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Novel pyrrolobenzodiazepine benzofused hybrid molecules inhibit nuclear factor- κ B activity and synergize with bortezomib and ibrutinib in hematologic cancers

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ABSTRACT

Chronic lymphocytic leukemia (CLL) and multiple myeloma are incurable hematologic malignancies that are pathologically linked with aberrant nuclear factor-kappa B (NF- κ B) activation. In this study, we identified a group of novel C8-linked benzofused pyrrolo[2,1-c][1,4]benzodiazepine monomeric hybrids capable of sequence-selective inhibition of NF- κ B with low nanomolar LD₅₀ values in CLL (n=46) and multiple myeloma cell lines (n=5). The lead compound, DC-1-192, significantly inhibited NF- κ B DNA binding after just 4 h of exposure, demonstrating inhibitory effects on both canonical and non-canonical NF- κ B subunits. In primary CLL cells, sensitivity to DC-1-192 was inversely correlated with RelA subunit expression ($r^2=0.2$) and samples with BIRC3 or NOTCH1 mutations showed increased sensitivity ($P=0.001$). RNA-sequencing and gene set enrichment analysis confirmed the over-representation of NF- κ B regulated genes in the downregulated gene list. Furthermore, *in vivo* efficacy studies in NOD/SCID mice, using a systemic RPMI 8226 human multiple myeloma xenograft model, showed that DC-1-192 significantly prolonged survival ($P=0.017$). In addition, DC1-192 showed synergy with bortezomib and ibrutinib; synergy with ibrutinib was enhanced when CLL cells were co-cultured on CD40L-expressing fibroblasts in order to mimic the cytoprotective lymph node microenvironment ($P=0.01$). Given that NF- κ B plays a role in both bortezomib and ibrutinib resistance mechanisms, these data provide a strong rationale for the use of DC-1-192 in the treatment of NF- κ B-driven cancers, particularly in the context of relapsed/refractory disease.

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Introduction

Nuclear factor kappa B (NF- κ B) denotes a family of homo- and heterodimeric transcription factors composed of five subunits: p65 (RelA), p50, RelB, p52 and c-Rel.¹ These subunits exert their effects via the canonical or non-canonical signaling pathways.² NF- κ B is maintained in an inactive state in the cytoplasm but following I κ B kinase (IKK) activation NF- κ B is shuttled into the nucleus where it exerts its transcriptional effects.³ NF- κ B regulates the transcription of genes that are essential for cell survival, proliferation, inflammation and invasion/metastasis. These processes are commonly dysregulated in cancers, including CLL and multiple myeloma, leading to the constitutive aberrant activation of NF- κ B.^{2,4} Indeed, NF- κ B has been shown to play a central role in disease progression and drug resistance in these hematologic cancers.^{5,6} While treatment with currently established therapies, such as the proteasome inhibitor bortezomib or the BTK inhibitor ibrutinib,

are initially effective in a significant proportion of patients,^{7,8} there is evidence to suggest that treatment with both of these agents causes an increase in NF- κ B activation which has been linked to drug resistance and treatment failure.^{9,10} Therefore, direct inhibition of NF- κ B could potentially resensitize tumor cells, thus highlighting this transcription factor as a potential therapeutic target.¹¹⁻¹³

Pyrrolo[2,1-c][1,4]benzodiazepines (PBD) are naturally occurring molecules produced by *Streptomyces* bacteria whose family members include anthramycin (Figure 1) and tomaymycin.^{14,15} PBD are a class of sequence-specific covalent DNA minor groove binding agents that are selective for GC-rich sequences, and have been evaluated as potential chemotherapeutic agents in clinical trials.^{16,17} More recently, members of the PBD family have been developed as cytotoxic payloads for attachment to antibodies to form antibody-drug conjugates, and a number of these are currently undergoing clinical evaluation for the treatment of leukemia and lung cancer.¹⁸

This study identified three lead compounds (DC-1-192, DC-1-92 and DC-1-170) (Figure 1) from a library screen of 87 novel synthetic C8-linked benzofused PBD monomeric hybrids based on their *in vitro* cytotoxicity. The compounds were then further evaluated for their biological properties, including differential toxicity, in malignant and age-matched normal B and T cells. In terms of their mechanism of action, PBD monomers can recognize and bind to specific sequences of DNA and therefore have the potential to act as competitive inhibitors of transcription factors. Previous research has shown that PBD monomers such as GWL-78 preferentially inhibit the transcription factor NF- κ B,¹⁹ while PBD monomers such as the DC-81-indole hybrid²⁰ and KMR-28-39 are potent NF- κ B inhibitors.²¹ The aim of this study was to determine the biological properties of these novel C8-linked benzofused PBD monomers by investigating their cytotoxic profiles in multiple myeloma cell lines, primary CLL cells and age-matched normal B- and T-lymphocytes. We went on to investigate their ability to inhibit NF- κ B and whether they could potentiate the effects of the targeted agents bortezomib and ibrutinib, currently used in the treatment of myeloma and CLL, respectively.

Methods

Detailed methods can be found in the *Online Supplementary Appendix*.

Cell lines, primary chronic lymphocytic leukemia cells and normal lymphocytes

Primary CLL cell lines (n=46) and age-matched normal B and T cells were obtained with informed consent in accordance with the ethical approval granted by South East Wales Research Ethics Committee (02/4806). In addition, five multiple myeloma cell lines, JJN3, U266, OPM2, MM.1S and H929, were obtained from the *Deutsche Sammlung von Mikroorganismen und Zellkulturen*. The provenance of the cell lines was verified by multiplex polymerase chain reaction of minisatellite markers; all were certified mycoplasma-free.

Measurement of *in vitro* apoptosis

Apoptosis was assessed using annexin V and propidium iodide labeling. Samples were analyzed using an Accuri C6 flow cytometer with CFlow software (BD Biosciences).

Enzyme-linked immunosorbent assay for NF- κ B subunits

Nuclear levels of p65, p50, p52 and RelB DNA binding were assessed in JJN3 and U266 cells treated for 4 h with DC-1-92, DC-1-170 (0nM 20 nM) and DC-1-192 (0nM 5 nM).

Synergy with bortezomib and ibrutinib

The synergy between the PBD monomers and bortezomib or ibrutinib was determined in JJN3 cells and primary CLL cells, respectively. Fixed molar ratios were derived from experimentally-determined median lethal dose (LD₅₀) values for each PBD and clinically achievable concentrations of bortezomib and ibrutinib.

RNA Isolation and sequencing

JJN3 cells were treated with 20 nM of either DC-1-170 or DC-1-192 for 4 h. RNA was extracted using an RNeasy mini-kit (Qiagen) in accordance with the manufacturer's instructions. Subsequently, 100-900 ng of high-quality total RNA (RNA integrity number >8) was depleted of ribosomal RNA, and sequencing libraries were prepared using the Illumina TruSeq Stranded Total RNA with Ribo-Zero Gold™ kit (Illumina Inc.).

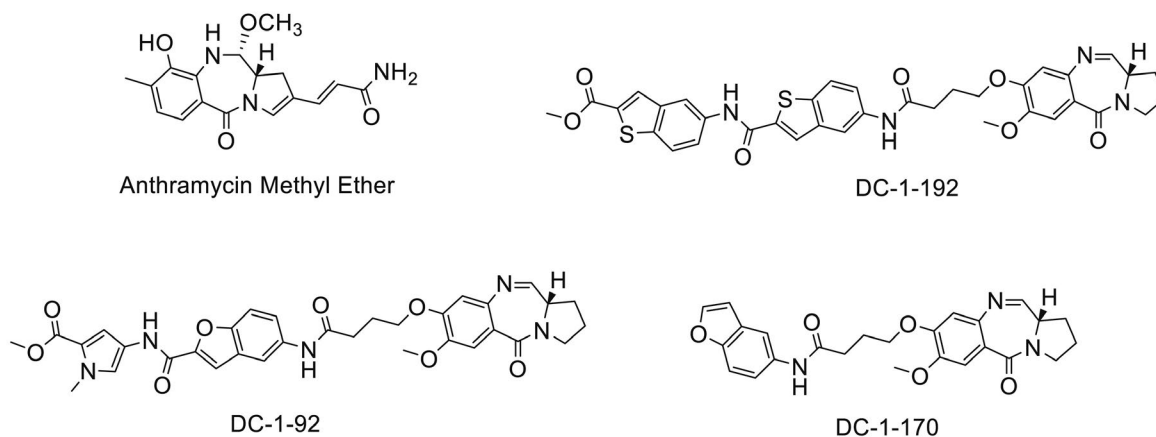


Figure 1. The structures of anthramycin and three structurally-related C8-linked benzofused PBD hybrids. Anthramycin (the first PBD to be isolated from a *Streptomyces* species), and the three synthetic PBD, DC-1-192, DC-1-92 and DC-1-170, identified as lead compounds in this study.

In vivo systemic xenograft model of myeloma in NOD/SCID mice

NOD/SCID mice were sublethally irradiated prior to tail vein inoculation with the human myeloma cell line RPMI8226 (1×10^7 cells) to initiate tumor development. The date of inoculation was denoted as day 0. Intravenous treatment with vehicle only; 0.05% dimethylsulfoxide in saline ($n=7$) or 1 mg/kg of DC-1-192 ($n=7$) was started at day 5. Survival was evaluated from the first day of treatment until death.

Statistical analysis

All statistical analyses were performed using Graphpad Prism 6.0 software (Graphpad Software). The normal distribution of the data was established using the omnibus K2 test. Univariate comparisons were made using the Student *t*-test for paired and unpaired observations. All toxicity data from drug treatment were used to produce sigmoidal dose-response curves from which LD_{50} values were calculated. Toxicity data from synergy experiments were processed using CalcuSyn software with the median effect method to subsequently calculate the combination index (CI) for each pair of agents; CI values less <1 were indicative of synergy.²²

Results

Cytotoxic screening of pyrrolo[2,1-c][1,4]benzodiazepine compounds identified three lead compounds

Initial cytotoxicity screening (trypan blue exclusion assay) of a library of 87 novel synthetic C8-linked benzofused PBD monomeric hybrids was carried out using the multiple myeloma cell line, JJN3. Three lead compounds were selected for further investigation based on their cytotoxic effects at nanomolar concentrations. The chemical structures of all three compounds, together with that of anthramycin on which they are based, are shown in Figure 1.

In vitro and in vivo cytotoxicity of the lead pyrrolo[2,1-c][1,4]benzodiazepine compounds in multiple myeloma cell lines

The relative cytotoxicity of the three lead compounds was then assessed in five different multiple myeloma cell lines, JJN3, U266, OPM2, MM.1S and H929 using an annexin V/propidium iodide apoptosis assay. The cells were cultured for 48 h in increasing concentrations (1 nM–100 nM) of DC-1-92, DC-1-170 and DC-1-192 and were compared with untreated controls. Each compound showed a dose-dependent increase in apoptosis; a representative example of the data generated is shown in Figure 2A. The dose-response curves for each compound were compared in each cell line using overlaid sigmoidal plots (Figure 2B) and the mean LD_{50} values were then calculated for each treatment and plotted on the bar chart shown in Figure 2C. Although each cell line showed differential sensitivity to the three compounds, in every case DC-1-192 was the most cytotoxic PBD with DC-1-170 showing the least cytotoxicity (Figure 2D). The LD_{50} values for DC-1-192 were compared with the published NF- κ B index value for each cell line.²³ The NF- κ B index is the average of the log₂ values for ten NF- κ B-regulated genes (excluding *BIRC3/dIAP2*); the higher the index value, the more NF- κ B-dependent the cell line is deemed to be. With the exception of JJN3 cells, sensitivity to DC-1-192 appeared to be inversely associated with the NF- κ B index, a concept we went on to explore in subsequent

experiments. In order to investigate the anti-tumor effects of DC-1-192 *in vivo*, we employed a systemic model of multiple myeloma in which NOD/SCID mice (2 groups of 7 mice) were inoculated with the human RPMI 8226 myeloma cell line (1×10^7 cells). Treatment was initiated 5 days after inoculation with either DC-1-192 (1 mg/kg) or vehicle control. DC-1-192 was administered once per day (5 days/week) for 3 weeks by intravenous injection and animals were monitored daily for morbidity and mortality. DC-1-192 significantly prolonged the survival of the mice; the median survival of the DC-1-192-treated mice was 68 days *versus* 56 days in untreated mice (hazard ratio [HR]=2.98; $P=0.017$) (Figure 2E).

Comparative cytotoxicity in primary chronic lymphocytic leukemia and normal B- and T-lymphocytes

Primary CLL cells and age-matched normal B- and T-lymphocytes obtained from healthy donors were treated with increasing concentrations of DC-1-92, DC-1-170 and DC-1-192. Apoptosis was measured using CD19/CD3/annexin V labeling to determine the percentage of apoptosis induced by the PBD compounds in CD19⁺ B cells and CD3⁺ T cells, as shown in *Online Supplementary Figure S1A*. *Online Supplementary Figure S1B* shows the comparative dose-responses for each of the cell types indicating that normal lymphocytes were less susceptible to the effects of the PBD. As was the case with the three multiple myeloma cell lines, DC-1-192 was the most potent cytotoxic agent in primary CLL cells. *Online Supplementary Figure S1C, D* shows that CLL cells were significantly more sensitive to the effects of the PBD than were age-matched normal B- and T-lymphocytes.

DC-1-192 shows preferential cytotoxicity in chronic lymphocytic leukemia cells carrying a NOTCH1 or BIRC3 mutation

All of the CLL samples treated with DC-1-192 ($n=46$) showed nanomolar LD_{50} values with a mean LD_{50} value for the entire CLL cohort of 3.8 nM (Figure 3A). We next examined whether sensitivity to DC-1-192 was associated with any of the known prognostic markers. There was no significant difference in mean LD_{50} value between *IGHV*-mutated and *IGHV*-unmutated samples (Figure 3B); CD38-positive and CD38-negative samples (\geq / $<20\%$) (Figure 3C) and samples with higher or lower β_2 -microglobulin concentrations (\geq / <3.5 mg/L) (Figure 3D). However, samples derived from patients with a BIRC3 ($n=3$) or NOTCH1 ($n=11$) mutation were significantly more sensitive to the effects of DC-1-192 (Figure 3E) suggesting that elevated NF- κ B signaling may be a determinant of sensitivity.^{24,25} In keeping with this concept, the nuclear expression of the NF- κ B subunit p65 (RelA) was inversely correlated with DC-1-192 LD_{50} values (Figure 3F).

Nuclear localization of NF- κ B subunits following treatment with pyrrolo[2,1-c][1,4]benzodiazepines

We have previously shown that PBD monomers, such as KMR-28-39, have NF- κ B inhibitory effects.²¹ We, therefore, determined the NF- κ B inhibitory properties of this new series of compounds in two myeloma cell lines JJN3 and U266. JJN3 cells overexpress both the canonical and non-canonical NF- κ B subunits and possess an *EFTUD2-NIK* fusion gene which lacks the TRAF3 binding domain resulting in the accumulation of a cytoplasmic EFTUD2-

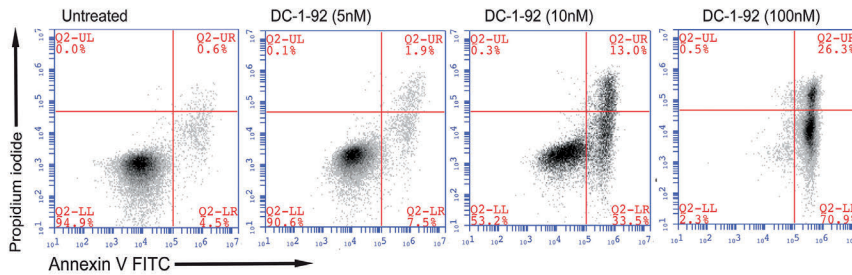
NIK fusion protein. U266 cells exhibit a TRAF3 mutation causing stabilization of wild-type NIK protein.^{23,24} Both cell lines were treated for 4 h with up to 20 nM of each agent and the relative change in nuclear p65 (RelA), p50, p52 and RelB DNA binding was determined as a function of the untreated control. Levels of c-Rel were not evaluated in this study as JJN3 cells show very low levels of this subunit relative to the dominant canonical subunits p65 and p50. In JJN3 cells, all the PBD showed significant inhi-

biton of p65, p50 and Rel B but no significant reduction in p52 (Figure 4A). In contrast, U266 cells showed a significant reduction in the nuclear DNA binding of all four subunits (Figure 4B).

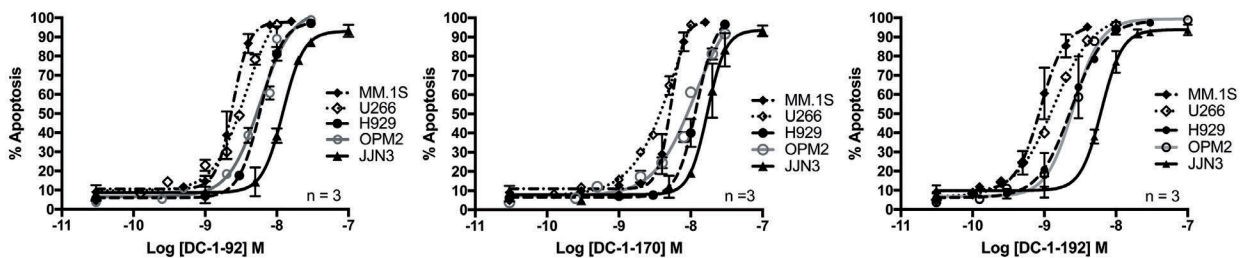
Transcriptional effects of DC-1-170 and DC-1-192 on JJN3 cells

As predicted, RNA-sequencing analysis of DC-1-170 and DC-1-192 revealed a dominant inhibitory effect on

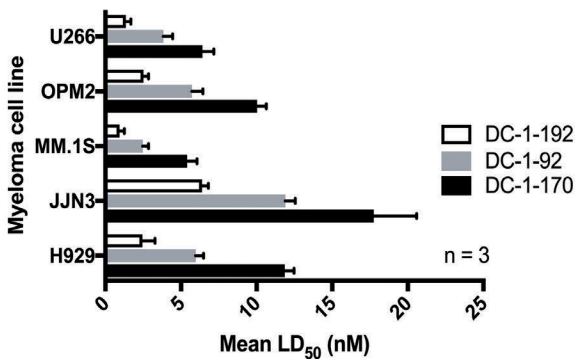
A



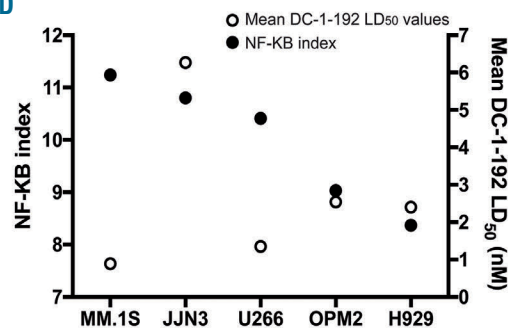
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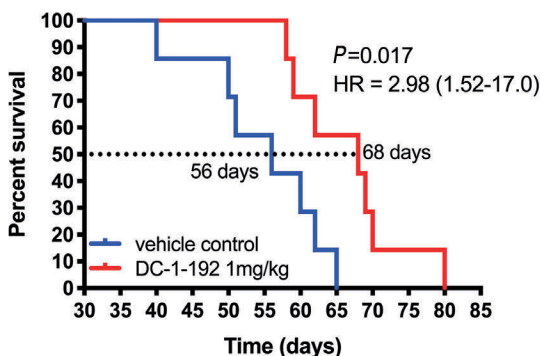


Figure 2. PBD induce apoptosis in multiple myeloma cell lines in a dose-dependent manner. (A) An example of annexin V and propidium iodide (PI) bivariate plots obtained from JJN3 cells treated with increasing concentrations of DC-1-92. A dose-dependent increase in the proportion of annexin V⁺/PI⁺ cells was observed. (B) Sigmoidal dose-response curves illustrating the comparative effects of each compound in U266, OPM2, H929, JJN3 and MM1.S multiple myeloma cell lines. (C) Comparative analysis of the three lead PBD in the five multiple myeloma cell lines revealed significant differential sensitivity to each compound and between each cell line but DC-1-192 was the most potent PBD in all five cell lines. (D) The relationship between the NF- κ B index of each of the cell lines and their respective mean DC-1-192 LD₅₀ value. (E) In order to investigate the *in vivo* antitumor effects of DC-1-192, NOD/SCID mice were systemically inoculated with the human RPMI 8226 myeloma cell line. DC-1-192 (1 mg/kg) significantly prolonged the survival of the mice when compared to that of untreated control mice. All *in vitro* experiments were performed in triplicate and data are presented as mean \pm standard deviation. The *in vivo* experiment was performed in treated and untreated mice (n=7 for each group).

gene transcription with a smaller subset of genes showing increased transcription following exposure to the drug. In unsupervised hierarchical clustering, the samples clustered according to treatment condition (Figure 5A). Strikingly, 4,040/5,077 (80%) of the genes altered by exposure to the drugs were common to both PBD compounds (Figure 5B) suggesting that their structural similarity resulted in the inhibition of a conserved set of genes. Furthermore, gene set enrichment analysis, using WebGestalt (WEB-based Gene Set Analysis Toolkit),²⁵ confirmed that NF- κ B-regulated genes were significantly over-represented in the downregulated gene list, with a normalized enrichment score of -1.7750 (Figure 5C, D). These data suggest that

inhibition of NF- κ B target genes may contribute to the cytotoxicity of the PBD compounds.

Synergy between DC-1-192 in combination with bortezomib or ibrutinib

Overexpression of NF- κ B is associated with chemotherapeutic drug resistance in both CLL and multiple myeloma.^{26,27} Having established that DC-1-192 inhibited nuclear NF- κ B DNA binding and downregulated NF- κ B target genes, we set out to determine whether these inhibitory properties could enhance the killing effect of bortezomib and ibrutinib in the JN3 myeloma cell line and primary CLL cells, respectively. To investigate syner-

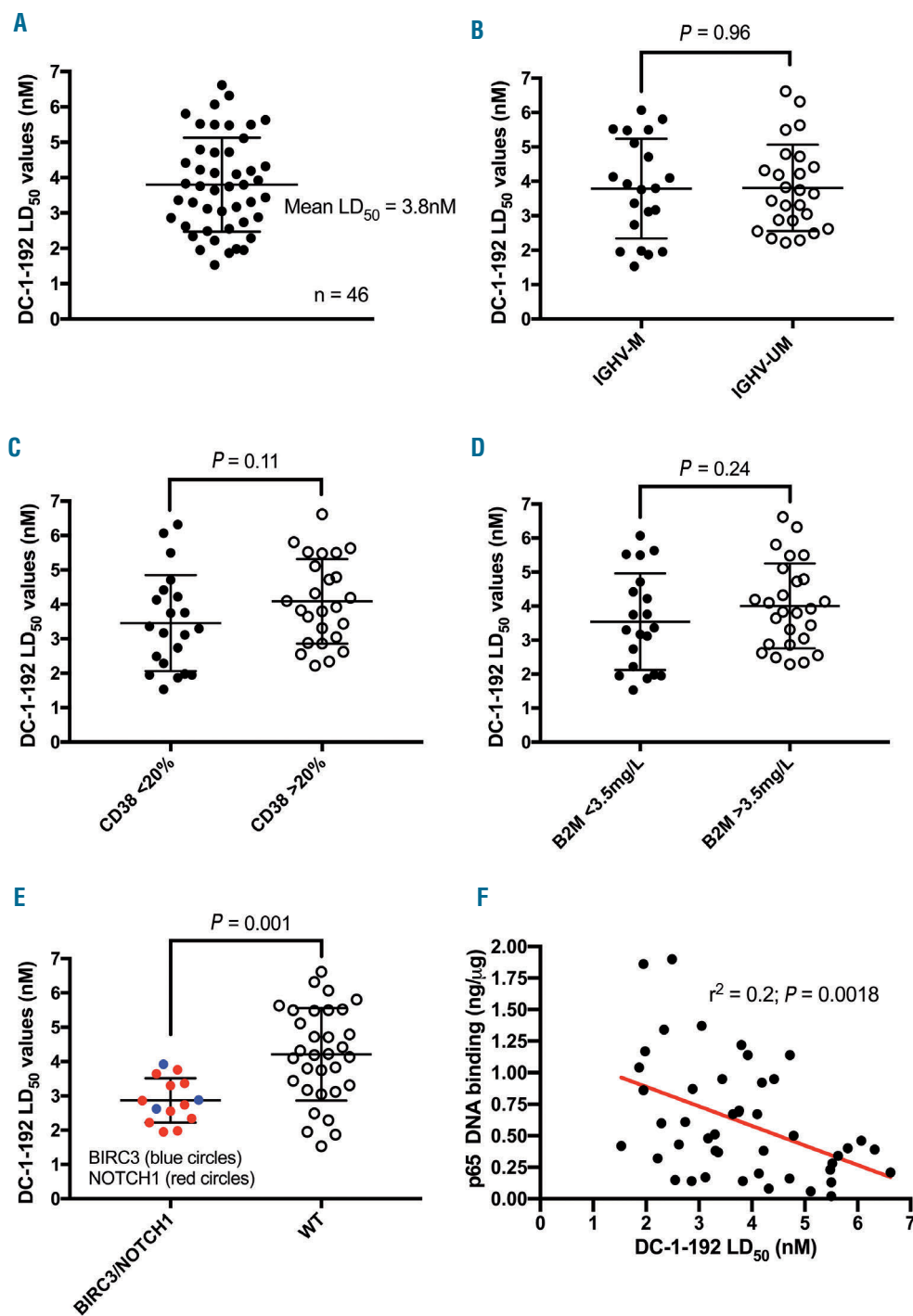


Figure 3. DC-1-192 was highly cytotoxic in primary chronic lymphocytic leukemia cells and showed preferential effects in BIRC3 and NOTCH1 mutated samples. (A) All 46 samples tested showed low nanomolar LD₅₀ values when treated with DC-1-192. (B-D) Analysis of prognostic subsets revealed that DC-1-192 was equipotent in IGHV mutated and unmutated samples (B), CD38-positive and CD38-negative samples (C) and samples with high or low concentrations of β -microglobulin (D). (E) In contrast, BIRC3 and NOTCH1 mutated samples showed significantly increased sensitivity to DC-1-192. (F) There was an inverse relationship between nuclear DNA binding of the canonical NF- κ B subunit, p65, and DC-1-192 LD₅₀ values.

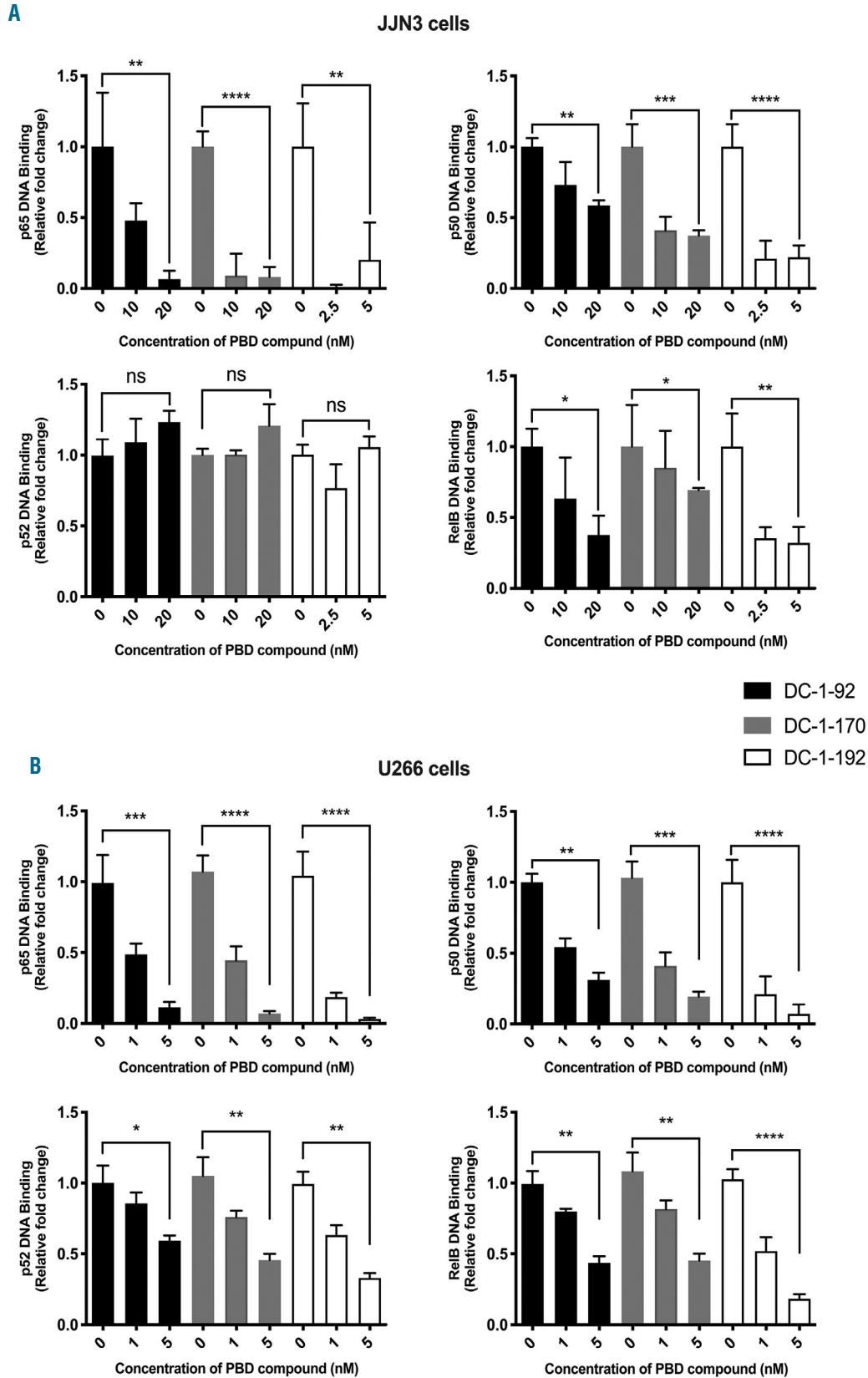


Figure 4. PBD show marked inhibitory effects on both canonical and non-canonical NF- κ B subunits. JJN3 and U266 cells were treated with DC-1-92, DC-1-170 and DC-1-192 for 4 h. Nuclear extracts were then generated from these samples and the amounts of p65, p50, p52 and Rel B were quantified and expressed as relative fold-change as a function of the untreated controls. (A) JJN3 cells showed significant reductions in nuclear expression of p65, p50 and RelB NF- κ B subunits but no change in p52 following exposure to DC-1-92, DC-1-170 and DC-1-192. (B) In contrast, U266 cells showed significant reductions in nuclear expression of all four NF- κ B subunits. All experiments were performed in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. ns: not statistically significant differences.

gy, JN3 and primary CLL cells (n=5) were treated with increasing concentrations of DC-1-192 both alone and in combination with bortezomib in JN3 cells and ibrutinib in CLL samples. The fixed molar ratios employed in the combination studies were determined experimentally

using the LD₅₀ values calculated from the previous toxicity data. The fraction affected plots and isobologram plots for the drugs and drug combinations in JN3 cells (Figure 6A), and in primary CLL cells (Figure 6B) show that the cytotoxic effects of DC-1-192 are potentiated by the

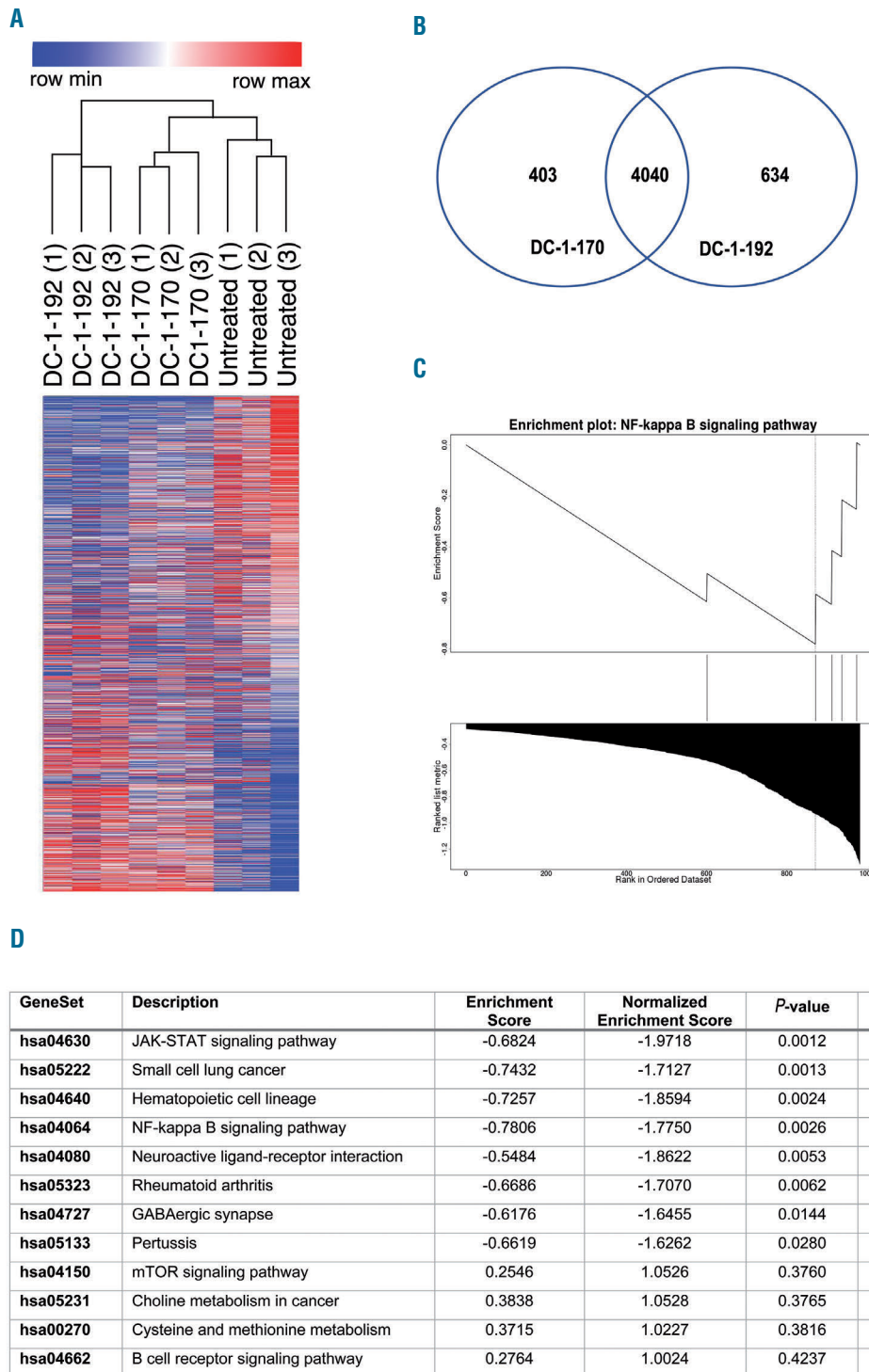


Figure 5. RNA sequencing and gene set enrichment analysis revealed that DC-1-170 and DC-1-192 preferentially inhibited NF-κB target genes. (A) Unsupervised hierarchical clustering revealed a strong drug-associated transcriptional signature for both DC-1-170 and DC-1-192. (B) The majority 4,418/5,077 (87%) of the significantly altered transcripts were downregulated in response to drug. Strikingly, 4,040/5,077 (80%) of the changes were common to both DC-1-170 and DC-1-192. (C) Gene set enrichment analysis showed over-representation of NF-κB target genes in the gene list commonly downregulated by exposure to DC-1-170 and DC-1-192. (D) The top 12 over-represented pathways in the commonly downregulated gene list following exposure to DC-1-170 and DC-1-192 are shown. The table also shows the normalized enrichment scores, P values and false discovery rates (FDR) for each canonical gene set.

addition of bortezomib and ibrutinib, respectively. Furthermore, the combination of DC-1-192 with bortezomib and ibrutinib showed synergy (CI values <1) at the level of LD₅₀, LD₇₅ and LD₉₀ with an incremental increase in synergistic effect from LD₅₀ to LD₉₀ (Figure 6C). Furthermore, DC-1-192 showed increased synergy with ibrutinib when primary CLL cells were co-cultured on CD40L-expressing fibroblasts (Figure 6D) suggesting that these agents may be particularly effective in the treatment of tissue-resident CLL cells.

Discussion

NF- κ B is a master regulator of a number of cellular processes that contribute to cancer progression, including cell survival and proliferation. Furthermore, it is often implicated in drug resistance, highlighting its potential as a therapeutic target.^{12,13} The interest in small molecular DNA-binding agents, such as the PBD monomers, has increased in recent years due to their ability to selectively bind to specific sequences within the minor groove of DNA, a characteristic that separates them from traditional

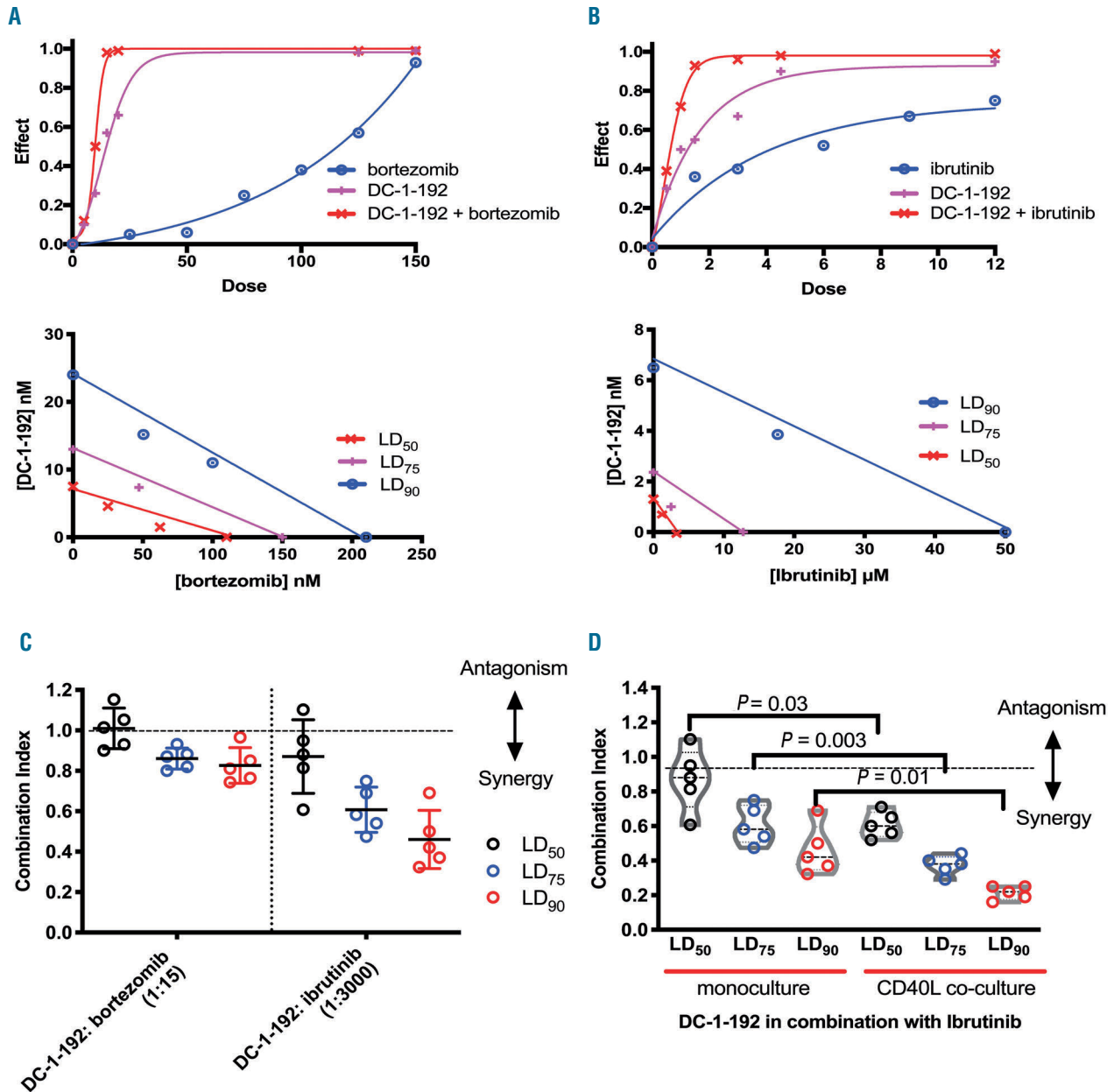


Figure 6. DC-1-192 demonstrates cytotoxic synergy with bortezomib and ibrutinib. Synergy between DC-1-192 and bortezomib was experimentally determined in JN3 cells and between DC-1-192 and ibrutinib in primary chronic lymphocytic leukemia (CLL) cells. The fixed molar ratios for each combination were derived from the mean LD₅₀ values for DC-1-192 and the clinically achievable doses of bortezomib and ibrutinib. Apoptosis was determined using the annexin V/propidium iodide assay. (A) The fraction affected plot and the isobologram plot for DC-1-192, bortezomib and their respective combination (1:15) in JN3 cells. (B) The fraction affected plot and isobologram plot for DC-1-192, ibrutinib and their combination (1:3000) in primary cells. (C) The combination indices for the combination DC-1-192 with bortezomib and DC-1-192 with ibrutinib at the level of LD₅₀, LD₇₅ and LD₉₀ in primary CLL cells (n=5). (D) Comparison of the combination indices generated by the combination of DC-1-192 and ibrutinib in monoculture and CD40L-expressing co-culture. All JN3 cell line experiments were performed in triplicate. All of the primary CLL experiments were performed on samples derived from five individual patients with data presented as the mean of duplicate experiments.

alkylating agents. This raises the possibility that they can selectively inhibit transcription factors,¹⁶ so this study set out to determine the *in vitro* and *in vivo* biological effects of a series of novel C8-linked PBD-benzofused hybrids.

Initially library screening identified three lead compounds. All three PBD showed high potency in five different multiple myeloma cell lines with LD₅₀ values in the low nanomolar range. Subsequently, the PBD showed similar high potency in a cohort of 46 primary CLL samples and significantly lower toxicity in normal age-matched B- and T-lymphocytes. The most cytotoxic PBD, DC-1-192, showed 2.4-fold and 4.6-fold differential toxicity in CLL cells suggesting that this compound has a positive therapeutic index. We went on to show that DC-1-192 was well tolerated in a systemic *in vivo* xenograft model of myeloma and significantly prolonged the survival of the mice.

Subset analysis of the CLL cohort data revealed that DC-1-192 was equipotent in poor prognostic groups including *IGHV* unmutated cases ($P=0.96$). Furthermore, samples derived from patients with *BIRC3* or *NOTCH1* mutations showed significantly increased sensitivity to DC-1-192. These mutations are known to cause aberrant activation of NF- κ B signaling and are associated with resistance to chemoimmunotherapy and inferior clinical outcome.²⁸⁻³² Although these mutations are linked with non-canonical NF- κ B activation, here we showed that nuclear expression of the canonical p65 subunit was a predictor of *in vitro* sensitivity to DC-1-192.

Given these findings, we plotted the previously published NF- κ B index for each of the myeloma cell lines²³ against their respective LD₅₀ for DC-1-192. Four of the five cell lines showed an inverse relationship between their NF- κ B index and DC-1-192 LD₅₀ value suggesting that response to DC-1-192 was influenced by how NF- κ B-dependent the cell lines were. JJN3 cells were the exception to this rule; these cells had a high NF- κ B index (10.8) but were relatively resistant, in comparison to the other four cell lines, to the cytotoxic effects of DC-1-192 (mean LD₅₀ = 6 nM). The reasons for this are likely to be multiple and may be unrelated to NF- κ B, but it is worthy of note that JJN3 cells possess a cytoplasmic *EFTUD2-NIK* fusion gene, which may alter p100 processing to p52. Indeed, when we assessed the impact of the PBD on nuclear NF- κ B subunit DNA binding in JJN3 cells, all three compounds showed significant inhibition of the p65 and p50 canonical subunits as well as the non-canonical subunit RelB after 4 h. In contrast, no significant change in p52 was observed following treatment with the PBD. We subsequently repeated the experiments using the U266 cell line, which has a *TRAF3* mutation leading to the cytoplasmic accumulation of NF- κ B inducing kinase (NIK).^{23,24} These cells showed a significant reduction in all four NF- κ B subunits including p52 following short-term treatment with PBD.

The rapid reduction in nuclear NF- κ B subunit expression indicates that NF- κ B inhibition precedes apoptosis in these cells and may contribute to the efficacy of the PBD. Given the DNA binding characteristics of these compounds, it seems possible that they compete for NF- κ B binding motifs, thereby inhibiting the transcription of NF- κ B target genes. The reduction in nuclear NF- κ B subunits observed in this study may have been caused by the shuttling of unbound NF- κ B back to the cytoplasm and/or targeted degradation.^{33,34}

Activation of NF- κ B has also been implicated in the development of resistance to chemotherapeutic drugs in myeloma and CLL.³⁵ Several DNA-damaging agents, including melphalan and fludarabine, have been shown to induce the activity of NF- κ B, thereby contributing to cellular resistance to the cytotoxic effects of these treatments.^{6,36} In myeloma, bortezomib has been shown to re-sensitize malignant cells to the effects of chemotherapy.³⁷ However, the emergence of bortezomib-resistant subclones ultimately leads to relapse in many patients.³⁸ One putative mechanism of bortezomib resistance is the constitutive expression of NF- κ B. Although bortezomib can prevent *de novo* activation of the canonical pathway, it has no significant effect on constitutive NF- κ B activity.²⁷ In this study, we showed that direct competitive inhibition of NF- κ B at the site of transcription led to the re-sensitization of multiple myeloma cells to the effects of bortezomib. This synergistic effect is likely to be multifactorial, but indicates that bortezomib and the PBD have different molecular targets.

Similarly, in CLL Bruton tyrosine kinase (BTK) has been shown to be a critical downstream mediator of B-cell receptor signaling that is often constitutively activated in CLL patients. The targeting of this kinase with the BTK inhibitor, ibrutinib, has shown notable effects in patients with relapsed CLL^{39,40} and this is mediated, at least in part, by the distal inhibition of NF- κ B.⁴¹ However, emerging evidence of resistance mechanisms to ibrutinib strongly implicate NF- κ B.⁴² Here, we show that the combination of DC-1-192 with ibrutinib produced cytotoxic synergy suggesting that the PBD and ibrutinib target NF- κ B through different mechanisms and/or that they have other, non-overlapping, molecular targets. Furthermore, synergy was enhanced when primary CLL cells were co-cultured on CD40L-expressing fibroblasts in order to mimic the lymph node microenvironment. This suggests that PBD may be particularly useful in targeting tissue-resident tumor cells.

In summary, the novel PBD compounds evaluated in this study showed low nanomolar toxicity in both primary CLL cells and myeloma cell lines. In addition, primary CLL cells carrying *BIRC3* or *NOTCH1* mutations were preferentially sensitive to the cytotoxic effects of DC-1-192 suggesting that this agent may be a potential therapeutic option for these poor-risk subsets. Mechanistically, the PBD demonstrated promising dual inhibitory properties on both the canonical and non-canonical NF- κ B pathways, a characteristic that has been previously linked to significant antitumor effects in multiple myeloma.⁴³ Furthermore, the PBD showed *in vitro* synergy with bortezomib and ibrutinib in multiple myeloma and CLL, respectively, providing a strong rationale for the use of these agents in the treatment of relapsed/refractory B-cell neoplasms.

Disclosures

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Contributions

TL performed experiments, analyzed data and drafted the

manuscript; DBC performed experiments, analyzed data and revised the manuscript; KMR and DET conceived and supervised the synthetic chemistry and revised the manuscript; PJG, KA, AGSP and EJW analyzed data and revised the manuscript; CDF provided vital reagents and revised the manuscript; CP conceived and supervised the cell biology experiments, analyzed data and revised the manuscript.

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