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Running title: G1T28, a novel CDK4/6 inhibitor, reduces myelototoxicity

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There are no conflicts of interest
Abstract

Chemotherapy-induced myelosuppression continues to represent the major dose-limiting toxicity of cytotoxic chemotherapy, which can be manifested as neutropenia, lymphopenia, anemia, and thrombocytopenia. As such, myelosuppression is the source of many of the adverse side effects of cancer treatment including infection, sepsis, bleeding, and fatigue, thus resulting in the need for hospitalizations, hematopoietic growth factor support, and transfusions (red blood cells and/or platelets). Moreover, clinical concerns raised by myelosuppression commonly lead to chemotherapy dose reductions, therefore limiting therapeutic dose-intensity, and reducing the anti-tumor effectiveness of the treatment. Currently, the only course of treatment for myelosuppression is growth factor support which is suboptimal. These treatments are lineage specific, do not protect the bone marrow from the chemotherapy-inducing cytotoxic effects, and the safety and toxicity of each agent is extremely specific. Here, we describe the preclinical development of G1T28; a novel potent and selective CDK4/6 inhibitor that transiently and reversibly regulates the proliferation of murine and canine bone marrow hematopoietic stem and progenitor cells (HSPCs) and provides multi-lineage protection from the hematologic toxicity of chemotherapy. Furthermore, G1T28 does not decrease the efficacy of cytotoxic chemotherapy on RB deficient tumors. G1T28 is currently in clinical development for the reduction of chemotherapy-induced myelosuppression in first and second line treatment of small cell lung cancer.
Introduction

The cell cycle is a highly conserved and regulated process by which genomic integrity and replicative capacity must be maintained for proper cell maintenance and proliferation. The cell cycle consists of four distinct phases: G1 or Gap1 phase where cells grow and synthesize proteins in preparation for DNA synthesis; S phase, where DNA synthesis occurs; G2 or Gap2 phase post synthesis where cells continue to synthesize proteins in order to increase mass in preparation for mitosis; and lastly, M phase in which the DNA divides and the parent cell undergoes cytokinesis to produce two daughter cells (1). Regulation of this process is maintained by a series of highly conserved proteins referred to as cyclins, and their catalytic binding partners, cyclin-dependent kinases (CDKs). The G1 to S checkpoint is a critical restriction point in the process of cell division. Cells are maintained in a quiescent state until the proper signal is achieved for re-entry into the cell cycle. Throughout G1, expression of the D-type cyclins (D1, D2, D3) increases until active complexes with CDK4/6 are formed. Active CDK4/6 complexes partially phosphorylate RB, which allows partial de-repression of transcription factor E2F. This induces additional transcript production including cyclin E. Cyclin E will bind CDK2 to form active complexes that results in the hyperphosphorylation of RB and drives the cells through late G1 into S phase. Inhibition of CDK4/6-cyclin D by the tumor suppressor p16^{INK4a} leads to a G1 arrest and cell cycle progression is halted (2).
Inhibitors of cyclin dependent kinases have been in clinical development for more than two decades (3). However, toxicity due to poor specificity has limited their therapeutic potential in oncology. More recently, potent and selective CDK4/6 inhibitors have received a significant level of attention with the report by Pfizer of robust progression free survival (PFS) data for postmenopausal women with estrogen receptor (ER) positive, human epidermal growth factor receptor 2 (HER2) negative metastatic breast cancer receiving the CDK4/6 inhibitor palbociclib in combination with letrozole, an aromatase inhibitor. This led to “Breakthrough Therapy” designation by the FDA and the subsequent approval of palbociclib in February 2015. Likewise, both Novartis and Eli Lilly and Company are advancing their proprietary CDK4/6 inhibitors into Phase 3 clinical trials for hormone responsive breast cancer as well as a variety of RB dependent tumor types.

While targeted therapies have been shown to be effective in appropriately defined settings, the use of cytotoxic chemotherapies is still the cornerstone for treating a large number of patients. However, the most effective chemotherapies are limited in their utility due to myelosuppression. Chemotherapy-induced myelosuppression often results in dose reductions and treatment delays that adversely affect efficacy and produce serious adverse toxicities such as febrile neutropenia. Bone marrow hematopoietic stem and progenitor cells (HSPCs) have been found to be highly dependent upon CDK4/6 for proliferation (4, 5). Additionally, the transient arrest of these cells with a potent, selective CDK4/6 inhibitor has been shown to protect blood cell counts and prolong survival in mice exposed to chemotherapy or lethal
doses of radiation (4, 6). While many tumors are CDK4/6 dependent, more than 300,000 patients are diagnosed every year whose tumors are functionally CDK4/6 independent (3). These include small cell lung cancer (7), triple negative breast cancer (8), bladder (9), human papilloma virus (HPV) associated head and neck (10), and prostate cancer (11, 12). The standard of care for many of these patients is myelosuppressive chemotherapy. The transient arrest of HSPCs by a CDK4/6 inhibitor during the administration of chemotherapy in these patients has the potential to protect the bone marrow and immune system from the cytotoxic effects of chemotherapy, while not adversely impacting the anti-tumor effects. This may translate to a decreased nadir and faster recovery of circulating blood cells, prevention of bone marrow exhaustion and the preservation of immune cell number and function, thereby allowing a more robust host immune response to the tumor.

G1T28 is a CDK4/6 inhibitor being developed to reduce chemotherapy-induced multi-lineage myelosuppression. This paper describes the identification of G1T28 as a potent and selective CDK4/6 inhibitor that inhibits the phosphorylation of RB and induces an exclusive, reversible G1 arrest. In vitro and in vivo, G1T28 protects RB competent cells from damage by chemotherapy as assessed by gamma-H2A.X (γH2AX) and apoptosis through caspase 3/7 activation. In vivo, G1T28 regulates the proliferation of HSPCs in both mouse and canine bone marrow, in a reversible, dose- and time-dependent manner. Pretreatment of mice with G1T28 allows a faster recovery of complete blood counts (CBCs) following chemotherapy. In addition, G1T28 does not protect RB deficient tumors from chemotherapy but, instead, adds
to the anti-tumor effect. G1T28 has recently been tested in a Phase 1, healthy volunteer pharmacokinetics and safety study (13, 14) and two Phase 1b/2a studies have been initiated in small cell lung cancer to assess the potential for reduction of chemotherapy-induced multi-lineage myelosuppression (15, 16).

**Materials and Methods:**

**Chemical**

G1T28 \[2'-(5-(4-methylpiperazin-1-yl)pyridin-2-yl)amino)-7',8'-dihydro-6'H-spiro[cyclohexane-1,9'-pyrazino[1',2':1,5]pyrrolo[2,3-d]pyrimidin]-6'-one\] was synthesized and characterized for purity and identity as an HCl salt at ChemoGenics BioPharma, LLC under the direction of G1 Therapeutics, Inc.

**Nanosyn CDK in vitro Assay**

Compounds were tested in CDK2-CYCLIN A, CDK2-CYCLIN E, CDK4-CYCLIN D1, CDK6-CYCLIN D3, CDK5-p25, CDK5-p35, CDK7-CYCLIN H-MAT1, and CDK9-CYCLIN T kinase assays by Nanosyn, Inc. 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 the Km for ATP. Phosphoacceptor substrate peptide concentration used was 1 μM and Staurosporine was used as the reference compound for all assays.

**KINOMEscan Primary Screen and Kd Determination**
G1T28 was profiled at DiscoveRx using their KINOMEscan and scanMAX screening technology (17). Briefly, G1T28 was tested at 100 and 1000 times the biochemical IC_{50} as described in Table 1. All target kinases that responded to greater than 90% inhibition were tested as individuals for $K_d$ determination.

**Cell Lines**

Cell lines were obtained from American Type Culture Collection (ATCC). HS68, A2058, WM2664, and MCF-7 were grown in Dulbecco’s Modified Eagle’s Medium (Life Technologies) containing 10% fetal bovine serum (HyClone) and 1x Glutamax (Life Technologies). ZR-75-1, NCI-H69, and SHP77 were grown in RPMI-1640 (CELLGRO) containing 10% fetal bovine serum and 1x Glutamax. The SUP-T1 cell line (Sigma) was grown in RPMI-1640 containing 10% fetal bovine serum and 1x Glutamax. Cell lines were authenticated using Short Tandem Repeat (STR) analysis at ATCC.

**Western Blots**

HS68, WM2664 and A2058 cells were treated with 300 nM G1T28 or DMSO (0.1%), for 4, 8, 16 or 24 hours. Whole cell extracts were prepared using 1x radioimmunoprecipitation assay buffer (RIPA) (ThermoFisher) containing 1x HALT® protease and phosphatase inhibitors (ThermoFisher). Total protein concentration was determined by using the bicinchoninic acid (BCA) Protein Assay Kit (PIERCE), according to manufacturer’s instructions. Fifteen micrograms of protein was heat denatured for 10 minutes at 70°C and resolved by Novex®
NuPAGE® SDS-PAGE gel system (ThermoFisher) and transferred to 0.45 μm nitrocellulose membrane by electroblotting. Membranes were blocked in LiCor Membrane Blocking Buffer and incubated overnight with rabbit anti-pRb (Ser807/811) antibody (Cell Signaling Technology) at a 1:1,000 dilution and mouse anti-MAPK antibody (Cell Signaling Technology) at a 1:2,000 dilution, as a loading control. Secondary antibodies (LiCor) were Goat anti-rabbit (680RD) and Goat anti-mouse (800CW) at a 1:15,000 dilution. Blots were incubated for one hour, washed and imaged using LiCor ImageStudio software (Version 4.0.21).

For H69, MCF7, SupT1, and ZR75-1 western blot, protein was processed as described previously. Antibodies to total RB (Cell Signaling Technology) and β-Tubulin (Cell Signaling Technology) run as a loading control were assessed. A goat anti-rabbit (680RD)(LiCor) secondary antibody was utilized at a dilution of 1:15,000.

**Cell Cycle Analysis**

HS68 cells were treated for 24 hours with G1T28 at 10, 30, 100, 300, or 1000 nM final concentration. Cells were harvested and fixed in ice-cold methanol (Sigma). Fixed cells were stained with 20 μg propidium iodide (Sigma), 50 μg RNase A (Sigma) in PBS-CMF + 1% Bovine Serum Albumin (BSA), Fraction V (Fisher Scientific). Samples were processed on Cyan ADP Analyzer (Beckman Coulter), and cell cycle analysis was completed using FlowJo software (Version 10.0.8; Tree Star).
Cell Proliferation

SupT1, MCF7, ZR-75-1, A2058 and H69 cells were seeded at 1000 cells per well in Costar 3903 96 well plates. After 24 hours, plates were dosed with G1T28 at a nine-point dose concentration from 10 μM to 1 nM. Cell viability was determined after four or six days using the CellTiter-Glo® assay (Promega) following manufacturer’s recommendations. Plates were processed on BioTek Synergy2 multi-mode plate reader and data analyzed using Graphpad Prism 5 statistical software.

γH2AX and Caspase 3/7 Activation

For the γH2AX assay, 30,000 HS68 cells were plated per well in 12-well plates and incubated for 24 hours at 37°C. Cells were incubated with 10, 30, 100, 300, or 1000 nM G1T28 or dimethyl sulfoxide (Sigma-Aldrich) as vehicle control for 16 hours. Plates were subsequently dosed with chemotherapy (5 μM Etoposide (Selleckchem), 1 μM Doxorubicin (Bedford Laboratories), 100 μM Carboplatin (APP Pharmaceuticals, LLC), 156 nM Camptothecin (Sigma) or 250 nM paclitaxel (Sigma)). For γH2AX, cells were harvested for analysis 8 hours after exposure to chemotherapy. Cells were fixed and stained using the H2AX Phosphorylation Assay Kit (Millipore) by the manufacturer’s instruction. γH2AX-positive HS68 cells were quantified using FACSCalibur Flow Cytometer (BD BioSciences) and FlowJo analysis software.
For the in vitro caspase 3/7 assays, HS68, H69 and SHP77 cells were seeded at 1000 cells per well in Costar 3903 96 well plates. Cells were incubated with 10, 30, 100, 300, or 1000 nM G1T28 or dimethyl sulfoxide (Sigma-Aldrich) as vehicle control for 16 hours. Plates were subsequently dosed with chemotherapy as previously described and were analyzed directly in the plates 48 hours after chemotherapy treatment. Caspase 3/7 induction was measured using Caspase-Glo 3/7 Assay System (Promega) by following the manufacturer’s recommended instructions.

**In vitro Washout Experiments**

Twenty-four hours after seeding on 60 mm dishes, HS68 cells were treated with G1T28 at a 300 nM final concentration for 24 hours. Wells were washed twice with PBS-CMF, and then replenished with fresh culture medium. The cells were further incubated for a series of time points (t= 16, 24, 40, 48 hrs. post washout). At the conclusion of the experiment, cells were harvested, fixed and stained for cell cycle analysis as described previously.

**Pharmacodynamic assessment of G1T28 in mouse bone marrow**

8-week-old female FVB mice (Jackson Labs) were given a single oral dose of vehicle alone (20% Solutol, Sigma) or G1T28 at 50, 100, or 150 mg/kg, followed 11 or 23 hours later by a single intraperitoneal (IP) injection of 100 µg 5-ethynyl-2′-deoxyuridine (EdU, Life Technologies) as the Institutional Animal Care and Use Committee (IACUC) approved at University of North Carolina, Chapel Hill. Mice were euthanized 1 hour after EdU injection (i.e., total G1T28 treatment of 12 or 24 hours), and Lineage-negative cells (Lin-) were isolated using biotin anti-mouse
lineage panel (BioLegend) and anti-biotin microbeads (Miltenyi Biotec). Lin- cells were stained for EdU following manufacture’s instructions.

**Peripheral Blood analysis of 5-FU and G1T28 in mice**

FVB female mice were given single oral doses of vehicle or G1T28 of 150 mg/kg, followed 30 minutes later by a single intraperitoneal dose of 5FU at 150 mg/kg. CBCs were measured every two days starting on day six. Data reported are from day 6 (Platelets), day 10 (white blood cells [WBC], neutrophils [Neu], lymphocytes [Lymph]), or day 16 (red blood cells [RBC]). The IACUC committee at the University of North Carolina, Chapel Hill, approved all protocols.

**Caspase 3/7 Activation in murine bone marrow**

C57Bl6 female mice were given single oral doses of vehicle, 50 mg/kg, or 100 mg/kg of G1T28 followed 30 minutes later by a single intraperitoneal dose of etoposide at 2 mg/kg. 6 hours post treatment, mice were euthanized and bone marrow harvested. Caspase 3/7 activation was assessed using 100,000 bone marrow cells per well as previously described. The IACUC committee at Charles River Laboratories approved all protocols.

**G1T28 and Topotecan efficacy in RB deficient tumors**

Female athymic nude mice were implanted with H69 cells and monitored until treatment initiation. Once tumors reached an acceptable size (150 mm³), mice were dosed in various combinations of G1T28 and topotecan for five days per week for
four weeks. Tumors were measured for up to 60 days post treatment. All mice that reached excessive tumor burden before 60 days were humanely euthanized. All protocols were IACUC approved and experiments were completed at South Texas Accelerated Research Treatments (START). Topotecan and G1T28 levels in blood plasma from the mice treated with G1T28 and/or Topotecan were processed and analyzed using established methods at Bioanalytical Systems, Inc.

Results

Identification of G1T28

Rational structure based drug design was utilized to create a novel, proprietary Tricyclic Lactam scaffold with activity against cyclin dependent kinases. Multiple rounds of structure activity relationship studies were conducted to optimize the potency, selectivity and cellular properties of this scaffold. To assess potency, biochemical profiling was completed against CDK4/cyclin D1 and CDK6/cyclin D3. To maximize selectivity, compounds were profiled against CDK2/cyclin A and CDK2/cyclin E. Compounds with 100 fold or greater selectivity for CDK4/cyclin D1 versus CDK2/cyclin E were profiled in cell based screens to look for G1 arrest in normal HS68 fibroblast cells, with a functioning RB pathway. Compounds with cellular EC50’s < 100 nM and an exclusive G1 arrest profile through 1 μM, were further investigated for inhibition of RB phosphorylation, caspase 3/7 activation and γH2AX induction. Based on physicochemical, absorption, distribution, metabolism and excretion (ADME) and pharmacokinetic properties, candidate
compounds were tested in vivo for pharmacodynamic activity (G1 arrest of HSPCs). G1T28, \[2'-(5-(4-methylpiperazin-1-yl)pyridin-2-yl) amino)-7',8'-dihydro-6'H-spiro[cyclohexane-1,9'-pyrazino[1',2':1,5]pyrrolo[2,3-d]pyrimidin]-6'-one\], was selected as the lead candidate small molecule reversible, kinase inhibitor which potently and selectively targets both CDK4/cyclin D1 and CDK6/cyclin D3 and has optimal in vivo biological activity as an ideal chemoprotectant (Figure 1A). All preclinical characterization was completed with the dihydrochloride salt form.

In microfluidic kinase detection technology (Caliper Assay Platform) assays (Nanosyn, Inc.), G1T28 reversibly inhibits CDK4/cyclin D1 and CDK6/cyclin D3 with an IC\textsubscript{50} of 1 nmol/L and 4 nmol/L, respectively. This activity is highly selective versus other CDK/cyclin complex family members (Table 1). In particular, inhibition of CDK2/cyclin A, CDK2/cyclin E, CDK5/p25, CDK5/p35, and CDK7/cyclin H/Mat1 is more than three orders of magnitude less than CDK4/cyclin D1 and CDK9/cyclin T is ~ 50 fold less than CDK4/cyclin D1. Kinase selectivity was further interrogated in a screen of 468 protein kinases (KINOMEscan, DiscoveRx) at 100 nM and 1\mu M (data not shown) concentrations. Profiling at 100 times the IC\textsubscript{50} shows high degree of selectivity using an S-Score of 35 (Figure 1B, right panel), with significant selectivity at an S-Score of 1 (Figure 1B, left panel). Those protein kinases with greater than 90% inhibition were followed up for K\textsubscript{d} determination (Supplementary Table 1). Additional kinases of interest involved in cell growth and proliferation were tested including AKT1, Aurora B, Erk1, and Wee1 with no appreciable activity observed.
**G1T28 reversibly pauses the cell cycle in the G1 phase in only CDK4/6 dependent cell lines.**

The cellular potency of G1T28 in producing a G1 cell cycle arrest was tested in CDK4/6 dependent (HS68, WM2664) and CDK4/6 independent (A2058) cell lines. G1T28 only inhibited the CDK4/6 dependent cells, with an EC$_{50}$ of 30 nM in HS68 cells (Figure 1C). In the CDK4/6 independent A2058 cell line, there is no decrease in S phase with G1T28 treatment (brown arrow, Figure 1C, left panel). However, in both the HS68 and WM2664 cell lines, there is a significant dose-dependent decrease in S phase with a concomitant increase in G1. In HS68 cells, up to 98% of cells are in G1 after 24 hours of treatment of either 300 nM or 1 μM (Figure 1C, right panel). This G1 arrest is maintained through 3 μM demonstrating that G1T28 elicits a clean G1 arrest for $>3000 \times$ the enzymatic IC$_{50}$.

In normal cycling cells, the CDK4/6-cyclin D complex phosphorylates RB as an immediate downstream effect. Once phosphorylated, RB dissociates from cell cycle promoting transcription factors, which then drive G1 to S phase transition (18). Conversely, inhibition of RB phosphorylation leads to G1 cell cycle arrest. Therefore, the CDK4/6 specificity of G1T28 was confirmed by phospho-RB western blot analysis. After incubation with HS68, WM2664, or A2058, G1T28 blocks RB phosphorylation in the RB dependent cell lines by 16 hours post exposure, while the CDK4/6-independent cell line (A2058) exhibits no RB or pRB expression, as
expected (Figure 1D). This observation was confirmed by comparing additional RB competent and deficient cell lines. As shown in Supplementary Figure 1, RB competent cell lines are sensitive to growth inhibition when incubated with G1T28 while RB deficient cell lines are resistant to growth inhibition.

To demonstrate the G1T28-induced G1 cell cycle arrest is transient and reversible, HS68 cells were treated with G1T28 at 300 nM for 24 hours and cell cycle analysis was completed at various times post treatment (0, 16, 24, 40, 48 hours). As previously seen, incubation with G1T28 for 24 hours induced a robust G1 cell cycle arrest (Figure 1E, time=0). By 16 hours after G1T28 washout, cells had re-entered the cell cycle and demonstrated cell cycle kinetics similar to untreated control cells (Figure 1E). These results demonstrate that G1T28 causes a transient, and reversible G1 arrest.

G1T28 protects CDK4/6 dependent cells from chemotherapy-induced damage 

in vitro.

To demonstrate G1T28-induced G1 arrest decreases chemotherapy-induced damage (i.e. apoptosis or DNA damage), HS68 cells (a surrogate model to represent CDK4/6-dependent HSPCs), were treated with G1T28 and an array of chemotherapies with differing mechanisms of action. Specifically, HS68 cells were pretreated with G1T28 or vehicle control for 16 hours, at which time indicated chemotherapies were. Cells were harvested 8 hours after treatment to measure γH2AX foci (DNA damage) and 48 hours post treatment to measure caspase 3/7 activity (apoptosis). Pretreatment
of G1T28 in all DNA damaging chemotherapies tested (carboplatin, doxorubicin, etoposide, camptothecin) demonstrated a dose-dependent decrease in γH2AX foci suggesting an attenuation of chemotherapy-induced DNA damage (Figure 2A). Additionally, treatment of HS68 cells with G1T28 prior to chemotherapy treatment (DNA damaging agents previously described, as well as a DNA intercalator; 5-FU and an anti-mitotic; paclitaxel) elicited a robust dose-dependent decrease in caspase 3/7 activation suggesting an attenuation of apoptosis (Figure 2B). The data show that a transient G1T28-mediated G1 cell cycle arrest in CDK4/6-sensitive cells decreases the in vitro toxicity of a variety of commonly used cytotoxic chemotherapy agents associated with myelosuppression.

**G1T28 induces a reversible cell cycle arrest in murine and canine HSPCs.**

To understand the temporal effect of G1T28 treatment on HSPC proliferation, the kinetics of G1T28-induced G1 cell cycle arrest and reversal in HSPCs was measured in young adult FVB/n female mice after a single oral gavage of G1T28 at 50, 100, or 150 mg/kg. G1T28 treatment resulted in a robust and dose-dependent suppression of proliferation in HSPCs at 12 hours, with EdU incorporation returning near baseline levels in a dose-dependent manner by 24 hours post administration (Figure 3A). The data demonstrate that a single oral dose of G1T28 can produce reversible cell cycle arrest in HSPCs in a dose-dependent manner in vivo.

To confirm the findings seen in murine bone marrow, the effect of G1T28 on dog bone marrow EdU incorporation was evaluated. Detailed methods can be found in
the supplementary methods. In summary, dogs were given single 30-minute IV infusions of G1T28 at 0, 1, 5, and 15 mg/kg. Bone marrow was taken from the dogs at 8, 16, and 24 hours after infusion. Blood samples were taken from dogs at pre-dose and 24, 48, 72, 168, 240, and 336 hours after the G1T28 dose to measure the plasma concentration of the drug. A dose-dependent decrease in bone marrow proliferation was observed, with higher dose levels producing a decrease in whole bone marrow proliferation similar to the decrease observed in mice (Supplementary Figure 2B).

The biological effect of the inhibition of bone marrow proliferation in both species was longer than the approximately 4-hour pharmacokinetic half-life of G1T28 in dogs and 5 hours in mice (Supplementary Figure 2A, data not shown). This suggests that factors other than drug concentration, such as cell cycling times of various progenitors, will impact the duration of HSPC G1 arrest. Finally, despite a robust G1 arrest of the bone marrow that persisted for up to 24 hours at a dose of 15 mg/kg G1T28, only subtle changes were noted in CBCs (Supplementary Figure 2C and 2D). This is further evidence the G1T28-induced G1 arrest is transient and reversible in vivo.

**G1T28 protects mouse bone marrow cells from chemotherapy-induced apoptosis and attenuates chemotherapy-induced myelosuppression in vivo.**

To directly measure the effect of transient G1 cell cycle arrest of the HSPCs on chemotherapy-induced bone marrow toxicity, we determined the ability of G1T28
to prevent etoposide-induced apoptosis of bone marrow cells in C57Bl6 mice. Mice received vehicle or G1T28 30 minutes by oral gavage prior to etoposide, and bone marrow was harvested 6 hours post etoposide treatment. Caspase 3/7 activation increased 3-fold in bone marrow from mice that received etoposide (Figure 3B), while mice that received G1T28 prior to etoposide showed a dose-dependent decrease in caspase 3/7 activation (Figure 3B). In fact, mice given 100 mg/kg G1T28 30 minutes prior to etoposide treatment, exhibited only background levels of caspase 3/7 activity. This data demonstrates that G1T28 can protect the bone marrow from chemotherapy-induced apoptosis in vivo.

To expand upon these findings, the ability of G1T28 to attenuate chemotherapy-induced myelosuppression was evaluated using a well-characterized single-dose 5-fluorouracil (5FU) regimen that is highly myelosuppressive in mice. FVB/n female mice were given single oral doses of vehicle or 150 mg/kg of G1T28, followed 30 minutes later by a single IP dose of 150 mg/kg of 5FU. Administration of G1T28 prior to 5FU produced a faster recovery of all hematopoietic lineages from 5FU-induced myelosuppression (Figure 3C). The data demonstrate that treatment with G1T28 prior to 5FU likely decreases 5FU-induced damage by chemotherapy in HSPCs, thus accelerating blood count recovery post-chemotherapy.

**RB-Deficient Cell Lines are Resistant to CDK4/6 Inhibition.**

To use G1T28 to selectively protect the HSPC, while not antagonizing the intended antitumor activity of the chemotherapy, the tumor must be CDK4/6-independent.
Since RB is the direct downstream effector of CDK4/6, loss of RB is one marker of CDK4/6-independence. Previous findings have shown that RB-deficient cells are resistant to CDK4/6 inhibition (19-21). To demonstrate this finding with G1T28, a panel of RB-null SCLC cell lines was treated with DMSO or G1T28 for 24 hours. All SCLC cell lines (H69, H82, H209, H345, SHP-77) were confirmed to be RB null by western blot analysis (Figure 4A). The effect of CDK4/6 inhibition on proliferation was measured by flow cytometry using propidium iodide (PI) staining. All SCLC cell lines were resistant to CDK4/6 inhibition, with no change in the percent of cells in the G1-phase upon treatment (data from two representative cell lines is shown in Figure 4B). These data are consistent with the previous findings that RB-deficient cells are resistant to CDK4/6 inhibition (4, 6, 22-25).

Co-Administration of G1T28 Does NOT Antagonize the Intended Chemotherapy-Induced Cytotoxicity of SCLC cells in vitro or in vivo.

To expand upon these findings and show that G1T28 does not antagonize the intended cytotoxicity of chemotherapy, the effect of co-administration of G1T28 with chemotherapy (cisplatin and etoposide) was evaluated in a panel of RB-null SCLC cell lines. As shown in data from two representative cell lines in Figure 4C, co-administration of G1T28 across a dose range of 10 nM to 1 µM had no impact on the cytotoxicity of cisplatin (5 µM) or etoposide (2.5 µM). These findings are consistent with previous results that RB-deficient cells are intrinsically resistant to CDK4/6 inhibition and thus are not protected from the intended cytotoxicity of chemotherapy.
To translate these findings in vivo, G1T28 was tested alone and in combination with topotecan in a SCLC xenograft model (H69) in athymic mice. H69 tumor bearing mice were treated with 100 mg/kg G1T28, 0.6 mg/kg topotecan, or 10, 50, or 100 mg/kg G1T28 thirty minutes before topotecan. As expected, H69 tumors were resistant to single agent G1T28, since the cells are Rb-null. While single agent topotecan produced a robust tumor regression of the H69 tumors, the combination of G1T28 at 10, 50 or 100 mg/kg with topotecan was superior. During and post dosing, G1T28 potentiated the statistically significant (p<0.05) effect of topotecan through study completion (Figure 5A). Overall, G1T28 was well tolerated and did not antagonize the effects of chemotherapy. In fact, G1T28 potentiated the anti-tumor effect of topotecan in the H69 model, which could not be explained by a potential drug-drug interaction since the plasma levels of G1T28 and topotecan were not affected by co-treatment (Supplementary Figure 3). Potential mechanisms for this enhancement of chemotherapy efficacy effect are currently being explored.

Discussion

Each year in the US, there are an estimated 300,000 new cases of cancer whose proliferation is controlled through a CDK4/6 independent pathway (3, 26). Of these, approximately 33,000 are cases of small cell lung cancer (SCLC), 95% of which are attributed to tobacco exposure (27, 28). SCLC represents approximately 20% of all lung cancer cases and is the most aggressive and lethal lung cancer subtype (3). It is
characterized by rapid tumor growth, early metastatic spread, and initial chemo-
responsiveness followed by disease progression with resistant disease. Despite
response rates of 70-85%, over 95% of patients with SCLC will die within 5 years of
diagnosis. Myelosuppressive chemotherapy (e.g. platinum/etoposide for first line
and topotecan as second line) is a cornerstone of SCLC treatment, however
chemotherapy treatments in these patients are often poorly tolerated leading to
dose reductions and treatment delays that minimize durable responses and
decrease long-term survival (29). Although growth factors such as G-CSF have
increased medical oncologists’ ability to deliver myelotoxic therapies in patients
with SCLC, neutropenia, anemia, and thrombocytopenia are still limiting toxicities of
SCLC regimens. Considerable improvement in patient outcomes could be realized
by maximizing the current treatment regimens through minimizing hematologic
toxicity.

The RB tumor suppressor is a major negative cell cycle regulator that is inactivated
in approximately 11% of all human cancers, and nearly 100% of SCLC (26, 30).
Moreover, activated CDK4/6 promote G1 to S traversal by phosphorylating and
inactivating RB. Importantly, cancers that inactivate RB do not require CDK4/6
activity for cell cycle progression (19-21). Since inactivation of RB is an obligate
event in SCLC development (7), this tumor type is highly resistant to CDK4/6
inhibitors and co-administration of CDK4/6 inhibitors with cytotoxic
chemotherapeutic agents such as those used in SCLC should not antagonize the
efficacy of such agents. Furthermore, CDK4 has also been shown to phosphorylate
SMAD3 thus inhibiting TGFβ-associated cell cycle progression in Rb inactivated tumors (e.g. SCLC) suggesting G1T28 treatment could arrest Rb null cells resulting in protection of the tumor from chemotherapy (31, 32). However, the in vitro and in vivo data presented herein shows a) G1T28 doesn’t cause a cell cycle arrest in Rb null cells (Figure 1) and b) G1T28 doesn’t protect Rb null tumor cells from chemotherapy-induced damage (Figure 4) demonstrating G1T28 is not inhibiting the TGFβ-associated cell cycle progression and any associated chemoprotection. In fact, G1T28/topotecan treatment of SCLC in vivo shows an increase in tumor efficacy when compared to topotecan alone demonstrating that G1T28 does not protect Rb incompetent SCLC from chemotherapy treatment (Figure 5).

Previous reports have shown that bone marrow cells are dependent upon CDK4/6 for proliferation (4, 6). In order to take advantage of this biology and preserve bone marrow and immune system function during chemotherapy administration for patients whose tumors are CDK4/6 independent, the ideal compound should have the following properties. First, it should demonstrate high potency and selectivity for CDK4/6 compared to CDK2/cyclin A. The active sites of CDK2 and CDK4/6 share significant homology. Until recently, it has proven very difficult to develop highly selective CDK4/6 inhibitors. Inhibiting CDK2/cyclin A would produce an S phase arrest in cells rather than a clean G1 arrest. Prolonged S phase arrest could be cytotoxic in of itself, e.g., the gastrointestinal toxicity seen with less selective CDK inhibitors, and could add to the cytotoxicity of chemotherapy. Second, an intravenously administered compound with a relatively short half life could be
beneficial so that the extent and duration of HSPC arrest can be tightly controlled, i.e., G1 arrest of HSPCs during and for several chemotherapy half lives after the last chemotherapy dose, followed by rapid re-entry of HSPCs into the cell cycle. The pharmacokinetic variability of orally delivered compounds that possess long half lives and accumulate on repeat dosing would arrest the bone marrow far too long and contribute to myelosuppression of the cytotoxic chemotherapies. Finally, the compound should demonstrate favorable physiochemical properties. Considering the Lipinski rule of five, the compound should have a molecular weight of less than 500, not more than five hydrogen bond donors, a LogP of less than 5, and fewer than 10 hydrogen bond acceptors (33).

The work described herein provides support for G1T28 having ideal properties to be used as an agent to preserve bone marrow and immune system function during chemotherapy. It is a highly potent and selective CDK4/6 inhibitor that is biologically active when delivered intravenously or orally. Assessment of kinase inhibition in vitro has been utilized successfully for many years to identify putative biological activities of small molecule inhibitors. These results must, however, be placed in context with the cellular and in vivo results observed. Albeit G1T28 appeared to potently bind additional kinases (Supp. Table 1) we did not observe the expected biological result of inhibition of these kinases. For example, we did not observed any increase in cell death or apoptosis as measured by cell cycle analysis (Figure 1C) or γH2AX induction (Figure 2A) which could result from inhibiting kinases such as GAK, MEK5, PRKD2, and PRKD3. We continue to assess the potential
for off target activity as well as complementary activity by other targets that G1T28 may bind.

Additionally, G1T28 inhibits the phosphorylation of RB and induces an exclusive, reversible G1 arrest. *In vitro* and *in vivo*, G1T28 protects cells from damage by chemotherapy as assessed by γH2AX and caspase 3/7 activation. *In vivo*, G1T28 reversibly and in a dose dependent manner, regulates the proliferation of HSPCs. Pretreatment of mice with oral G1T28 allows for the faster recovery of CBCs following chemotherapy treatment. Likewise, oral G1T28 does not protect RB deficient tumors from chemotherapy but adds to the anti tumor effect. While this effect was found in athymic mice that lack T lymphocytes, it is still possible that the enhanced efficacy is due to preservation of other immune cell types such as natural killer cells. While G1T28 protects immune cell numbers through it’s affect on the bone marrow, the effect of G1T28 on immune cell function is currently unknown but is an area of active research.

G1T28 has recently been tested in a Phase 1, healthy volunteer pharmacokinetics and safety study (NCT02243150) (14) and two Phase 1b/2a studies in small cell lung cancer (NCT02499770 and NCT02514447) have been initiated. The proof of concept studies will provide data on the ability of G1T28 to reduce chemotherapy-induced myelosuppression and improve patient outcomes.

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References

## Table

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Bisi Table 1
Figure Legends

Figure 1.
Chemical structure, kinome specificity and biochemical properties of G1T28. A, Structure, molecular formula and formula weight (free base and HCl salt) of G1T28. B, Kinome binding specificity of G1T28 was measured by site-directed competition-binding assays (S-Score= 1, left, S-Score= 35, right). C, G1T28 reversibly inhibits the cell cycle in only CDK4/6 dependent cell lines (HS68 and WM2664). D, Western blot analysis demonstrating a time course of G1T28-dependent inhibition of RB phosphorylation at Serine 807/811. E, The CDK4/6-dependent cell line (HS68) was treated with 300 nM G1T28 for 24 hours and cells were harvested at the indicated times following washout of G1T28 with media. The percentage of cells in the G1 phase is shown.

Figure 2.
G1T28 protects CDK4/6 dependent cells from chemotherapy-induced DNA damage and apoptosis in vitro. HS68 cells were incubated with G1T28 at the indicated doses for 16 hours followed by exposure to the indicated chemotherapy agents for 8 hours (γH2AX) or 48 hours (Caspase 3/7) prior to harvest. A, Direct DNA damage was measured through γH2AX flow cytometry staining. B, Apoptosis was measured through caspase 3/7 activation in HS68 cells. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 Error bars are SEM.
Figure 3.

G1T28 inhibits cellular proliferation in bone marrow leading to chemoprotection in vivo. A, FVB Mice received a single dose of 50, 100, or 150 mg/kg G1T28 by oral gavage, and were pulsed with 100 µg of EdU 1 hour before harvest. Bone marrow was harvested at either 12 or 24 hours after G1T28 administration and Lineage-negative cells (Lin-) were isolated. Data shows relative proliferation of Lin- cells at the indicated times following G1T28 administration. B, Caspase activation in mouse bone marrow post Etoposide treatment. Mice were given either 3 mg/kg etoposide, only, or etoposide and G1T28 (50 or 100 mg/kg) 30 minutes prior to etoposide. Bone marrow harvested 6 hours post treatment shows a decrease in caspase 3/7 activation with G1T28 treatment. C, FVB mice were treated with G1T28 150 mg/kg or vehicle control by oral gavage thirty minutes prior to administration of 5FU 150 mg/kg by ip injection. CBCs were measured every two days starting on day six. Data reported are from day 6 (Platelets), day 10 (white blood cells [WBC], neutrophils [Neu], lymphocytes [Lymph]), or day 16 (red blood cells [RBC]). CBC analysis shows mice treated with 150 mg/kg 5-FU and 100 mg/kg G1T28 (30 minutes prior to 5-FU) show a faster recovery in all peripheral blood lineages.

**p≤0.01, ***p≤0.001, ****p≤0.0001. Error bars depict SEM.

Figure 4.

Cells that are CDK4/6- independent are RB null and G1T28 does not cause protection from chemotherapy treatment. A, Western blot analysis of SCLC cell lines (H69, H82, H209, H345, Shp77) demonstrate these cells are RB-null, whereas
known CDK4/6-dependent cells (HS68 and SupT1) express RB. Two representative CDK4/6-independent cell lines (SHP77 and H69) were treated with G1T28 or DMSO for 24 hours. Cell cycle analysis was evaluated by propidium iodide incorporation and flow cytometry and demonstrates that the proportion of cells in the G1 phase does not change with G1T28 exposure. C, The CDK4/6 independent H69 and SHP77 SCLC cell lines were treated with the indicated doses of G1T28 or DMSO and either cisplatin or etoposide. Caspase 3/7 activity was measured and demonstrates no change with G1T28 exposure. All samples were normalized to the DMSO + DMSO treatment. Error bars depict SEM.

**Figure 5.**

G1T28 does not protect the effect of chemotherapy on CDK4/6 independent, RB null, SCLC *in vivo*. H69 SCLC cells were implanted into immune-deficient mice and the study initiated at a mean tumor volume of approximately 150-250 mm³. G1T28 administered alone or thirty minutes before topotecan was well tolerated with no additive weight loss or toxicity. G1T28 was dosed at 10, 50, or 100 mg/kg/dose. Topotecan 0.6 mg/kg/dose was administered by I.P. injection daily for 5 days of each week for four consecutive weeks. G1T28 does not decrease the efficacy of topotecan in the RB null H69 SCLC cell line.

* p≤0.05, topotecan only compared to 50 mg/kg G1T28+Topotecan. ∨ p≤0.05, topotecan only compared to 100 mg/kg G1T28+Topotecan. Error bars depict SEM. Yellow bar indicates duration of dosing.
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