CYC065, a novel CDK2/9 inhibitor: molecular basis for clinical development in basal-like Triple-Negative Breast Cancer.

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CYC065 is a novel CDK inhibitor, which inhibits CDK2 and 9 with IC_{50} 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.

Triple negative breast cancers (TNBC), particularly the basal subtype, often exhibit aggressive characteristics. Despite good initial responses to chemotherapy, patients experience early relapse and diminished 5 year survival. Molecular features of basal-like TNBC include amplification or overexpression of cyclin E and MYC, suggesting potential utility for a CDK2/9 inhibitor such as CYC065. CYC065 is effective in cyclin E-overexpressing tumors, such as uterine serous carcinoma[1] and trastuzumab-resistant Her2+ breast cancer[2]. Moreover, CDK inhibition has also been reported to be synthetic lethal with overexpressed MYC[3]. This led us to assess the potency and mechanism of action of CYC065 in basal-like TNBC models to evaluate the potential for CYC065 development in this indication.

In vitro cell-based experiments support twice weekly pulse dosing using submicromolar concentrations of CYC065 to achieve maximum impact on cell growth in the majority of breast cancer cell lines tested. Preclinical toxicology data indicate that such levels and durations of exposure are achievable and well tolerated.

As a single agent, CYC065 treatment in breast cancer cells resulted in inhibition of RNA-Pol II phosphorylation, down-regulation of Mcl-1, up-regulation of p53 and rapid induction of apoptosis. The impact of CYC065 on CDK2 targets, cyclin E and MYC was also explored. Interestingly immortalized cell lines obtained from non-malignant tissue displayed similar effects on RNA Pol II, Mcl-1, and p53 but did not undergo apoptosis and consequently exhibited relative resistance to CYC065, indicative of a potential therapeutic window. Cell cycle analysis demonstrated that CYC065 treatment induced an increase in G1 population with no significant induction of cell death in non-malignant derived cell lines, compared to cancer cell lines, in which there was significant induction of cell death.

CDKs have a role in DNA repair which can be exploited to enhance the effectiveness of DNA damaging agents. Seliciclib, an oral, first generation CDK2/9 inhibitor, can be effectively combined with DNA damaging agents, such as the oral nucleoside analogue sapacitabine, or its active metabolite CNDAC. A Phase 1 clinical trial is currently underway to evaluate this combination (NCT00999401). Similarly to seliciclib, we demonstrate that CYC065 is synergistic in combination with CNDAC when given sequentially across multiple breast cancer cell lines. CNDAC-induced double strand breaks persisted for longer when cells were subsequently treated with CYC065, supporting the conclusion that these CDK inhibitors suppress DNA double-strand break repair capacity, which may contribute to the observed synergy.

Taken together the data establish CYC065 as a promising anti-cancer agent in basal-like TNBC, with the potential to be combined effectively in this indication with DNA damaging agents.

1. Cocco, E. *et al.* AACR, 2015, Abstract 3103.
2. Scaltriti, M. *et al.* Proc Natl Acad Sci USA. 201, 108, 3761-6.
3. Horiuchi, D. *et al.* J Exp Med. 2012, 209, 679-96.

CYC065, a novel CDK2/9 inhibitor: molecular basis for clinical development in basal-like triple-negative breast cancer

Abstract # P5-03-10

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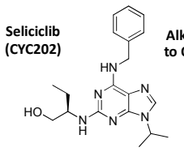
Introduction

- CYC065: 2nd-generation CDK inhibitor (CDKi) in first-in-human phase 1 clinical trial in patients with advanced cancer (NCT02552953)
- CYC065 inhibits CDK2 and 9 (IC_{50} = 5 and 26 nM, respectively)
- Good pharmaceutical properties. High solubility and oral bioavailability; suitable for intravenous and oral administration routes
- Target malignancies include those driven by CDK9-dependent oncogenic and leukemogenic pathways, such as acute leukemias driven by mixed lineage leukemia rearrangements (MLLr)[1] and Myc overexpressing tumors[2]
- CYC065 efficacy demonstrated *in vitro* and *in vivo* in Cyclin E amplified and drug resistant uterine and breast tumor models[3,4]
- Molecular characteristics of basal-like triple negative breast cancer (TNBC) include amplification or overexpression of Cyclin E and Myc suggesting potential utility for a CDK2/9 inhibitor such as CYC065
- Study Aims: Evaluate the potential for CYC065 development in basal-like TNBC, elucidate the mechanism of action as a single agent and in clinically relevant combinations

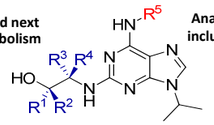
CYC065, a second generation CDK inhibitor

Seliciclib (CYC202)

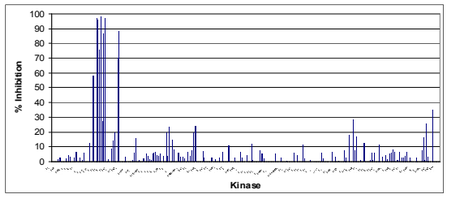


Alkyl substituents introduced next to OH group to modify metabolism

Analogue series including CYC065



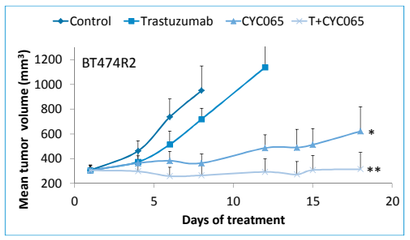
- Tri-substituted purine seliciclib (CYC202, R-Roscovitine) inhibits CDKs 2, 7 and 9 via the ATP binding site
- Seliciclib inhibits proliferation and transcription, and induces apoptosis
- CYC065 was synthesised as part of a seliciclib analogue series to improve potency and alter metabolism



Enzyme	IC_{50} (nM)
CDK2	5
CDK5	21
CDK9	26
CDK3	29
CDK7	193
CDK4	232
CLK2	252
CLK1	549
CDK1	578

- 1 µM CYC065 was tested in 256-kinase panel (Carna Biosciences) at ATP [Km]. Nine kinases (all CDK and CDK-like) were inhibited >50% by CYC065 at this concentration. CYC065 IC_{50} values were determined for these nine kinases

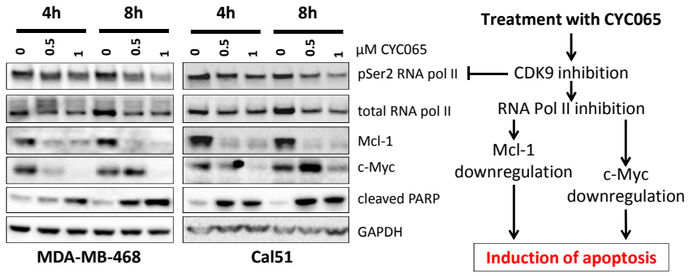
CYC065 reverses trastuzumab resistance in cyclin E amplified *in vivo* models



- BT474R is a trastuzumab-resistant derivative of HER2 amplified breast cancer cell line BT474[4]
- BT474R trastuzumab resistance is caused by *CCNE1* amplification leading to CDK2 activation[4]
- CYC065 alone or in combination with trastuzumab is highly effective against BT474R proliferation *in vitro* and *in vivo*[4]
- CYC065 induces apoptosis and reverses trastuzumab resistance in BT474R[4]

BT474R2 mouse xenograft model treated with: trastuzumab (10 mpk ip biw), CYC065 (22.5 mpk po qd) or combination. *P = 0.0019 vs. trastuzumab; **P = 0.00085 vs. trastuzumab d12.

CYC065 can target Mcl-1 or c-Myc dependent tumors



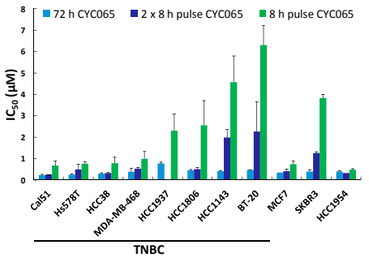
- CYC065 inhibits CDK9, resulting in reduced RNA Polymerase II CTD Ser2 phosphorylation. Short half-life RNA Polymerase II-dependent transcripts and proteins such as Mcl-1 and c-Myc are diminished and consequently cells undergo apoptosis

SABCS, 8-12 December 2015, San Antonio, TX.

CYC065 is an effective inhibitor of TNBC cell line proliferation

Cell Line	Classification	72h IC_{50} (µM)	2 x 8h pulse IC_{50} (µM)	8h pulse IC_{50} (µM)
Cal51	TNBC	0.23	0.25	0.68
Hs578T	TNBC	0.25	0.50	0.76
HCC38	TNBC	0.30	0.33	0.78
MDA-MB-468	TNBC	0.38	0.53	0.99
HCC1937	TNBC	0.77	ND	2.30
HCC1806	TNBC	0.46	0.51	2.56
HCC1143	TNBC	0.41	1.99	4.57
BT-20	TNBC	0.47	2.27	6.31
MCF7	ER positive	0.35	0.41	0.73
SKBR3	HER2 amplified	0.40	1.26	3.83
HCC1954	HER2 amplified	0.40	0.33	0.48

CYC065 IC_{50} values determined by resazurin proliferation assays



- As 72 h continuous exposure of CYC065 shows broad anti-proliferative activity, short pulse exposure was used to identify highly sensitive cell lines or genetic backgrounds
- An 8 h pulse of submicromolar CYC065 potently inhibits proliferation of the majority of the breast cancer cell panel
- A repeat 8 h pulse of CYC065 (2 day interval) results in significantly enhanced inhibition of proliferation in all breast cancer cell lines tested, producing IC_{50} values in the sensitive cell lines equivalent to those for 72 h exposure

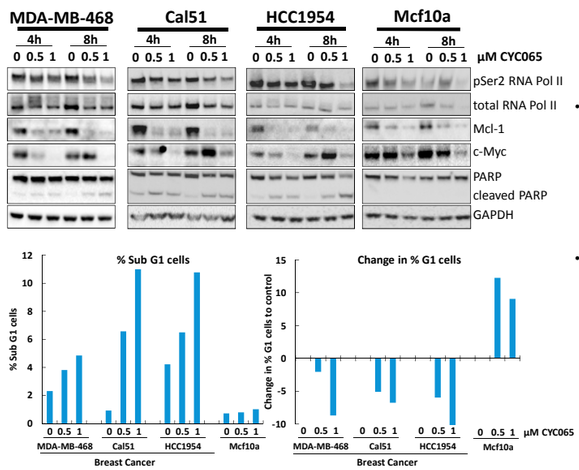
Mcl-1 dependency in breast cancer may predict sensitivity to CYC065

- Levels of Bcl2 family members are predictive of CYC065 sensitivity in AML[5]
- Mcl-1 and Bcl-xL levels are predictive of apoptosis induced by the CDK inhibitor dinaciclib in solid tumor cell lines[6]
- There is a positive correlation between published data on Mcl-1 dependency, determined by viability after Mcl-1 knockdown[7], and CYC065 8 h pulse IC_{50} values in the breast cancer cell lines tested (Pearson correlation coefficient 0.98)

Cell Line	% viability after Mcl-1 siRNA[7]	8 h CYC065 IC_{50} (µM)
Mcf10a	85	3.89
MDA-MB-231	30	2.13
MDA-MB-468	20	0.99
MCF7	10	0.73
HCC1954	5	0.48

Differential cell line response indicates CYC065 selectivity for cancer cells

Cell Line	Classification	CYC065 8h pulse IC_{50} (µM)
Mcf10a	Non-malignant	3.89
184A1	Non-malignant	3.87
Breast cancer cell panel median		0.99



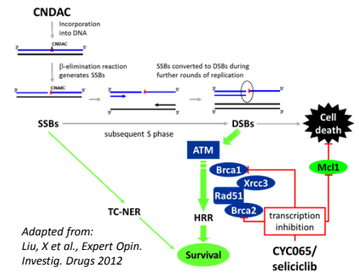
- Non-cancer breast cell lines exhibit relative resistance to pulse treatments of CYC065
- CYC065 treatment results in reduced levels of phosphorylated RNA Polymerase II, Mcl-1 and c-Myc in both cancer and non-cancer breast cell lines
- CYC065-dependent apoptotic induction, indicated by PARP cleavage and accumulation of a sub-G1 cell population, is observed in cancer cell lines but not in non-cancer cell lines tested
- Instead CYC065 treatment of non-cancer cell lines increased the population of cells in the G1 phase of the cell cycle

Lower panels: Cells were treated with an 8 h pulse of CYC065 followed by a 24 h recovery before cell cycle distribution (DNA content by PI staining) was determined.

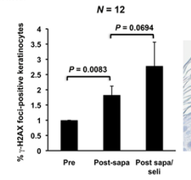
CYC065 and sapacitabine combine effectively in breast cancer

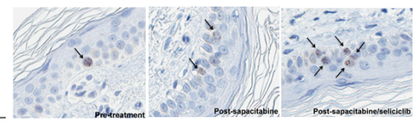
- Seliciclib and sapacitabine combination is being evaluated in a phase 1 study in patients with advanced cancer (NCT00999401) demonstrating durable partial responses in breast, ovarian and pancreatic cancer patients[9]
- Homologous recombination repair deficient cells have been shown to be hypersensitive to CNDAC, the active metabolite of sapacitabine[10]
- Potential mechanisms of synergy include:
  - CDKi-dependent inhibition of repair of CNDAC-induced DNA double-strand breaks
  - Enhancement of CNDAC-induced apoptosis by CDKi-dependent reduction of Mcl-1 levels
- Skin biopsies from patients on this trial exhibited a statistically significant increase in γH2AX staining (a marker of DNA double-strand breaks) post-sapacitabine and a further increase post-seliciclib[9]

Potential mechanisms of synergy between CYC065 or seliciclib and sapacitabine/CNDAC



Skin biopsies from patients given seliciclib and sapacitabine were stained for γH2AX to assess DNA damage following sapacitabine (d8 vs pre-treatment) and seliciclib (d11 vs d8) exposure[9].

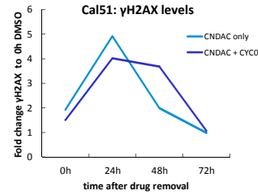




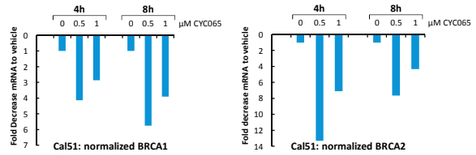
Cell Line	Seliciclib + CNDAC		CYC065 + CNDAC	
	Median CI CNDAC first	Median CI Seliciclib first	Median CI CNDAC first	Median CI CYC065 first
Cal51	0.84	0.96	0.93	0.94
Hs578T	0.89	1.00	0.88	0.79
MDA-MB-468	0.82	0.95	1.00	0.89
HCC1937	0.89	0.88	0.99	1.06
HCC1143	0.78	1.01	0.85	0.99
MCF7	1.04	1.06	0.94	0.95
SKBR3	0.74	0.96	0.74	0.96

Breast cancer cells were treated with a 7x7 drug concentration matrix for 24 h drug 1 followed by 72 h drug 2, and cell viability was determined by resazurin proliferation assays. Combination Index (CI) values were calculated using the method of Chou & Talalay[8]. Additivity is indicated by CI values of 0.9 - 1.1. CI values less than 0.9 are indicative of synergy.

- Sequential treatment of breast cancer cell lines with CNDAC and either CYC065 or seliciclib is additive or synergistic regardless of order of addition
- CYC065 treatment results in reduction of BRCA1 and BRCA2 mRNA levels
- CNDAC-induced DNA double-strand breaks persist in cells subsequently treated with CYC065



Cells were treated for 18 h with 5 µM CNDAC followed by 6 h 1 µM CYC065 or DMSO. Median γH2AX levels were measured in the cell population by flow cytometry analysis after staining cells with a FITC-conjugated γH2AX antibody. Median γH2AX levels are plotted as the fold increase relative to the 0 h DMSO sample.



BRCA1 and BRCA2 mRNA levels were determined by qPCR for cells treated with CYC065. Values are normalized to the levels of a housekeeping gene and are plotted as the fold decrease in mRNA relative to the respective vehicle only treatments.

Conclusions

- **CYC065 directs a pro-apoptotic mechanism in breast cancer cell lines, which includes transcriptional down-regulation of key pro-survival and oncogenic regulators, including Mcl-1 and c-Myc**
- **Short pulse treatments of CYC065 potently inhibit proliferation of breast cancer cell lines and twice weekly pulse treatments achieve maximal inhibition in sensitive lines**
- **CYC065 rapidly induces cell death in breast cancer cell lines, but causes G1 arrest in non-malignant breast cell lines**
- **Combination of seliciclib and sapacitabine has demonstrated durable partial responses in breast, ovarian and pancreatic cancer patients[9]**
- **Similarly to seliciclib, CYC065 combined positively with CNDAC/sapacitabine, likely via multiple mechanisms including:**
  - **BRCA1/2 reduction → persistence of CNDAC-induced DNA double-strand breaks**
  - **Mcl-1 reduction → enhanced CNDAC-induced apoptosis**
- **Basal-like TNBC represents an unmet clinical need lacking targeted therapeutic options. CDK2/9 inhibitor CYC065 potently induces cell death in TNBC cell lines and has the potential to be combined effectively with DNA damaging agents in this indication**

(1) Dou, Int J Haematol. 2008, 87:10. (2) Horiuchi, J Exp Med. 2012, 209:679. (3) Cocco, AACR 2015, Abs 3103. (4) Scaltriti, PNAS 2011, 9:3761. (5) Saladino, AACR 2015, Abs 1650.
(6) Booher, PLoS ONE 2014, 9:e108371. (7) Modugno, Exp Cell Res. 2015, 332:267. (8) Chou, Cancer Res. 2010, 70:440. (9) Shapiro, AACR 2013, Abs LB-202. (10) Liu, Blood. 2010, 116:10.