Molecular rationale for clinical development of CYC065 in hematology.

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Context: Cyclin-dependent kinase 9 (CDK9) activity is essential for sustained expression of anti-apoptotic protein Mcl-1, oncogenic transcription factor myc and rearranged MLL target genes - Hoxa9 and Meis1. This led us to hypothesise that inhibition of CDK9-driven transcription by CYC065 would lead to rapid loss of these short half-life transcripts, offering a therapeutically potent and selective method to eradicate these CDK9-dependent tumors. CYC065 is a novel CDK inhibitor, which inhibits CDK2 and 9 with IC50 values of 5 and 26 nM, respectively. Following completion of IND-enabling studies, CYC065 has been cleared by FDA for first-in human Phase 1 clinical trials.

Objective: To establish the mechanistic rationale and dosing schedule for clinical development of CYC065 in subsets of leukemias and lymphomas with key molecular features, representing an unmet clinical need.

Design: CYC065 was examined in three cell line panels, derived from AML, ALL and B-cell lymphoma with representation of different Mcl-1 expression levels/dependence, *myc* overexpression/amplification and MLL status. Cell lines were profiled to determine cellular sensitivity parameters that may form the basis of patient selection markers in the clinic. The in vivo activity of CYC065 was demonstrated in two AML xenograft models.

Results: As expected from the target inhibitory profile, CYC065 caused a rapid decrease in phosphorylation of RNA polymerase II and downregulation of key target proteins, Mcl1 and MLL target genes, triggering rapid induction of apoptosis. Short pulse treatments were sufficient to cause ≥ 90% cell death in sensitive cell lines. AML and ALL cell lines with MLL rearrangements were more sensitive to CYC065 than WT cells; the sensitivity of WT AML correlated with the level of Bcl-2 family proteins. Combining CYC065 with Bcl-2 inhibitors or cytarabine was synergistic.

Conclusions: By exploiting the rapid and robust downregulation of key oncogenic transcripts, CYC065 has potential in a variety of leukemia and lymphoma indications with unmet clinical need, including MLL-translocated and FLT3-ITD leukemia, and myc-driven lymphoma. CYC065 has been effectively combined with standard cytotoxic agents such as cytarabine, and agents targeting other key components of the apoptotic pathway, such as the bcl-2/bclXL inhibitors.

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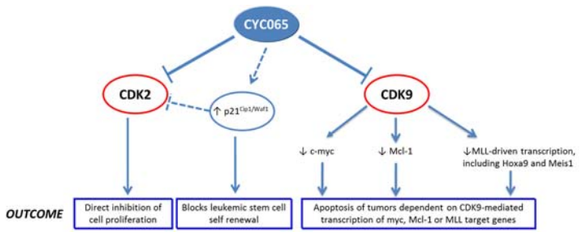
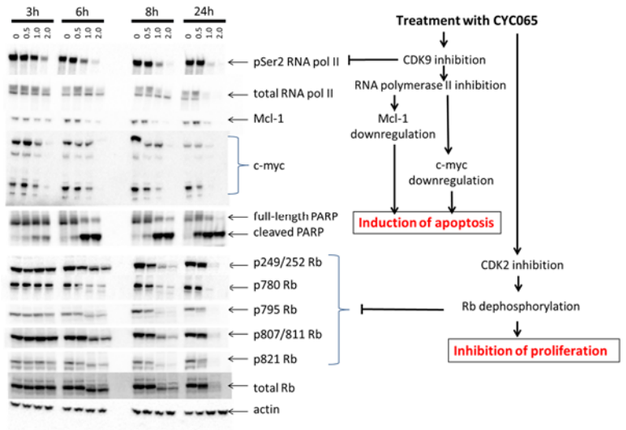
Introduction

- CYC065 cleared by FDA for first-in human Phase 1 clinical trial
- CYC065 is a novel cyclin-dependent kinase (CDK) inhibitor; inhibits CDK2 and 9 (IC_{50} = 5 and 26 nM, respectively); causes rapid induction of apoptotic cell death of cancer cells at submicromolar concentrations
- Good pharmaceutical properties. High solubility and oral bioavailability permits intravenous and oral administration routes
- Antitumor activity in *in vivo* xenograft models of hematological and solid tumors with well tolerated once a day oral dosing
- **Context**: CDK9 activity essential for sustained expression of key pro-survival and proliferative regulators, such as anti-apoptotic protein Mcl-1, oncogenic transcription factor c-myc and rearranged MLL target genes - Hoxa9 and Meis1
- **Hypothesis**: Inhibition of CDK9-driven transcription by CYC065 leads to rapid loss of short half-life transcripts, offering therapeutically selective method to target CDK9-dependent tumors. Other target indications include poor prognosis, drug resistant tumors overexpressing cyclin E[1,2]
- **Objectives**: Establish mechanistic rationale and dosing schedule for clinical development of CYC065 in subsets of leukemias and lymphomas with key molecular features, representing an unmet clinical need

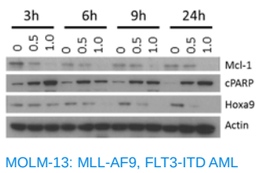
Inhibition of CDK9 by CYC065 targets CDK9-dependent tumors



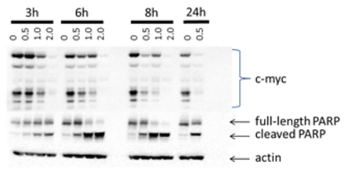
- CYC065 inhibits the complexes of CDK2/cyclin E and CDK2/cyclin A, leading to inhibition of cell proliferation; CYC065 induces p21 in cells with wild-type p53, which directly blocks leukemic stem cell self renewal[3]
- CYC065 inhibition of CDK9 leads to a reduction in phosphorylation of Ser2 of RNA polymerase II and inhibition of transcription. Transcripts and proteins with short half-lives are lost, including c-myc, Mcl-1, and MLL target genes, Hoxa9 and Meis1
- Cells rapidly undergo cell death upon treatment with CYC065



Kasumi-1: AML



MOLM-13: MLL-AF9, FLT3-ITD AML



HL60: c-myc amplified AML

1. Scaltriti et al., PNAS, 2011, 9:3761. 2. Cocco et al., AACR 2015 Abs 3103. 3. Wong et al., 2010, 70(9):3833. 4. Glaser et al., Genes Dev. 2012, 26(2):120. 5. Frame et al., SOHO 2014 Abs 209. 6. Chou et al., Cancer Res. 2010, 70:440.

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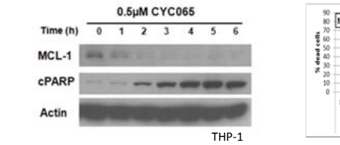
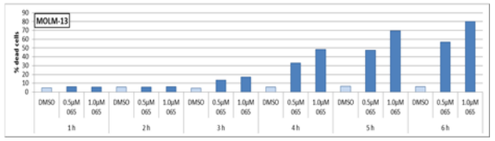
A short pulse of CYC065 is sufficient to achieve maximum cytotoxicity

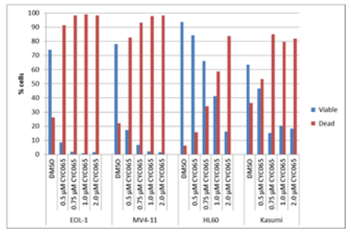


THP-1

- Mcl-1 knockdown precedes the induction of apoptosis



- Cells treated with compound for time indicated, then compound removed; percentage of dead cells measured 24 h after initiation of pulse treatment. In AML-MLL cell line, MOLM-13, proportion of dead cells reaches maximal following a 6h pulse



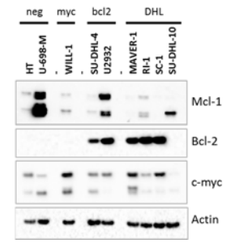
Cell line	EOL-1	MOLM-13	MV4-11	THP-1	HL60	Kasumi-1
6h pulse	0.35	0.26	0.50	1.1	0.79	0.32
72h	0.37	0.24	0.26	0.57	0.44	0.23

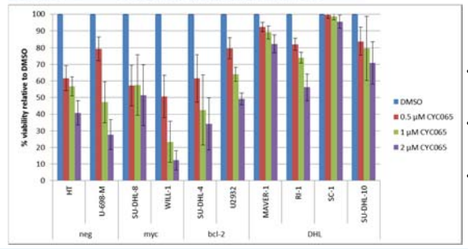
- IC_{50} values determined at 72 h after one 6 h pulse or continuous treatment with CYC065 establish short pulses sufficient to achieve maximal cytotoxicity
- Rapid induction of apoptosis in AML cell lines leading to dose-dependent elimination of the majority of the cell population (% viability analysed at 48 h following 6 h pulse of CYC065), including AML: EOL-1 (MLL-PTD), MV4-11 (MLL-AF4, flt-3-ITD & trisomy chr 8), HL60 (myc amplified), and Kasumi-1

CYC065 efficacy in lymphomas

Cell line name	Bcl2 rearrangement	Myc rearrangement
HT	-	-
U-698-M	-	-
SU-DHL-8	-	t(8;22); IgL-myc
WILL-1	-	t(8;14); IgH-myc
SU-DHL-4	t(14;18); bcl2 MBR-IgH	-
U2932	Extensive genomic amplification of bcl2 region	-
MAVER-1	Amplification of bcl2 region	Non-IgH rearrangement of myc
RI-1	der 18; bcl2 amplification	t(4;8); myc rearrangement
SC-1	t(8;14;18) translocation affecting genomic co-amplification of bcl2 and myc; IgH-bcl2	
SU-DHL-10	Concurrent rearrangements of IgH with myc and bcl-2	

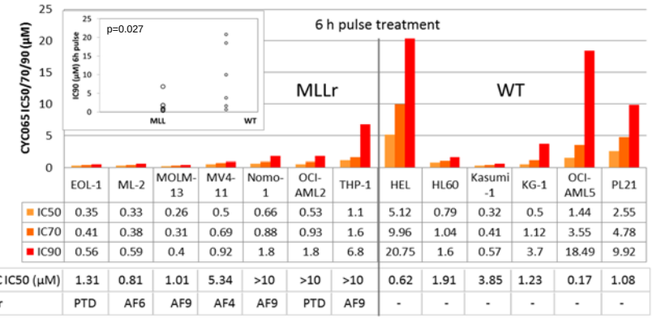
- Panel of B-cell lymphoma cell lines representing a variety of genomic alterations, including poor prognosis double hit lymphoma (DHL)





- Basal levels of bcl-2 protein are significantly upregulated in several cell lines carrying genomic amplification of bcl-2 (50-450 fold higher)
- Cell lines with myc rearrangement show slightly elevated levels of c-myc (up to 4.5 fold higher)
- Dose-dependent reduction in viability (% viability analysed at 48 h following 8 h pulse of CYC065); DHL may benefit from treatment with CYC065 in combination with a bcl2-inhibitor (see below)

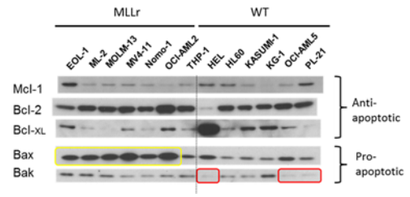
Reliance of AML on Mcl-1 confers sensitivity to CYC065

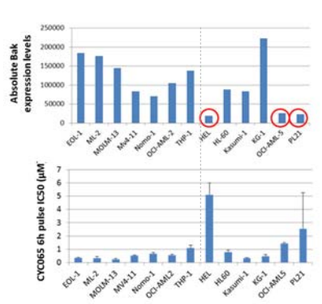


	EOL-1	ML-2	MOLM-13	MV4-11	Nomo-1	OCI-AML2	THP-1	HEL	HL60	Kasumi-1	KG-1	OCI-AML5	PL21
IC50	0.35	0.33	0.26	0.5	0.66	0.53	1.1	5.12	0.79	0.32	0.5	1.44	2.55
IC70	0.41	0.38	0.31	0.69	0.88	0.93	1.6	9.96	1.04	0.41	1.12	3.55	4.78
IC90	0.56	0.59	0.4	0.92	1.8	1.8	6.8	20.75	1.6	0.57	3.7	18.49	9.92
AraC IC50 (μM)	1.31	0.81	1.01	5.34	>10	>10	>10	0.62	1.91	3.85	1.23	0.17	1.08
MLLr	PTD	AF6	AF9	AF4	AF9	PTD	AF9	-	-	-	-	-	-

- AML survival is highly dependent on Mcl-1[4], making these tumors highly sensitive to CYC065 treatment
- AML cell lines with MLLr are especially sensitive to CYC065; similar results were obtained in ALL cell line panel[5]
- Cytosine arabinoside (AraC), widely used in the treatment of acute leukemia, selected as reference compound. Several of the MLLr AML cell lines are resistant to AraC , and all are less sensitive to AraC than CYC065
- Other CDK9-dependent cell lines with genetic features conferring a poor prognosis using existing therapies are also effectively treated with CYC065, including cell lines with Flt3-ITD (MOLM-13, MV4-11) or c-myc amplification (HL60)

Bcl2-family member protein level correlates with sensitivity to CYC065

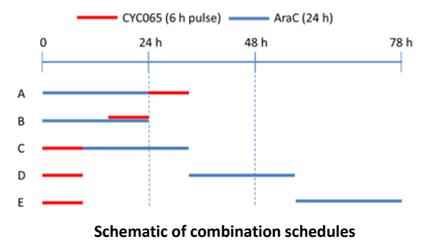




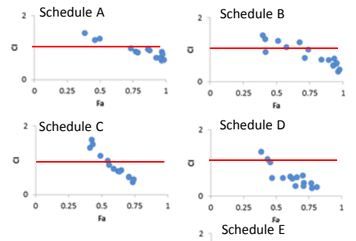
- AML cell lines with MLLr express high levels of Bax, which could enhance their apoptotic response following CYC065-mediated Mcl-1 downregulation
- In WT AML cell lines, less sensitive cells have higher expression of Bcl_{XL} (Pearson correlation coefficient 0.6-0.9) and negligible Bak levels (Pearson correlation coefficient approximately -0.6)

Bcl_{XL} and Mcl-1 have an overlapping function in sequestering Bak; depletion of Mcl-1 releases Bak to induce apoptosis; however overexpression of Bcl_{XL} can compensate for loss of Mcl-1, sequestering Bak, and preventing apoptosis
Cells less sensitive to CYC065 have negligible levels of Bak; loss of Bak expression prevents efficient induction of apoptosis

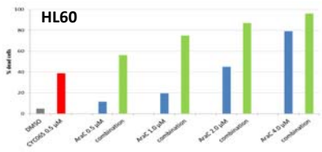
CYC065 can be effectively combined with cytarabine or ABT-199



Schematic of combination schedules



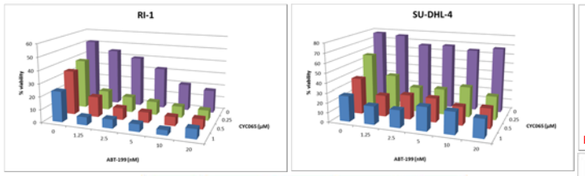
MV4-11: Fa-CI plots

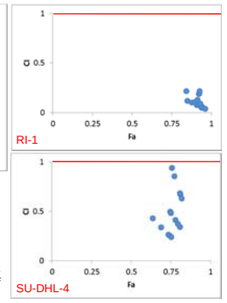


Median CI values

Schedule/ Cell line	A	B	C	D	E
HL60	1.41	1.18	0.86	0.84	0.80
MV4-11	0.86	0.73	0.86	0.55	0.62

- Clinically applicable dosing schedules for cytarabine (AraC) and CYC065 combination explored in HL60 and MV4-11 AML cell lines (schematic above). CYC065 6 h pulse at concentrations between 0.5 and 2 μM; cytarabine between 0.5 and 4 μM for 24 h. Cells analyzed at end of experiment using Viacount (PI exclusion) to measure proportion of viable and dead cells
- CI values for each point within the 5 x 3 dosing matrix for each schedule were calculated using Chou and Talalay[6]. The Fa-CI plots (above) show representative data for MV4-11, a cell line that is resistant to cytarabine as a single agent, with median CI values for the combination matrices for both cell lines highlighted in table.
- Combining CYC065 and cytarabine synergistically induces cell death, especially when CYC065 precedes cytarabine (CYC065 – 24h gap – AraC schedule in HL60 depicted on left)





	Bcl-2		DHL	
Cell line	U2932	SU-DHL-4	RI-1	MAVER-1
Median CI	0.059	0.431	0.102	0.328

- B-cell lymphoma cells treated with 5x3 concentration matrix for up to 24 h; cell viability determined by Viacount; concentrations of CYC065 used were 0.25 μM, 0.5 μM and 1 μM and for ABT-199 were from 1.25 nM to 20 nM (RI-1 & MAVER-1) or 12.5 nM to 200 nM (U2932, SU-DHL-4) in 2-fold increments; CI values calculated using method of Chou & Talalay[6]. CI values <1 indicative of synergy, <0.3 indicates strong synergy
- Significant impact on cell viability following concomitant treatment with CYC065 and ABT-199 in several DLBCL and AML cell lines (not shown)

Conclusions

- **Apoptosis is the dominant mechanism for CYC065 in hematological malignancies via direct inhibition of CDK9, resulting in reduced phosphorylation of RNA Pol II, inhibition of CDK9-dependent transcription and down-regulation of key pro-survival and oncogenic regulators, including Mcl-1, c-myc and Hoxa9, Meis1**
- **An exposure of 6-8 h is sufficient to cause cytotoxicity in sensitive cell lines; preclinical data have shown that such levels and durations of exposure are achievable and well tolerated**
- **CYC065 effectively induces apoptosis and loss of cell viability in a range of CDK9-dependent AML, ALL and DLBCL cell lines with different genetic characteristics correlating with poor prognosis, including MLLr, flt3-ITD, c-myc amplification/overexpression**
- **AML and ALL cell lines with MLL rearrangements are highly sensitive to CYC065; the effect on Meis1 may be of particular importance as this gene is a rate-limiting determinant of MLL leukemia stem cell biology[3]**
- **Bak and Bcl_{XL} levels may be predictive for CYC065 response in AML; further exploration of these potential stratification markers is required**
- **By exploiting the rapid and robust downregulation of key oncogenic transcripts, CYC065 has potential in a variety of leukemias and lymphomas with unmet clinical need, including MLLr leukemia, and myc-driven lymphoma. CYC065 has been combined effectively with standard cytotoxic agents such as cytarabine, and with agents targeting apoptotic regulators, such as bcl-2/bclXL inhibitors**