Synergistic induction of cell death in haematological malignancies by combined phosphoinositide-3-kinase and BET bromodomain inhibition.

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Running title: Cell death with PI3K and BET inhibition

Key words: MYC, PI3K, BRD4, JQ1, apoptosis

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There has been a marked increase in the development of targeted therapies for the treatment of malignant disease. Responses may be compromised by the presence of pre-existing or treatment-induced resistance mechanisms. The phosphoinositide-3-kinase (PI3K) pathway has been a major focus of drug development due to its function as a key regulator of cell growth and survival. PI3Ks may be dysregulated by mechanisms including mutations of the negative regulator phosphatase and tensin homologue (*PTEN*), of regulatory and catalytic subunits, and of upstream regulators such as tyrosine kinases and the *RAS* family.(Fruman and Rommel 2014) Key targets of PI3K include the serine/threonine kinase AKT and the mechanistic target of rapamycin (MTOR) pathway.

Drugs targeting the PI3K/AKT/MTOR axis include pan-PI3K inhibitors, isoform-selective PI3K inhibitors, rapamycin and analogues, active-site MTOR inhibitors, dual-PI3K-MTOR and AKT inhibitors.(Fruman and Rommel 2014) Early clinical trials with these agents have shown varying activity with incomplete understanding for the lack of response seen in some settings.(Fruman and Rommel 2014, Klempner, *et al* 2013) A large body of work shows that PI3K and v-myc avian myelocytomatosis viral oncogene homolog (MYC) form interlinked but overlapping signalling pathways.(Dang 2012) Both pathways are often dysregulated in acute leukaemias, lymphomas and myeloma. We and others have shown that MYC upregulation can impair the response to PI3K inhibitors and may be a frequent mechanism underlying resistance to this class of drugs.(Klempner, *et al* 2013, Shepherd, *et al* 2013) Until recently, it has been difficult to target MYC using pharmacological agents, but this has changed

with the identification and development of bromodomain and extra terminal domain (BET) inhibitors such as JQ1 and I-BET762.(Dawson*, et al* 2012) BETs, such as bromodomain containing 4 (BRD4), function as chromatin readers to alter transcription - inhibition leads to the loss of MYC expression with activity in several haematological tumours. (Delmore*, et al* 2011, Zuber*, et al* 2011)

Inhibition of BRD4 and PI3K is individually associated with consistent reductions in cancer cell proliferation, but with variable effects on cell death.(Delmore*, et al* 2011, Fruman and Rommel 2014, Zuber*, et al* 2011) We postulated that the combination of PI3K and BRD4 inhibition may show enhanced cytotoxicity. As induction of cell death in *ex vivo* assays, rather than of cytostasis, is a key predictor of clinical activity, (Faber*, et al* 2012)we focussed on this parameter.

We screened a panel of acute myeloid leukaemia (AML) cell lines with the BRD4 inhibitor JQ1, the dual PI3K/MTOR inhibitor BEZ235, or the combination to investigate effects on cell survival. Figure 1A shows enhanced killing with the combination of BEZ235 and JQ1 compared with either agent alone. The combination was effective in cells with differing cytogenetic and molecular abnormalities (*MLL* translocated – ML2, MOLM13, MV4;11; *FLT3* mutated – MOLM13, MV4;11, PL21; *KIT* mutated – HMC1.2; *CBL* mutated – GDM1). Formal combination effect analysis showed synergy in the majority of cell lines. (Figure 1B)

Next, we extended the analysis to cells of different haematopoietic cell origin including T-cell acute lymphoblastic leukaemia (T-ALL), myeloma and Burkitt lymphoma. We found similar effects to those seen in AML, with dual PI3K/MTOR and BRD4 inhibition leading to synergistically increased levels of cell death. (Figure 1B and C) We confirmed our results using an alternative BRD4 inhibitor, I-BET151, and an alternative PI3K/MTOR inhibitor, PI103 (data not shown).

As data from cell lines may not be representative of results in primary tumours, we carried out dose ranging experiments in six separate primary AML samples. Figure 1D shows that the combination of JQ1 and BEZ235 is more potent than the individual agents. This is statistically significant at higher concentrations – e.g. survival is 47±10%, 66±8% and 24±7% of control for JQ1 (2μM), BEZ235 (1μM) and the combination respectively (p=0.01 by one-way ANOVA).

As BEZ235 inhibits both PI3K and MTOR, we wanted to assess which of these components may be responsible for the synergistic effects seen. We compared the effect of BEZ235 with that of a PI3K inhibitor with minimal direct anti-MTOR activity (ZSTK474), and with an active-site MTOR inhibitor with no anti-PI3K activity (WYE354). Figure 1E shows that although each inhibitor has activity in conjunction with JQ1, neither agent can fully replicate the effect of BEZ235, indicating that both anti-PI3K and anti-MTOR activities are required. To assess the role of MYC, we ectopically expressed it in SUP-T1 cells and repeated the cytotoxicity experiment. (Figure 1F) MYC can only partly rescue the combined effects of BEZ235/JQ1, indicating that other pathways regulated by BRD4 are also involved in this process. Recent results indicate that BRD4 can mediate the

expression of a number of oncogenes, regulated by so called super enhancers.(Loven, *et al* 2013)

To analyse the potential pathways involved in mediating cytotoxicity, we carried out Western blotting for relevant downstream targets. In the majority of the cell lines, incubation with JQ1 eliminated the expression of MYC. As expected, BEZ235 inhibited phosphorylation of PI3K and MTOR targets, AKT, eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1) and ribosomal protein S6 (RPS6). (Figure 2) The effects of single or combined inhibition on key apoptosis regulators showed variable effects. Some cell lines, such as RPMI8402 and MM1S, upregulated BCL2-like 11 (apoptosis facilitator) (BCL2L11, also known as BIM), whereas it was unaffected in others (HPBALL, KMS28BM). (Figure 2) Effects on BCL2-like 1 (BCL2L1, also known as BCL-X) were also variable. However, the majority of cell lines showed significant effects on the anti-apoptotic protein myeloid cell leukemia 1 (MCL1). In AML and T-ALL cells, full length MCL1 was downregulated, especially in response to combined JQ1 plus BEZ235. (Figure 2A, B and D) In myeloma cells, consistent upregulation of the short isoform of MCL1, which has pro-apoptotic activity antagonistic to full-length MCL1, was seen.

In conclusion, we have shown that the combination of PI3K/MTOR and BRD4 inhibition can synergise across a range of haematological malignancies to induce high levels of cell death. Both classes of agents are in clinical trials and these results provide a rational basis for testing this combination.

Authorship

AK was the principal investigator and takes primary responsibility for the paper. ST, KM and CS performed laboratory work. AK wrote the letter which was reviewed and revised by all the authors.

Disclosures

All authors report no conflict of interest.

Figure legends

Figure 1

The effect of combined PI3K/MTOR and BRD4 inhibition on malignant haematopoietic cell survival.

A. The indicated AML cell lines were incubated with the PI3K/MTOR inhibitor BEZ235 (1µM) ± varying concentrations of the BRD4 inhibitor JQ1 for 72 hours. The live cell fraction (as a % of untreated control cells) was quantified by flow cytometry with Annexin V/propidium iodide staining (live cells defined as Annexin/PI negative).

B. The indicated cell lines were incubated with varying concentrations of BEZ235 and JQ1 at a fixed dose ratio, assayed for survival after 72 hours as above, and combination indices calculated using Calcusyn software, which utilises the Chou-Talalay method for evaluating synergy. A value of less than 0.9 is indicative of a synergistic response.

C. T-ALL (HPB-ALL, RPMI8402), myeloma (H929, MM1S), and Burkitt lymphoma (Ramos, Daudi) cells were incubated with BEZ235 and JQ1 and the live cell fraction measured after 72 hours as above.

D. Primary AML cells from six separate individuals were incubated with increasing concentrations of JQ1 (range 0.25 to 2µM), BEZ235 (range 0.125 to 1 µM) or both for 72 hours and live cell fraction quantified as above. Cells were cultured in 5% oxygen in serum-free medium supplemented by cytokines as described.(Meja, *et al* 2014)

E. HMC1.2 cells were incubated with the PI3K/MTOR inhibitor BEZ235 (1μM), the PI3K inhibitor ZSTK474(1μM), or the MTOR inhibitor WYE354 (1μM) ± varying concentrations of JQ1 for 72 hours and the live cell fraction measured.

F. SUPT1 T-ALL cells stably expressing MYC, or empty vector control cells, were incubated with BEZ235 ± varying concentrations of JQ1 for 72 hours and the live cell fraction measured.

Figure 2

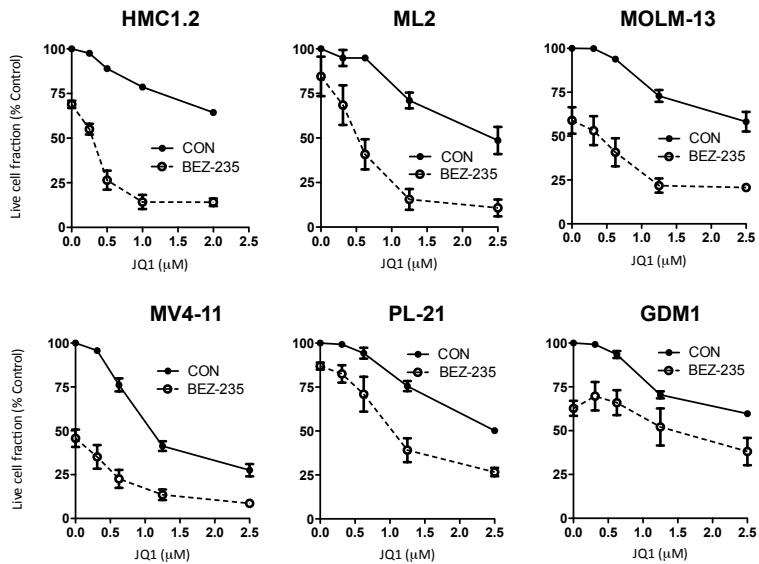**The effect of combined PI3K/mTOR and BRD4 inhibition on cell signalling and survival pathways.**

A. AML cells (HMC1.2), B. T-ALL cells (HPB-ALL, RPMI8402), C. myeloma cells (MM1S, KMS28BM) or D. primary AML cells were incubated with either the PI3K/MTOR inhibitor BEZ235, the BRD4inhibitor JQ1, or both for 16 hours and cell lysates analysed by Western blotting using the indicated antibodies.

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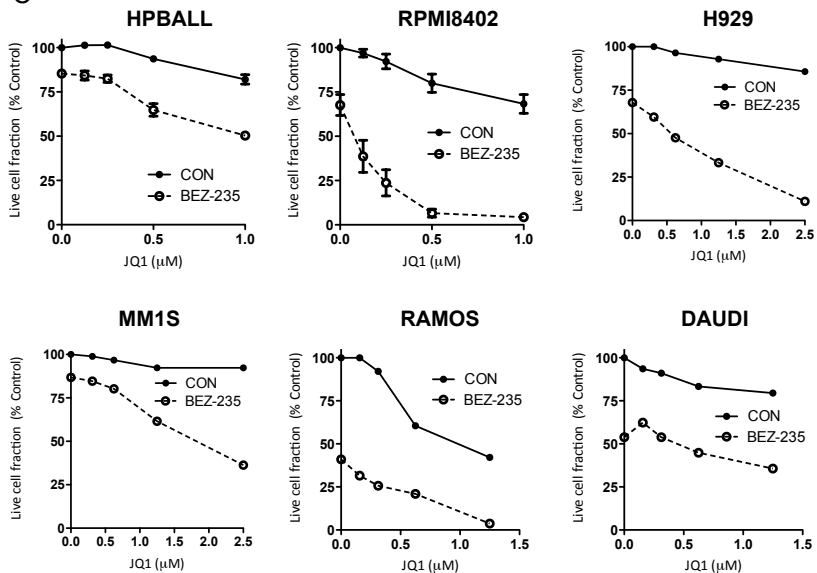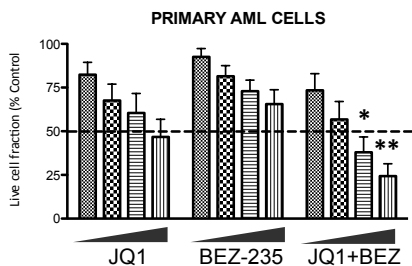
FIGURE 1

FIGURE 2

A

HMC1.2

				PARP1
				MYC
				p-AKT
				p-RPS6
				p-EIF4EBP1
				MCL1
				BCL2L1
				◄EL ◄L BCL2L11
				GAPDH

| - | + | - | + | **JQ1** |
| - | - | + | + | **BEZ-235** |

B

HPB-ALL　　**RPMI8402**

									PARP1
									MYC
									p-AKT
									p-RPS6
									p-EIF4EBP1
									MCL1
									BCL2L1
									◄EL ◄L BCL2L11
									GAPDH

| - | + | - | + | - | + | - | + | **JQ1** |
| - | - | + | + | - | - | + | + | **BEZ-235** |

C

MM1S　　**KMS28BM**

								MYC
								p-RPS6
								p-EIF4EBP1
								◄L MCL1 ◄S
								◄EL BCL2L11 ◄L ◄S
								BCL2L1
								GAPDH

| - | + | - | + | - | + | - | + | **JQ1** |
| - | - | + | + | - | - | + | + | **BEZ-235** |

D

PRIMARY AML

				MYC
				p-RPS6
				p-EIF4EBP1
				MCL1
				GAPDH

| - | + | - | + | **JQ1** |
| - | - | + | + | **BEZ-235** |