PRE-CLINICAL CHARACTERIZATION OF G1T28, A NOVEL CDK4/6 INHIBITOR FOR PROTECTION OF BONE MARROW FROM CYTOTOXIC CHEMOTHERAPIES

JOHN E. BISI , HANNAH S. WHITE, JESSICA A. SORRENTINO, PATRICK J. ROBERTS, JAY C. STRUM
G1 THERAPEUTICS, INC., RESEARCH TRIANGLE PARK, NC

ABSTRACT

G1T28 is a clinical stage, small molecule inhibitor of cyclin dependent kinases 4/6 (CDK4/6). Hematopoietic stem and progenitor cells (HSPC) require CDK4/6 for proliferation, and CDK4/6 inhibition allows the transient arrest of HSPC in the G1 phase of the cell cycle. This arrest may reduce the sensitivity of HSPC to DNA damaging chemotherapies by limiting G1 to S-phase progression in the setting of unrepaired DNA damage. Reducing HSPC death may reduce chemotherapy-induced myelosuppression (CIM), the major dose-limiting toxicity of most cytotoxic anti-cancer agents. G1T28 was specifically designed with high potency, exquisite selectivity, and favorable pharmacology to induce a predictable and well-defined transient arrest of HSPC as compared to less potent and selective CDK4/6 inhibitors. Biochemical profiling demonstrates that G1T28 is a competitive inhibitor of CDK4/6 at low nanomolar concentrations, and that G1T28 is highly selective for CDK4/Cyclin D1 and CDK6/Cyclin D3 as compared to CDK2/Cyclin A or CDK2/Cyclin E. G1T28 induces a clean G1 arrest in CDK4/6-dependent cell lines *in vitro*. Since the down-stream target of CDK4/6 is the retinoblastoma protein (Rb), we investigated the ability of G1T28 to inhibit Rb phosphorylation. In CDK4/6-dependent cell lines, G1T28 exposure fully blocks Rb phosphorylation by 16 hours, while no effect on Rb in CDK4/6-independent cells is observed. To determine the duration and reversibility of G1T28's effects on cells in culture, Rb competent cells were treated with G1T28 for 24 hours, and then the drug was washed out. While cells were arrested at 24 hours, they re-entered the cell cycle by 16 hours after washout and maintained normal cell cycle kinetics thereafter. To demonstrate that the G1 arrest induced by G1T28 decreases DNA damage and apoptosis following exposure to chemotherapeutic agents, Rb competent cells were pre-treated with G1T28 or vehicle control for 16 hours followed by incubation with various chemotherapies. Cells were then assayed for γ-H2AX formation and caspase activation. Treatment with G1T28 prior to DNA damaging agents attenuates DNA damage as measured by γ-H2AX formation and decreased caspase 3/7 activation in a dose-dependent manner, indicating a decrease in chemotherapy-induced apoptosis. In Rb-deficient cancers, such as small cell lung cancer (SCLC), G1T28 may effectively protect HSPCs without affecting the chemotherapy's efficacy. *In vitro* testing with SCLC cell lines confirmed that chemotherapy efficacy was unaffected by the addition of G1T28. In summary, G1T28 is a novel potent and selective CDK4/6 inhibitor that induces a transient and reversible G1 cell cycle arrest in CDK4/6-dependent cells. However, the anti-tumor activity of chemotherapy in CDK4/6-resistant cells is unaffected by G1T28.

METHODS

CDK *in vitro* Kinase Assay: Compounds were tested in CDK2/ CycA, CDK2/ CycE, CDK4/CyclinD1, CDK6/ CycD3, CDK5/ p25, CDK5/ p35, CDK7/CycH/Mat1, CDK9/CycT kinase assays by Nanosyn (Santa Clara, CA). The assays were completed using microfluidic kinase detection technology (Caliper Assay Platform). The compounds were tested in 12-point dose response format in singlicate at Km for ATP. G1T28 was also profiled using DiscoveRx (Fremont, CA) technology against 456 kinases (KinomeScan) for single point binding at 100 nM and 1000 nM concentrations representing 100 and 1000 fold concentrations over the K_i for CDK4, respectively.

Cell Cycle Analysis: For determination of cellular fractions in various stages of the cell cycle following treatment, cells are stained with propidium iodide staining solution and analyzed on Dako Cyan Flow Cytometer. The fraction of cells in G1 phase of DNA cell cycle was determined using FlowJo 7.2 .2 analysis software.

Western Blot Analysis: Cells were incubated with G1T28 for varying times. Whole cell extracts were resolved by Novex® NuPAGE® SDS-PAGE gel system and analyzed with antibodies to either Rabbit phospho—Rb (Ser807/811) (Cell Signaling, 9308s) or phospho-Rb (Ser780) (Cell Signaling, 9307) at 1:1,000. Mouse anti-MAPK (Cell Signaling, 9107) at 1:2,000 was used as a loading control. Secondary antibodies from LiCor were employed as Goat anti-rabbit(680RD) at 1:15,000 or Goat anti-mouse (800CW) at 1:15,000. Western blots were imaged using LiCor Odyssey software.

γ-H2AX DNA Damage Assay/Caspase 3/7 Apoptosis Assay: The tHS68 cells were treated with varying concentrations of G1T28 16 hours before treatment with 5 μM etoposide, 1 μM doxorubicin, 100 μM carboplatin, 250 nM paclitaxel or 156 nM camptothecin. The tHS68 cells were then assayed for γ-H2AX formation with Millipore (Billerica, MA) H2A.X Flow Cytometry Assay Kit and caspase 3/7 activation via Promega (Madison, WI) Caspase Glo 3/7 Assay System.

Murine SCLC Chemoprotection Assay: H69 xenograft murine models (S.T.A.R.T., San Antonio, TX) were treated with vehicle (twice weekly for 3 weeks), G1T28 (P.O. 100mg/kg twice weekly for 3 weeks), Carboplatin (I.P. 40mg/kg once a week for 3 weeks)/Etoposide (I.P. 4mg/kg twice weekly for 3 weeks), or G1T28/Carboplatin/Etoposide. Dosing for G1T28 was determined from previous tumor efficacy studies in Rb competent cell lines as well as oral PK data. Tumor size and murine survival were measured for 50 days post tumor injection.

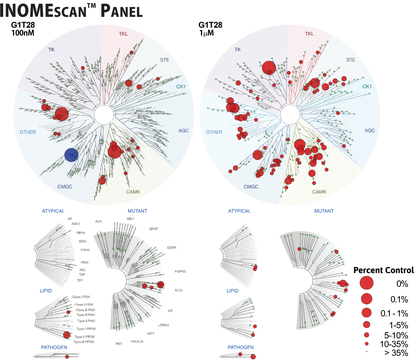
RESULTS

FIGURE 1. G1T28 IS A POTENT AND SELECTIVE INHIBITOR OF CDK4/6

	CDK4/ CycD1 μM	CDK6/ CycD3 μM	CDK2/ CycA μM	CDK2/ CycE μM	CDK5/ p25 μM	CDK5/ p35 μM	CDK7/ CycH/Mat1 μM	CDK9/ CycT μM
Mean	0.001	0.004	1.285	2.505	1.707	1.243	4.637	0.052
SEM	0.0005	0.001	0.098	0.248	0.480	0.044	0.385	0.006
n	9	3	6	6	3	3	3	3

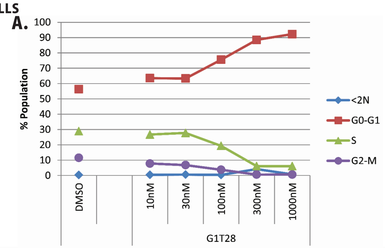
Biochemical profiling of G1T28 in Nanosyn (Santa Clara, CA) kinase screen for CDK's. Results are displayed as micromolar concentrations for IC_{50}'s against putative target. "n" represents individual batch preparations.

FIGURE 2. G1T28 IS HIGHLY SELECTIVE FOR CDK4 IN DISCOVERX KINOMESCAN™ PANEL

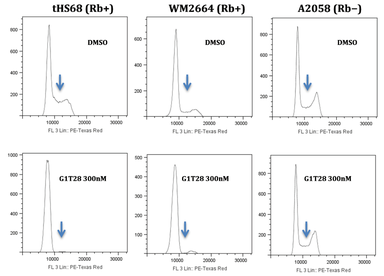


Assessment of G1T28 at 100 and 1000 times the biochemical IC_{50} across 468 and 456 kinases, respectively, in the DiscoveRx KINOMEscan™ kinase panel exhibits high degree of selectivity in the CGMC family. Blue circle represents CDK4. Only 2 other kinases, NEK10 and PRKD3, demonstrate high affinity for G1T28.

FIGURE 3. G1T28 INDUCES CLEAN G1 ARREST IN RB COMPETENT CELLS

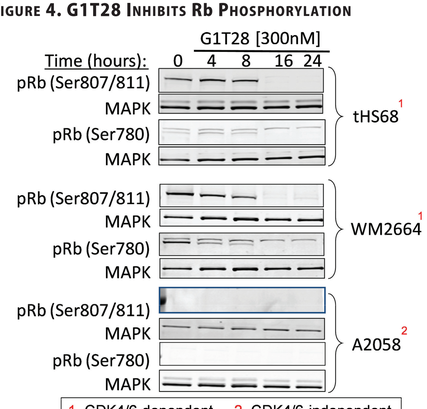


B.



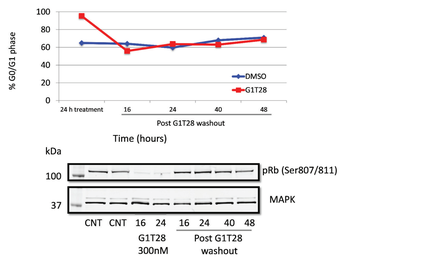
CDK4/6-dependent (tHS68 and WM2664) and CDK4/6-independent cell lines (A2058) were treated with indicated concentrations of G1T28 for 24 hours. Following treatment cells were harvested and analyzed for cell cycle distribution. **(A)** tHS68 cells show a clean G1 arrest with corresponding percent decrease in the number of cells in S-phase. **(B)** Cell Cycle histograms for treatment with G1T28 causes a loss of the S-phase population (indicated by blue arrow) seen in dimethyl sulfoxide (DMSO) treated CDK4/6-dependent cell lines (tHS68 and WM2664), but not in the CDK4/6- independent cell line (A2058).

FIGURE 4. G1T28 INHIBITS RB PHOSPHORYLATION



To confirm the on-target activity of G1T28, a cell system consisting of two CDK4/6-dependent cell lines and one CDK4/6-independent cell line was used to evaluate changes in Rb-phosphorylation.

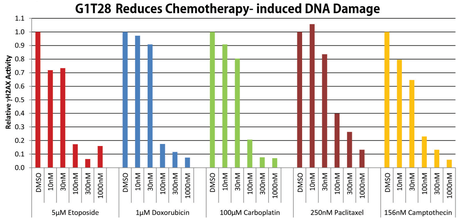
FIGURE 5. TREATMENT OF CDK4/6-DEPENDENT FIBROBLAST CELLS WITH G1T28 INDUCES REVERSIBLE G1 CELL CYCLE ARREST



The G1 cell cycle arrest observed in CDK4/6-dependent fibroblasts treated with 300 nM G1T28 for up to 24 hours can be reversed by washing G1T28 out of the culture media. Cells return to normal cell cycling 16-24 hours post washout. Expression of pRb, which is decreased at 16 and 24 hours after treatment with G1T28, returns to control levels 16-24 hours after G1T28 washout as well.

FIGURE 6. G1T28 PROTECTS AGAINST CHEMOTHERAPY-INDUCED γ-H2AX FORMATION AND APOPTOSIS

A. Inhibition of H2AX Formation by G1T28: Analysis of tHS68 Cells Exposed to Various Chemotherapeutic Agents



B. Inhibition of Caspase 3/7 Activation by G1T28: Analysis of tHS68 Cells Exposed to Various Chemotherapeutic Agents

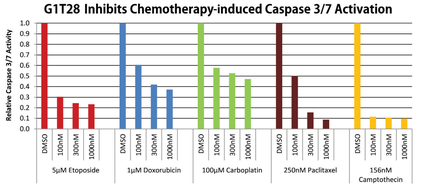
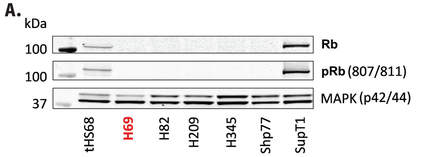
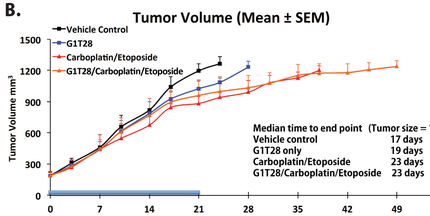


FIGURE 7. G1T28 TREATMENT DOES NOT AFFECT CHEMOTHERAPY TREATMENT OF RB-DEFICIENT TUMORS *IN VIVO*

A.



B.



A. Western blot analysis showing Rb deficiency in SCLC tumor cell lines. Total Rb, pRb and MAPK (control) were measured in various tumor cells lines. H69, an Rb-deficient small cell lung cancer line (red), was used to test the effectiveness of G1T28 treatment in a xenograft model.

B. Rb-deficient cell lines are not affected by G1T28 and efficacy of carboplatin/ etoposide is not reduced by G1T28 treatment. Median time to end point (when tumor size is 1 cm) is not extended or shortened when G1T28 is given with the carboplatin/etoposide regimen. Since G1T28 does not decrease chemotherapy efficacy in a CDK4/6-independent tumor, it could be used to protect the bone marrow from chemotherapy toxicity in patients with CDK4/6 independent tumors, such as SCLC. Blue bar indicates treatment duration.

SUMMARY

- G1T28 is a potent, selective CDK4/6 inhibitor that leads to a decrease in phosphorylation of Rb in CDK4/6-dependent cell lines.
- Treatment with G1T28 leads to a clean, robust G1 cell cycle arrest, but is transient when compound is washed out.
- Combined treatment of G1T28 and cytotoxic chemotherapies leads to a decrease in DNA damage and apoptosis when compared to chemotherapy treatment alone *in vitro*.
- *In vivo*, G1T28 treatment of Rb-deficient SCLC tumors does not protect the cells from the intended chemotherapy-induced cytotoxicity. Therefore, G1T28 treatment can be used in combination with chemotherapy to protect the bone marrow while not antagonizing the intended anti-tumor effect in patients with CDK4/6-independent tumors (e.g., SCLC).
- A Phase 1a, first-in-human (FIH) clinical trial has been completed. Based on these results, I.V. G1T28 will be investigated in Phase 1b/2a studies in patients with CDK4/6-independent tumors to evaluate its potential as a targeted bone marrow chemoprotectant.