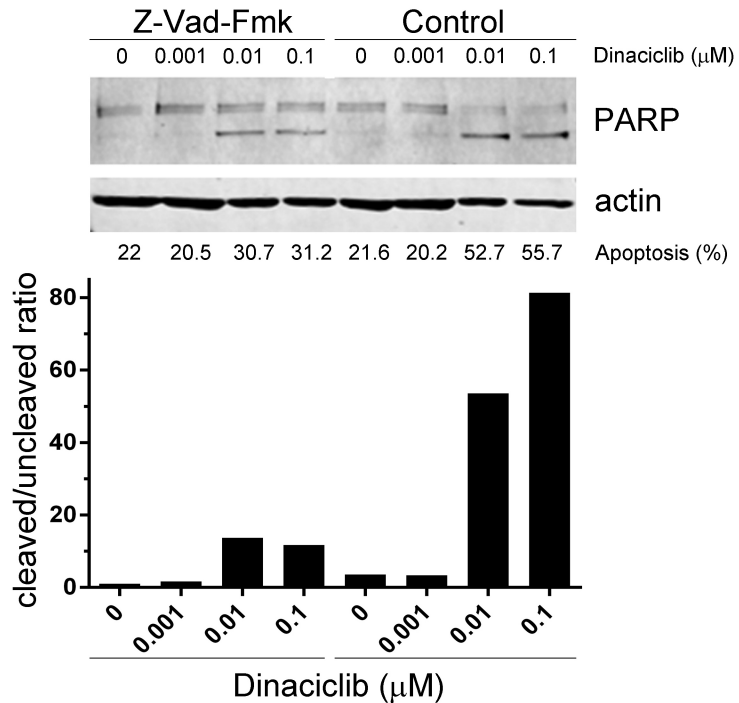


Figure 2

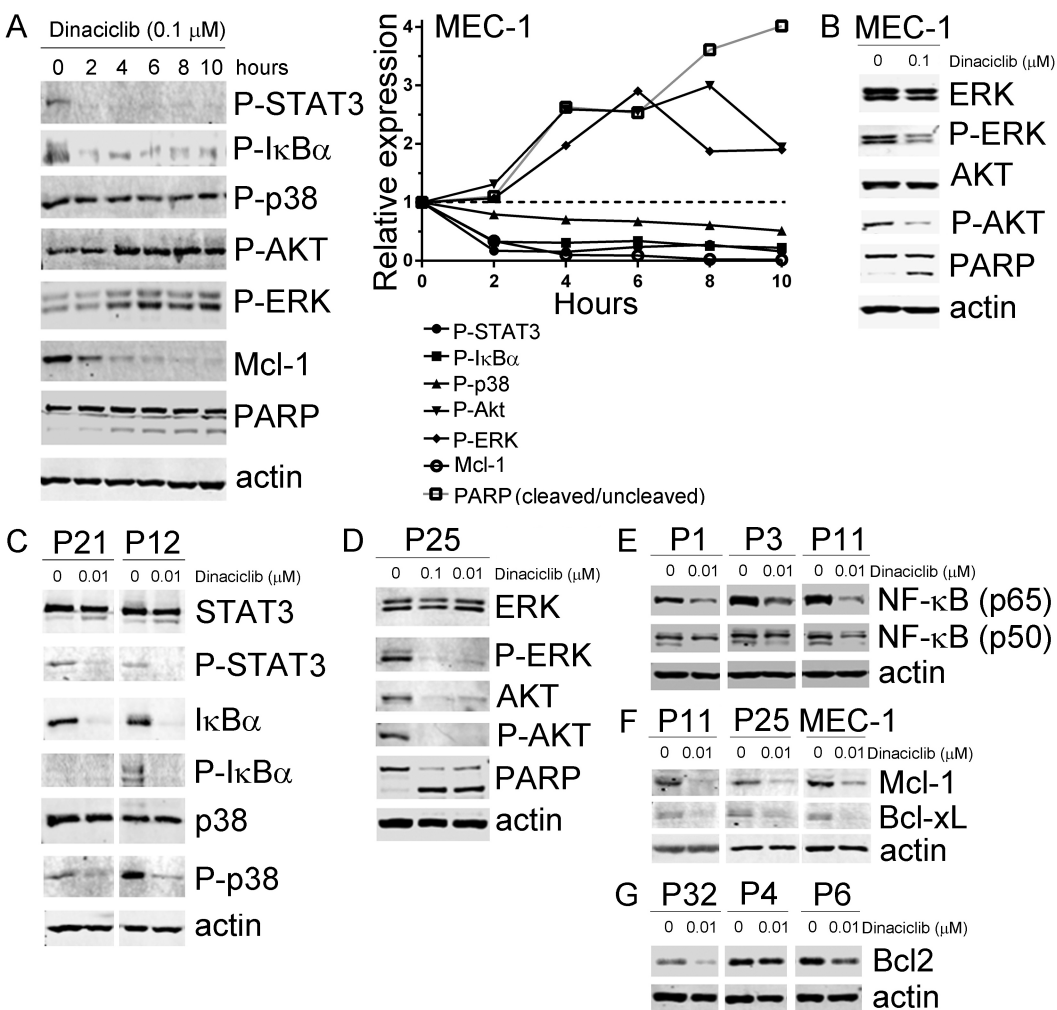


Figure 3



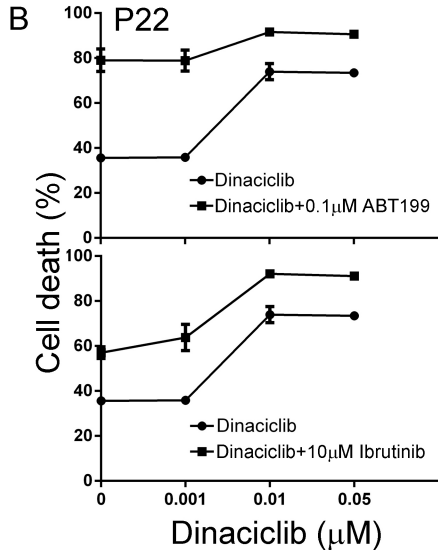
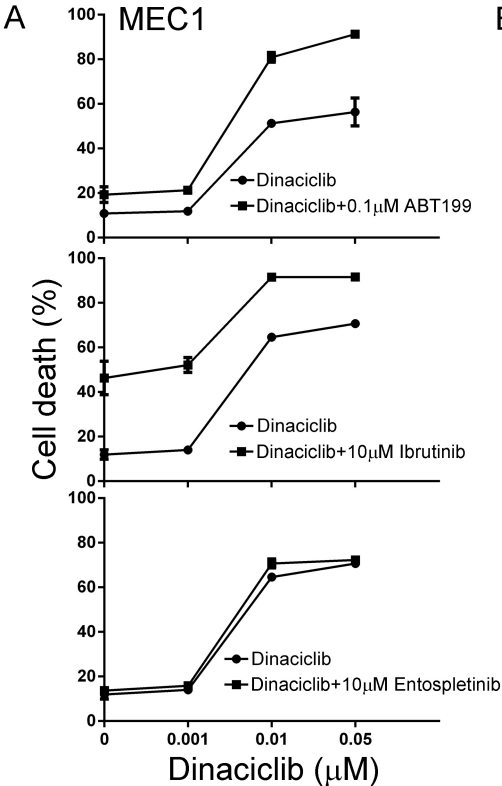


Figure 4