NOVEL CDK9 INHIBITORS TO TREAT CDK4/6 RESISTANT TUMORS

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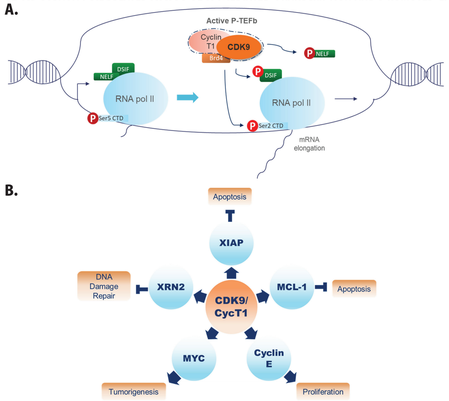
INTRODUCTION

The family of cyclin-dependent kinases (CDKs) are serine/threonine protein kinases important for cell cycle control and/or regulation of transcription. CDK9/CyclinT1 is a key regulator of transcription in eukaryotic cells and has been shown to be dysregulated in both hematologic and solid tumors. CDK9/CyclinT1 forms the active P-TEFb complex and phosphorylates Ser2 residues in the carboxy-terminal domain of RNA polymerase II (RNAP II) to initiate elongation of mRNA transcripts. CDK9 activity regulates transcription of a variety of short-lived transcripts that promote survival and directly suppress apoptosis in cancer cells, including MYC, CCNE, XRN2, MCL-1, and XIAP. MYC-driven tumor types with Rb-loss or high expression levels of cyclin E, such as triple negative breast cancer (TNBC), are difficult to treat and are resistant to existing CDK4/6 inhibitors. Likewise, ER+ breast cancer acquires resistance to CDK4/6 inhibitors by upregulation of Cyclin E, which allows G1 to S cell cycle progression through CDK2. Since CDK9 is upstream of these oncogenic drivers, inhibition of CDK9 could potentially bypass innate and acquired resistance mechanisms and induce cell death in TNBC or ER+ breast cancer through decreasing the expression of MYC or Cyclin E. G1 Therapeutics has developed a focused library of potent and selective CDK9 inhibitors to assess whether CDK9 is a target for treating tumor types resistant to CDK4/6 inhibitors.

RESULTS

FIGURE 1. CDK9 ACTIVITY REGULATES RNA POL II MEDIATED TRANSCRIPTION AND PROMOTES CANCER



(A) Graphical depiction of the active CDK9/Cyclin T1 (P-TEFb) function in RNAP II mediated transcription elongation. (B) Graphical representation of pro-tumor signaling events that occur downstream of CDK9/Cyclin T1 activity.

TABLE 1. BIOCHEMICAL PROFILING OF CDK9i COMPOUNDS REVEAL HIGH POTENCY AND SELECTIVITY FOR CDK9

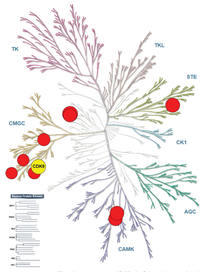
	CDK1/ Cyclin B1 IC_{50} [nM]	CDK2/ Cyclin E IC_{50} [nM]	CDK2/ Cyclin A IC_{50} [nM]	CDK3/ Cyclin E IC_{50} [nM]	CDK4/ Cyclin D1 IC_{50} [nM]	CDK5/ p35 IC_{50} [nM]	CDK6/ Cyclin D3 IC_{50} [nM]	CDK7/ Cyclin H IC_{50} [nM]	CDK9/ Cyclin T IC_{50} [nM]
CDK9i-1	273	76	195	258	4	134	23	52	1
CDK9i-2	83	34	8	11.3	14	60	47	16	5
CDK9i-3	1,290	123	35	147	1	203	2.5	716	65
CDK9i-4	24	24	38	9.6	12	18	37	4.4	1
CDK9i-5	509	164	486	371	14	1,080	49	111	4
CDK9i-6	90	114	193	238	13	470	72	173	3
CDK9i-7	270	137	259	240	11	310	47	86	3
CDK9i-8	154	56	160	342	10	227	45	125	1
CDK9i-9	396	65	150	292	16	233	53	76	2
CDK9i-10	4,360	3,120	12,800	7,240	2,900	30,400	28,200	13,700	11
CDK9i-11	290	148	226	262	4	120	24	70	2
CDK9i-12	220	85	92	261	13	116	48	119	3
CDK9i-13	195	35	73	247	19	84	75	181	3
CDK9i-14	111	94	41	180	5.2	122	43	213	5
CDK9i-15	235	63	146	231	12.5	216	34	69	2

Analysis of CDK9i biochemical IC_{50}s was determined using Caliper technology by Nanosyn, Inc.

RESULTS

FIGURE 2. CDK9i-1 IS A HIGHLY POTENT AND SELECTIVE CDK9 INHIBITOR

CDK9i-1 100nM S-SCORE (20)



Graphical depiction of all kinases inhibited >80% by CDK9i-1 at 100nM. Biochemical kinome screening was performed by Thermo Fisher Scientific SelectScreen Kinome Profiling Services across 485 kinases. Assays were completed at a concentration 100 times the biochemical IC_{50} of CDK9. CDK9 is represented by the yellow circle.

KINASES INHIBITED >80% BY CDK9i-1

Target	Average % inhibition
CDK9 (Inactive)	101
CDK9 / Cyclin K	95
CDK9 / Cyclin T1	96
CDK13 / Cyclin K	86
CDK4 / Cyclin D1	81
CDK16(PCTK1) / Cyclin Y	80
ERN1	104
ERN2	91
MAPK15 (ERK7)	85
TAOK1	97
PRKD1 (PKC mu)	95
PRKD2 (PKD2)	96

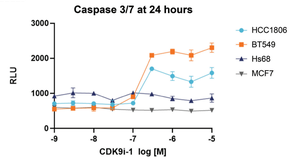
List of all kinases inhibited >80% by CDK9i-1 at 100nM. Results generated by Thermo Fisher Scientific SelectScreen Kinome Profiling Services.

TABLE 2. 6-DAY CONTINUOUS TREATMENT WITH CDK9i-1 REDUCES VIABILITY OF ALL TESTED CELL TYPES

Cell Line	Disease/ Cell Type	CDK9i-1 IC_{50} [nM]	RB1 Status
A2058	Melanoma	56	Null
A549	NSCLC	121	Wild-type
BT549	TNBC	51	Null
HCC1806	TNBC	33	Wild-type
HCC70	TNBC	99	Mutant
Hs68	Primary fibroblast	57	Wild-type
MCF7	ER+ breast cancer	15	Wild-type
MOLT-4	ALL	122	Wild-type
WM2664	Melanoma	23	Wild-type

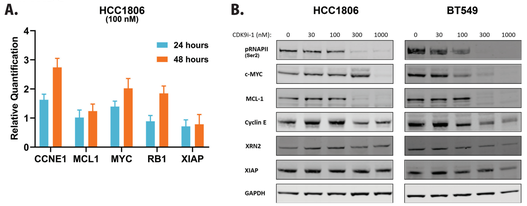
CDK9i-1 IC_{50} results of various normal and tumor cell lines was achieved by 6-Day CellTiter Glo analysis. Cells were plated 24 hours prior to CDK9i-1 treatment (1.0nM- 10μM).

FIGURE 3. CDK9i-1 INDUCES CASPASE 3/7 IN TNBC CELLS



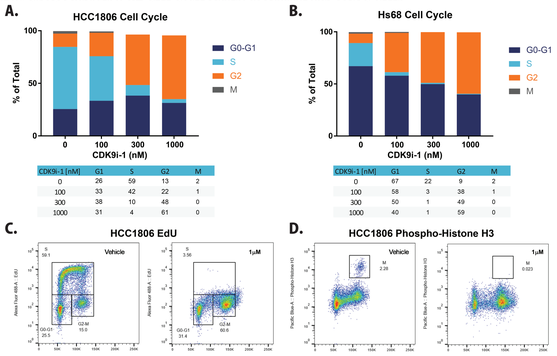
Caspase3/7 Glo results for HCC1806, BT549, primary human fibroblasts (Hs68) and MCF7 cells at 24 hours were incubated with increasing concentrations of CDK9i-1.

FIGURE 4. CDK9i-1 MODULATES mRNA AND PROTEIN EXPRESSION OF PRO-SURVIVAL ONCOGENES REGULATED BY RNAP II IN TNBC



(A) CDK9i-1 upregulates Cyclin E1, MYC1, and RB1 transcripts after 48-hour treatment at 100 nM in HCC1806 cells, revealed by qRT-PCR. At 48 hours, XIAP and MCL1 transcripts levels are unaffected by CDK9i-1. Fold changes are shown relative to DMSO-treated control. Genes were normalized to β-actin. Error bars represent ± SD. Relative quantification = $2^{-\Delta\Delta C_t}$. (B) Western blot analysis of HCC1806 (expresses functional Rb) and BT549 (Rb-null) TNBC cell lines treated with increasing concentrations of CDK9i-1 for 24 hours show a concentration-dependent reduction in phosphorylation of RNAP II at Ser2, and a decrease in MCL1, MYC, and Cyclin E protein levels. GAPDH was used as a loading control.

FIGURE 5. CDK9i INDUCES A G2 CELL CYCLE ARREST IN NORMAL AND TNBC CELLS

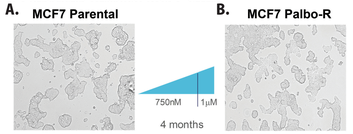


CDK9i-1 [nM]	G1	S	G2	M
0	26	59	13	2
100	33	42	22	1
300	38	10	48	0
1000	31	4	61	0

CDK9i-1 [nM]	G1	S	G2	M
0	67	22	9	2
100	58	3	38	1
300	50	1	49	0
1000	40	1	59	0

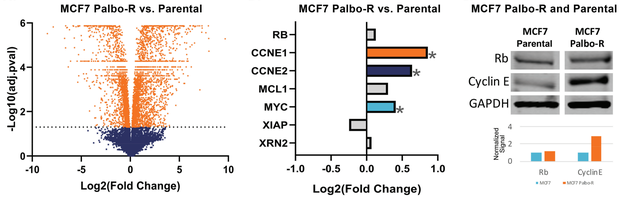
(A) HCC1806 and (B) Hs68 cell lines treated with CDK9i-1 for 24 hours. Cell cycle profiles were evaluated following CDK9i-1 treatment via Flow Cytometry using FlowJo (v10.0) software. Both cell lines show a dose-dependent decrease in S and increase in G2. (C) and (D) representative flow gating schematic for FxCycle DNA stain, Click-iT™ EdU, and Phospho-Histone H3 conjugated antibody, respectively.

FIGURE 6. DEVELOPMENT OF PALBOCICLIB-RESISTANT MCF7 CELLS



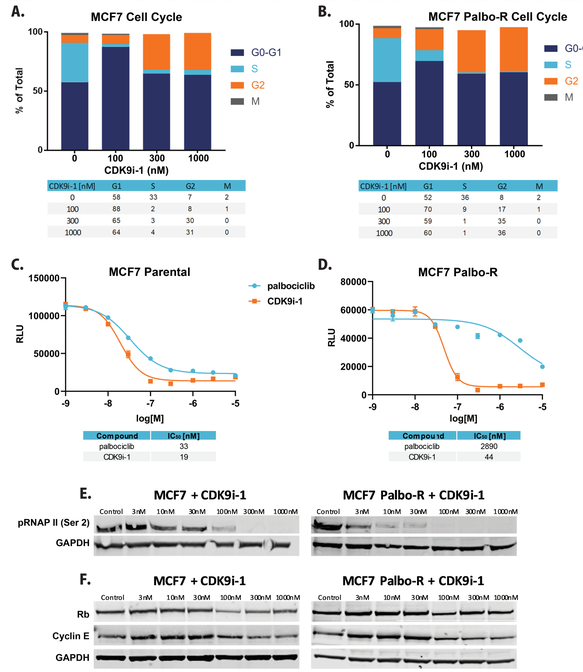
(A) MCF7 Parental cells (ATCC) were maintained in culture for four months in complete media (EMEM/ 10% FBS/ glutamax/ insulin) as control. (B) MCF7 Palbo-R cells were maintained in complete media plus palbociclib for three months at IC_{50} (750nM) followed by one month at 1μM. MCF7 Palbo-R cells maintain morphology and growth characteristics of parental cells.

FIGURE 7. CHARACTERISTICS OF PALBOCICLIB-RESISTANT CELLS



Whole transcriptome profiling was performed on control and palbociclib-resistant (Palbo-R) MCF7 cells by RNA-Seq. Libraries were prepared using the Illumina TruSeq Stranded mRNA assay and paired-end sequenced (2x50bp) on the Illumina HiSeq platform. (A) Pairwise comparison of transcript level in MCF7 Palbo-R vs. control. 6039 genes out of 17383 detectable genes were differentially expressed (orange), with adjusted p-value <0.05. (B) Log2 Fold change of downstream targets of CDK9 are shown. * denotes statistical significance, adj. p-val < 0.05. (C) Western blot analysis of MCF7 Parental and MCF7 Palbo-R cells demonstrated an increase in the ratio of Cyclin E to Rb levels in palbociclib-resistant cells.

FIGURE 8. CDK9i-1 INDUCES A G2 ARREST IN BOTH MCF7 PARENTAL AND MCF7 PALBOCICLIB-RESISTANT CELLS



CDK9i-1 [nM]	G1	S	G2	M
0	58	33	7	2
100	88	2	8	1
300	65	3	30	0
1000	64	4	31	0

CDK9i-1 [nM]	G1	S	G2	M
0	52	36	8	2
100	70	9	17	1
300	59	1	35	0
1000	60	1	36	0

Compound	IC_{50} [nM]
palbociclib	33
CDK9i-1	19

Compound	IC_{50} [nM]
palbociclib	2890
CDK9i-1	44

(A) MCF7 and (B) MCF7 Palbo-R cell lines were treated with CDK9i-1 for 24 hours. Cell cycle profiles following treatment were evaluated via Flow Cytometry with FxCycle DNA stain, Click-iT™ EdU, and Phospho-Histone H3 conjugated antibody. Profiles show a dose-dependent decrease in S and increase in G2. 6-Day CellTiter Glo results for (C) MCF7 parental and (D) MCF7 palbociclib-resistant cell lines demonstrate CDK9i-1 inhibits cell proliferation independent of CDK4/6. CDK9i-1 inhibits (E) pRNAP II (Ser2) and its downstream target (F) Cyclin E.

SUMMARY

1. **The lead CDK9i-1 is a potent and selective CDK9 inhibitor *in vitro*.**
2. **CDK9i-1 inhibits RNAP II CTD Ser2 phosphorylation resulting in decreases in MYC, MCL-1 and Cyclin E protein levels.**
3. **CDK9i-1 induces a G2 cell cycle arrest and apoptosis in a concentration and time dependent manner in both normal and tumor cells independent of Rb status.**
4. **Palbociclib-resistant MCF7 cells have upregulated Cyclin E mRNA and protein relative to parental MCF7 cells.**
5. **CDK9i-1 inhibits RNAP II CTD Ser2 phosphorylation in palbocilcib-resistant MCF7 cells resulting in a decrease of Cyclin E protein levels.**
6. **Our results suggest that CDK9 is a potential new target for the treatment of tumor types that have intrinsic or acquired resistance to CDK4/6 inhibitors.**