

Targeting of cyclin-dependent kinases in atypical teratoid rhabdoid tumors with multikinase inhibitor TG02

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OBJECTIVE Atypical teratoid rhabdoid tumors (ATRTs) are aggressive pediatric brain tumors with no current standard of care and an estimated median patient survival of 12 to 18 months. Previous genetic analyses have implicated cyclin D1 and enhancer of zeste homolog 2 (EZH2), a histone methyltransferase that is implicated in many cancers, as key drivers of tumorigenicity in ATRTs. Since the effects of EZH2 and cyclin D1 are facilitated by a host of cyclin-dependent kinases (CDKs), the authors sought to investigate the potential therapeutic effects of targeting CDKs in ATRTs with the multi-CDK inhibitor, TG02.

METHODS Human ATRT cell lines BT12, BT37, CHLA05, and CHLA06 were selected for investigation. The effects of TG02 on cell viability, proliferation, clonogenicity, and apoptosis were assessed via Cell Counting Kit-8 assays, cell counting, clonogenic assays, and flow cytometry, respectively. Similar methods were used to determine the effects of TG02 combined with radiation therapy (RT) or cisplatin. Synergism indices for TG02-cisplatin combination therapy were calculated using CompuSyn software.

RESULTS TG02 was observed to significantly impair ATRT cell growth in vitro by limiting cell proliferation and clonogenicity, and by inducing apoptosis. TG02 inhibited ATRT cell proliferation and decreased cell viability in a dose-dependent manner with nanomolar half maximal effective concentration (EC_{50}) values (BT12, 207.0 nM; BT37, 127.8 nM; CHLA05, 29.7 nM; CHLA06, 18.7 nM). TG02 (150 nM) dramatically increased the proportion of apoptotic ATRT cells 72 hours posttreatment (TG02 8.50% vs control 1.52% apoptotic cells in BT12, $p < 0.0001$; TG02 70.07% vs control 15.36%, $p < 0.0001$). Combination therapy studies revealed that TG02 acted as a potent radiosensitizer in ATRT cells (BT12 surviving fraction, RT 51.2% vs RT + TG02 21.7%). Finally, CompuSyn analysis demonstrated that TG02 acted synergistically with cisplatin against ATRT cells at virtually all therapeutic doses. These findings were consistent in cell lines that cover all three molecular subgroups of ATRTs.

CONCLUSIONS The results of this investigation have established that TG02 is an effective therapeutic against ATRTs in vitro. Given the lack of standard therapy for ATRTs, these findings help fill an unmet need and support further study of TG02 as a potential therapeutic option for patients with this deadly disease.

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KEYWORDS atypical teratoid rhabdoid tumor; TG02; cyclin-dependent kinase; inhibition; oncology

ATYPICAL teratoid rhabdoid tumors (ATRTs) are highly aggressive pediatric CNS malignancies, accounting for approximately 1% to 2% of all pediatric brain tumors. With a median age of diagnosis of 1.2 years, ATRTs account for up to 20% of brain tumors in children less than 3 years of age.^{1,2} Related to rhabdoid tumors of the kidney, ATRTs in the CNS were first defined in 1996 as an embryonal tumor that is radiologically indistinguishable but immunohistochemically distinct from

medulloblastoma.³ Despite the morphological similarities between ATRTs and medulloblastomas, ATRTs historically have a more dismal prognosis with a median survival of 12 to 18 months.^{4,5}

While multimodal chemotherapy has produced modest improvements in overall survival, the 2-year survival rate remains around 50%, with a 5-year survival rate of less than 20%.^{6–8} Furthermore, ATRTs treated with multimodal chemotherapy typically recur at approximately 6 months.^{9,10}

ABBREVIATIONS AnnV = annexin-V; ATRT = atypical teratoid rhabdoid tumor; CCK-8 = Cell Counting Kit-8; CDK = cyclin-dependent kinase; CI = combination index; DRI = drug reduction index; EC_{50} = half maximal effective concentration; EZH2 = enhancer of zeste homolog 2; GBM = glioblastoma; PBS = phosphate-buffered saline; PI = propidium iodide; RT = radiation therapy.

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Radiation therapy (RT) has also been associated with improved survival in ATRTs when administered early to the entire craniospinal axis.^{11,12} However, craniospinal irradiation is generally contraindicated in children younger than 3 years of age due to undesired long-term cognitive and neuroendocrine sequelae.¹³ Given the early median age of diagnosis for ATRTs, balancing prompt initiation of RT with mitigation of long-term adverse sequelae has proven to be particularly difficult. Taken together, these treatment challenges have made ATRT a formidable malignancy with no consensus on a therapeutic regimen.^{8,14}

Recent efforts to characterize the genetic landscape of ATRTs have provided insight into potentially promising targets for new therapeutics. In particular, mutations in *SMARCB1*, a core component of the switch/sucrose nonfermentable (SWI/SNF) chromatin remodeling complex, have been shown to amplify the expression of cyclin D1, which interacts with cyclin-dependent kinases (CDKs) to promote ATRT cell proliferation and survival.^{15,16} *SMARCB1* downregulation is also promoted by enhancer of zeste homolog 2 (EZH2), a downstream effector of CDK1/2 and a catalytic subunit of the polycomb repressive complex 2 (PRC2).^{17,18} Due to the importance of CDKs in the activation and expression of both cyclin D1 and EZH2, CDK inhibition has become a growing strategy for antitumor therapy. TG02 is a novel multikinase inhibitor that has high affinity for multiple CDKs at nanomolar concentrations. While first studied in leukemia with encouraging preclinical results and ongoing clinical trials, TG02 has more recently been investigated for CNS malignancies. Su et al. investigated the use of TG02 in glioblastomas (GBMs) and found that TG02 achieved good blood-brain barrier penetration, inhibited cellular proliferation, synergized with temozolomide, and ultimately prolonged survival in orthotopic GBM mouse models.¹⁹ Given its ability to achieve effective concentrations in the brain and documented efficacy in GBMs, TG02 remains a potentially promising but understudied therapeutic for CNS malignancies.

Methods

Cell Lines and Cell Culture

Human ATRT cell lines BT12, BT37, CHLA05, and CHLA06 were obtained from the Rubens Laboratory (Johns Hopkins University). BT12 and BT37 cells were maintained in RPMI 1640 medium (ThermoFisher Scientific Inc.) supplemented with 10% fetal bovine serum (Lonza) and 1% glutamine (ThermoFisher Scientific Inc.) at 37°C in 5% CO₂-humidified incubators. CHLA05 and CHLA06 cells were maintained with a 7:3 mixture of DMEM (ThermoFisher Scientific Inc.) and Ham's F12 media (ThermoFisher Scientific Inc.) supplemented with 1% glutamine, 2% B27 without vitamin A (ThermoFisher Scientific Inc.), 20 ng/mL epidermal growth factor (PeproTech Inc.), 20 ng/mL fetal growth factor 2 (PeproTech Inc.), and 5 µg/mL heparin sodium salt (Sigma-Aldrich). When appropriate, cells were treated with desired concentrations of TG02 (MedChemExpress) or phosphate-buffered saline (PBS) (ThermoFisher Scientific Inc.) as a vehicle control.

Cell Proliferation Assay

ATRT cells were treated as indicated with desired concentrations of TG02 or PBS as a vehicle control. BT12 and BT37 cell lines were treated with 25 nM, 50 nM, and 100 nM TG02. CHLA05 and CHLA06 cell lines were treated with 5 nM, 10 nM, and 25 nM TG02. Initially, 2.5 × 10⁴ cells were plated, then collected and counted using a Malassez slide (Invitrogen) on posttreatment days 2, 4, and 6. Photographs of cells on posttreatment day 6 were taken with a camera-mounted microscope (Zeiss).

Cell Counting Kit-8 Assay

ATRT cells were seeded in 96-well plates at 1 × 10⁴ cells per well. Cells were then treated with TG02, cisplatin (Sigma-Aldrich), or a combination of both drugs and were incubated for 48 hours (BT12, BT37, and CHLA06) or 96 hours (CHLA05). ATRT cell viability was determined using the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies Inc.) protocol and quantified using a microplate reader (PerkinElmer Inc.).

Clonogenic Cell Survival Assay

The clonogenic cell survival assay was performed as previously described.²⁰ ATRT cells were treated with TG02 and/or radiation (CIXD irradiator, Xstrahl) at indicated doses. Cells were incubated for 10 days, fixed with 100% methanol (Sigma-Aldrich), and subsequently stained with a solution of 0.5% crystal violet (Sigma-Aldrich) in 25% methanol. Plating efficiency and surviving fraction were normalized and quantified as described previously.²⁰

Flow Cytometry

ATRT cells were seeded in 6-well plates and treated with 150 nM TG02 for 48 or 72 hours. They were then collected, washed in PBS, and prepared for flow cytometry according to the manufacturer's instructions. Cells were labeled with APC-AnnexinV/Dead Cell Apoptosis Kit (Invitrogen) for cell death. Cells were then analyzed on a flow cytometer (FACS Calibur, Becton-Dickinson). Subsequent analyses were performed using FlowJo software (FlowJo LLC).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 9.1.0 (GraphPad Software, Inc.). Data are shown as mean ± standard error and are representative of at least three independent experiments. Significance is represented where *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. One-way ANOVA or independent Student t-tests were used for statistical comparisons. Drug synergy was analyzed using CompuSyn software (CompuSyn Inc.).²¹

Results

TG02 Inhibits ATRT Cell Proliferation and Induces Cell Apoptosis

In this study, we tested the effects of TG02 on cell proliferation in multiple ATRT cell lines by treating cells with varying concentrations of TG02 and counting viable

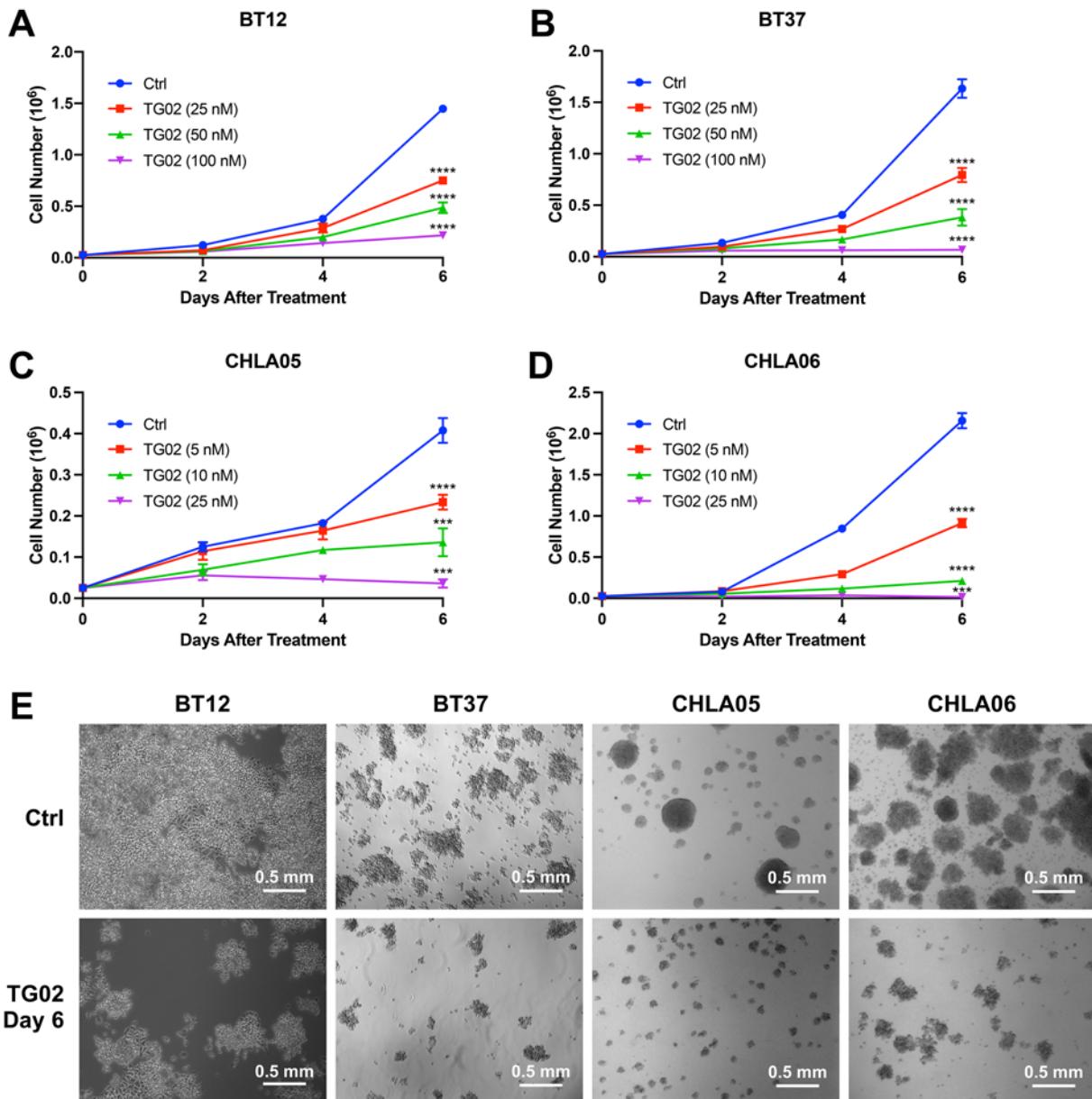


FIG. 1. TG02 inhibits ATRT cell proliferation in a dose-dependent manner. **A** and **B**: Cell growth/viability assays performed with BT12 and BT37 cells demonstrate a decreased cell number after treatment with TG02 (blue curve indicates control [Ctrl] PBS vehicle, red curve indicates TG02 25 nM, green curve indicates TG02 50 nM, and purple curve indicates TG02 100 nM). **C** and **D**: Cell growth/viability assays performed with CHLA05 and CHLA06 also showed decreased cell numbers after treatment (blue curve indicates control PBS vehicle, red curve indicates TG02 5 nM, green curve indicates TG02 10 nM, and purple curve indicates TG02 25 nM). **E**: Representative images of ATRT cells treated with TG02 (50 nM for BT12 and BT37, 10 nM for CHLA05 and CHLA06) or the control for 6 days. Bar = 0.5 mm. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ TG02 versus control, $n = 3$.

cells at posttreatment days 2, 4, and 6. By day 6, cell proliferation was significantly inhibited by TG02 in a dose-dependent fashion for BT12 (control, 1.45×10^6 ; 25 nM, 7.50×10^5 ; 50 nM, 4.87×10^5 ; 100 nM, 2.17×10^5 ; $p < 0.0001$), BT37 (control, 1.63×10^6 ; 25 nM, 7.93×10^5 ; 50 nM, 3.83×10^5 ; 100 nM, 6.67×10^4 ; $p < 0.0001$), CHLA05 (control, 4.08×10^5 ; 5 nM, 2.33×10^5 ; 10 nM, 1.36×10^5 ; 25 nM, 3.58×10^4 ; $p < 0.0001$), and CHLA06 (control, 2.16×10^6 ; 5 nM, 9.13×10^5 ; 10 nM, 2.12×10^5 ; 25 nM, 1.58×10^4 ; $p < 0.0001$) (Fig. 1A–D). The corresponding images of the

treatment groups taken on day 6 showed a clear decrease in viable cell number with respect to TG02 (50 nM for BT12 and BT37, 10 nM for CHLA05 and CHLA06) (Fig. 1E). To further characterize the effects of TG02 on ATRT cell growth and proliferation, we then conducted cell viability analyses (Fig. 2A–D). The half maximal effective concentration (EC_{50}) of TG02 was 207.0 nM in BT12, 127.8 nM in BT37, 29.7 nM in CHLA05, and 18.7 nM in CHLA06 cells.

Using flow cytometry with annexin-V (AnnV) and

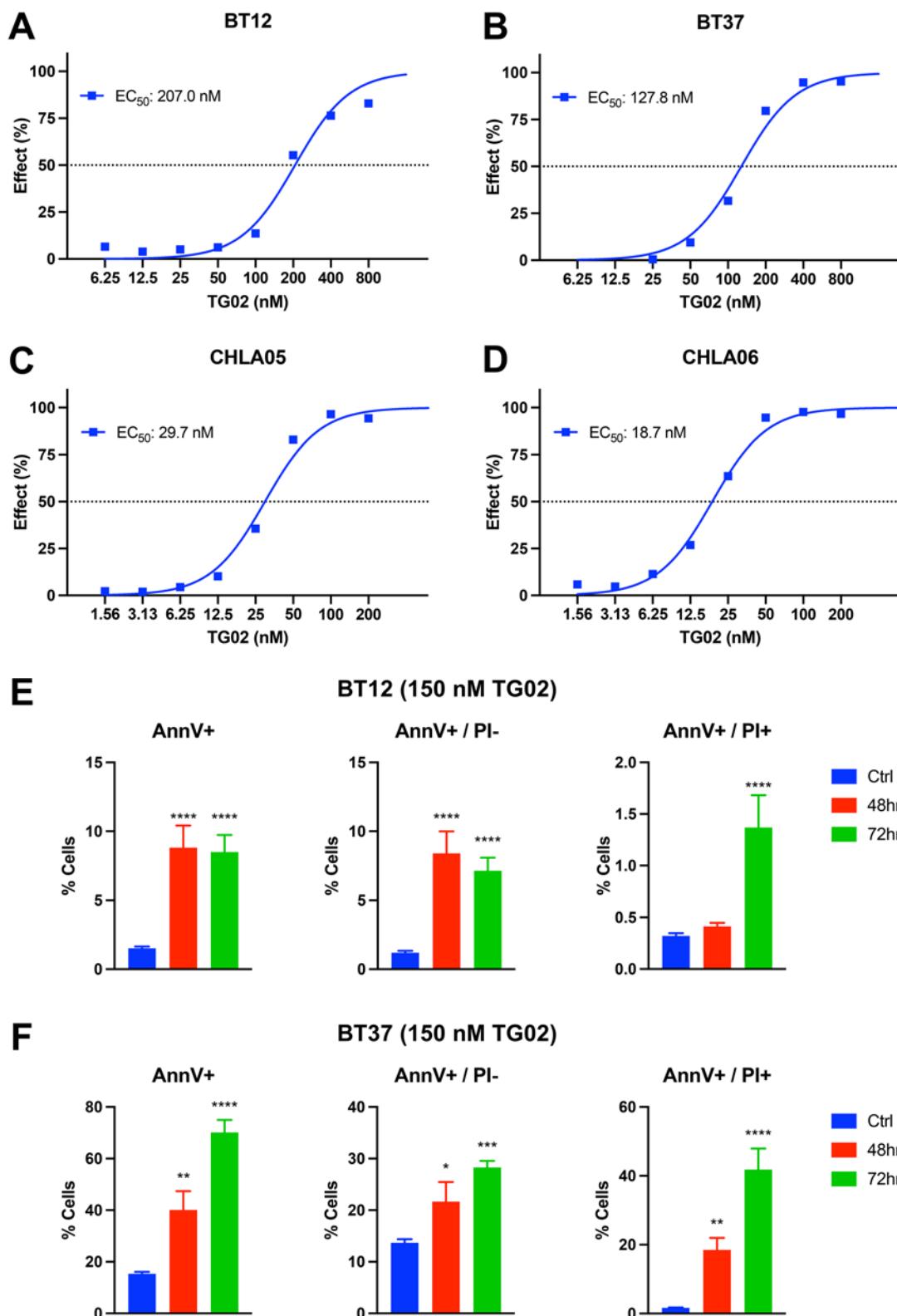


FIG. 2. TG02 achieves effective cell death at nanomolar concentrations and induces apoptosis in ATRT cells. **A–D:** CCK-8 assays performed with BT12, BT37, CHLA05, and CHLA06 cells show effective cell death after 48 hours (or after 96 hours for CHLA05) of treatment with TG02 at varying concentrations. EC₅₀ values represent the concentration of TG02 needed to achieve 50% cell death for each cell line. **E and F:** Quantification of cell death for BT12 and BT37 cells using flow cytometry and AnnV/PI staining 48 and 72 hours after treatment with TG02 (150 nM). TG02 significantly increased the percentage of total apoptotic (AnnV+) cells by 72 hours of treatment in both cell lines. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 TG02 versus control, n = 3.

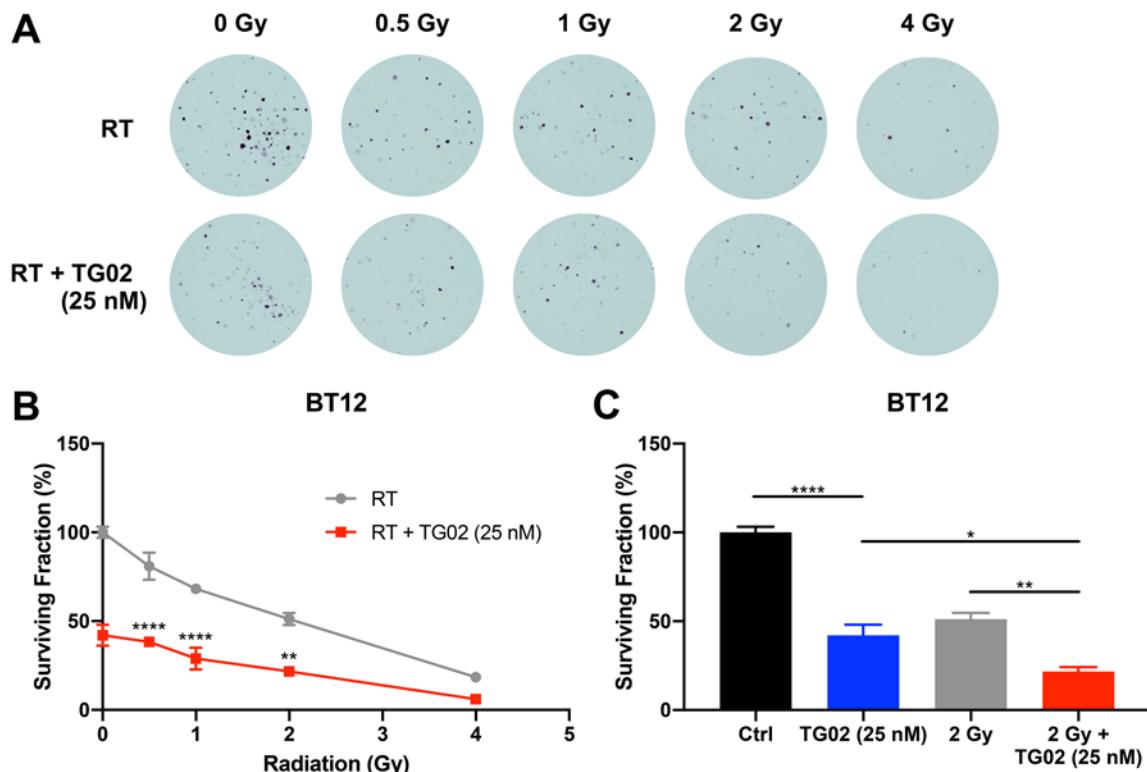


FIG. 3. TG02 sensitizes ATRT cells to radiation. **A:** Representative images of BT12 cells treated with RT (0 Gy, 0.5 Gy, 1 Gy, 2 Gy, 4 Gy) or TG02 (25 nM) + RT. RT led to a decreased number of colonies (> 50 cells) in a dose-dependent fashion. Combination treatment further decreased the number of colonies compared with RT alone. **B:** Quantification of clonogenic assays for BT12 cells following treatment with TG02 (25 nM) and varying doses of RT. The addition of TG02 significantly decreased the number of colonies compared with RT alone. **C:** Quantification of clonogenic assays for BT12 cells following treatment with TG02 (25 nM), RT (2 Gy), or combination therapy. TG02 + RT significantly decreased the number of colonies compared with either treatment alone. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ TG02 versus control, $n = 3$.

propidium iodide (PI) staining on BT12 cells (Fig. 2E), we demonstrated that treatment with 150 nM TG02 significantly increased the total percentage of AnnV+ cells after 48 hours of treatment (control 1.52%; TG02 48 hours 8.80%, TG02 72 hours 8.50%; $p < 0.0001$). We then performed separate analyses on AnnV+/PI- and AnnV+/PI+ cells to delineate cell populations undergoing early and late apoptosis, respectively. There was a significant increase in early apoptotic (AnnV+/PI-) cells by 48 hours of treatment (control 1.20%; TG02 48 hours 8.39%, TG02 72 hours 7.13%; $p < 0.0001$), whereas late apoptotic (AnnV+/PI+) cells were significantly increased by 72 hours of treatment (control 0.32%; TG02 48 hours 0.41%, TG02 72 hours 1.37%; $p < 0.0001$). Although there was a decrease in the mean percentage of AnnV+/PI- BT12 cells 72 hours after treatment compared with 48 hours after treatment, this difference was not statistically significant. These findings were consistent in BT37 cells (Fig. 2F), which showed a robust increase in early apoptotic (control 13.68%; TG02 48 hours 21.63%, $p < 0.05$; TG02 72 hours 28.27%, $p < 0.001$) and late apoptotic (control 1.684%; TG02 48 hours 18.47%, $p < 0.01$; TG02 72 hours 41.80%, $p < 0.0001$) cells after treatment with 150 nM TG02. Altogether, these findings suggest that TG02 inhibits ATRT proliferation and subsequently induces apoptosis.

TG02 Acts as a Radiosensitizer in ATRT Cells

To investigate the radiosensitizing effects of TG02 on ATRTs, we conducted clonogenic assays on BT12 cells, which were treated with RT (0.5, 1, 2, and 4 Gy), TG02 (25 nM), or combination therapy 24 hours after plating and were stained and quantified 7 days after treatment. Visually, RT alone inhibited the clonogenic capacity by decreasing the percentage of surviving colonies (> 50 cells) in a dose-dependent fashion (Fig. 3A). Combination therapy with RT + TG02 significantly decreased BT12 clonogenicity compared with RT alone, decreasing the percentage of quantified colonies to 41.4% that of the RT-alone group (Fig. 3B). In particular, BT12 cells treated with RT (2 Gy) + TG02 (25 nM) were found to have a significantly decreased percentage of surviving colonies compared with RT or TG02 monotherapies (control 100% vs TG02 42.1% vs RT 51.2% vs RT + TG02 21.7%) (Fig. 3C).

TG02 Acts Synergistically With Cisplatin in ATRT Cells

To investigate drug-drug interactions between TG02 and cisplatin, we conducted CCK-8 assays on BT12, BT37, and CHLA05 cells comparing combination therapy with TG02 and cisplatin monotherapies (Fig. 4A). The addition of TG02 to a constant cisplatin-to-TG02 concentration ra-

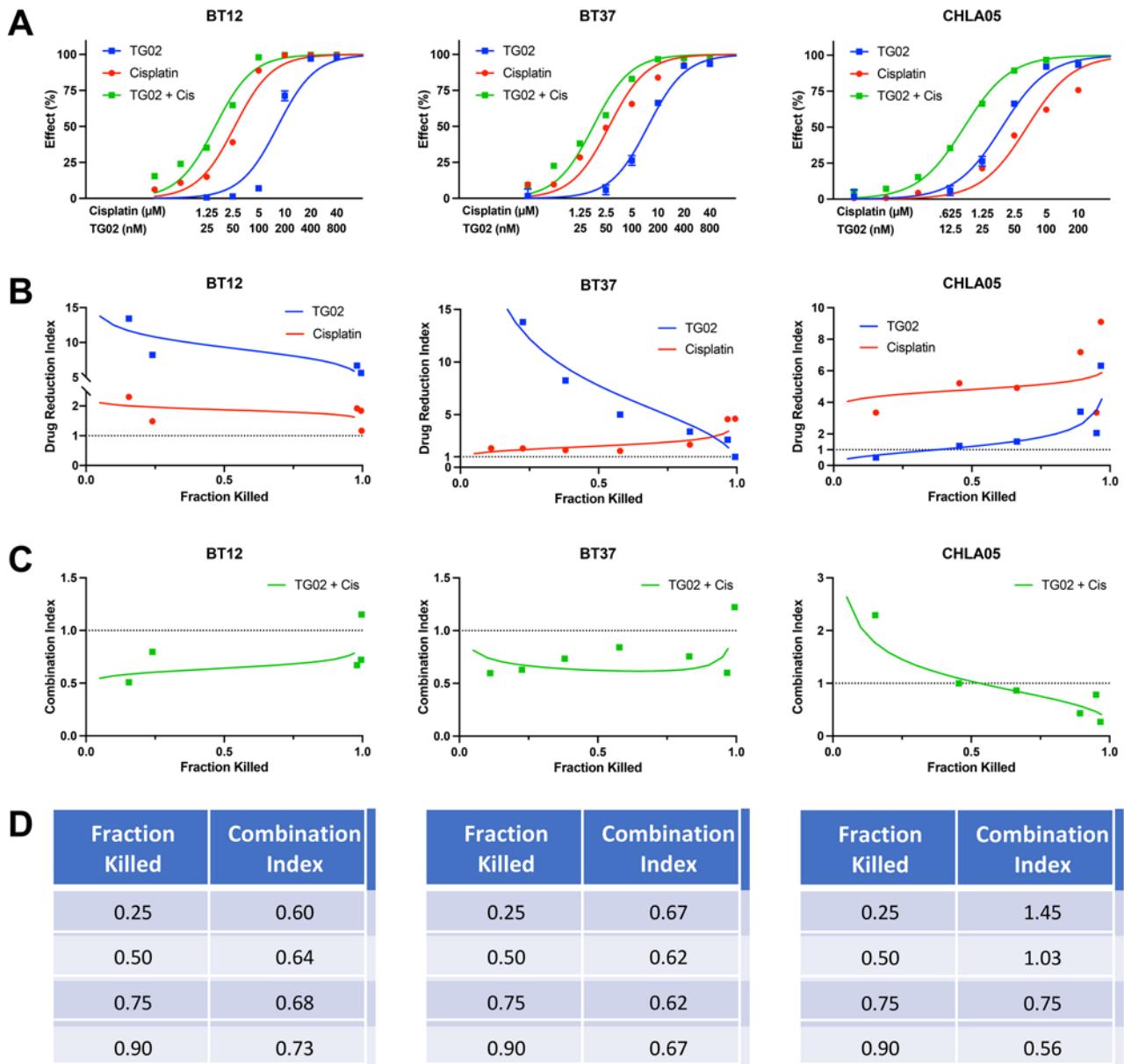


FIG. 4. TG02 acts synergistically with cisplatin in ATRT cells. **A:** CCK-8 assays for BT12, BT37, and CHLA05 cells after 48 hours (or after 96 hours for CHLA05) of treatment with TG02 (blue curve), cisplatin (red curve), or combination therapy in a cisplatin (Cis)–to-TG02 concentration ratio of 50:1 (green curve). **B:** DRI calculations made by CompuSyn software show that addition of TG02 consistently lowers the concentration of cisplatin needed to achieve cytotoxic effects in BT12, BT37, and CHLA05 cells. DRI > 1 is a reduction, DRI = 1 is maintenance, and DRI < 1 is an increase in the concentration of drug needed in combination therapy to achieve the same effect compared with monotherapy. **C** and **D:** Synergistic effects of TG02 and cisplatin combined in BT12, BT37, and CHLA05 cells were determined by CI calculations with CompuSyn software. CI < 1 is a synergistic, C = 1 is an additive, and C > 1 is an antagonistic effect of two combined drugs.

tio of 50:1 was found to be a synergistic combination and significantly decreased the EC_{50} in ATRT cells compared with cisplatin alone (BT12 cisplatin 2.643 μM vs combination 1.569 μM ; BT37 cisplatin 2.727 μM vs combination 1.787 μM ; CHLA05 cisplatin 3.298 μM vs combination 0.847 μM). Drug reduction index (DRI) calculations for TG02 and cisplatin were performed using CompuSyn to compare combination therapy with the monotherapy doses needed to achieve desired cytotoxic effects (Fig. 4B).

On average, combination therapy was able to match the cytotoxic effects of cisplatin or TG02 monotherapy using 46.3% less cisplatin and 89.3% less TG02 for BT12 (cisplatin average DRI, 1.86; TG02 average DRI, 9.34), 53.3% less cisplatin and 88.8% less TG02 for BT37 (cisplatin average DRI, 2.14; TG02 average DRI, 8.96), and 79.5% less cisplatin and 34.6% less TG02 for CHLA05 (cisplatin average DRI, 4.88; TG02 average DRI, 1.53). Of particular relevance, to achieve 97% cell death, combina-

tion therapy used 38.5% less cisplatin for BT12 (DRI 1.63), 70.8% less cisplatin for BT37 (DRI 3.43), and 76.2% less cisplatin for CHLA05 (DRI 5.88) compared with cisplatin monotherapy.

Combination index (CI) calculations were then performed using CompuSyn, as previously described,²¹ to assess for potential synergistic effects between TG02 and cisplatin (Fig. 4C and D). Calculated CI values were consistently below 1 for BT12 and BT37 cell lines, indicating synergism between TG02 and cisplatin across virtually all combinations of doses tested. CI values for CHLA05 were below 1 for concentrations that achieved greater than 50% cell death.

Discussion

Study Rationale

ATRTs are of the most common and aggressive CNS malignancies in infants and currently have no consistent therapeutic regimen. Despite challenges in treatment, recent efforts to characterize the molecular profile of ATRTs have demonstrated consistent loss or mutation of *SMARCB1*. Abrogation of the *SMARCB1* axis is associated with overexpression of cyclin D1 and EZH2, driving tumor growth in ATRTs. Previous genetic analyses have similarly implicated cyclin D1 as a key driver of tumorigenicity in ATRTs, with overexpression of cyclin D1 reported in virtually all cases.^{22,23} Cyclin D1 has been well characterized as a protein that facilitates progression through the G1 phase of the cell cycle by forming a complex with CDK4 and CDK6.^{16,24} With the traditional association between cyclin D1 and CDK4/6 in mind, other groups have previously studied the effect of CDK4/6 inhibition in ATRTs.^{25,26} However, phase I clinical trials involving CDK4/6 inhibition (identifier no. NCT01747876, ClinicalTrials.gov) did not demonstrate efficacy. Our group surmised from these results that CDK4/6 targeting alone does not provide sufficient therapeutic effects in ATRTs. There are, however, other CDKs that are associated with cyclin D1 expression and activity. Of importance, levels of cyclin D1 have been correlated with activity levels of CDK9, a kinase that forms a super-enhancer complex with cyclin T1 to recruit RNA polymerase II.²⁷ This same mechanism of transcription induction is also responsible for the expression of other oncogenes such as myeloid cell leukemia sequence 1 (MCL1) and MYC.²⁸ Interestingly, a recent study demonstrated that cyclin D1 forms inactive complexes with CDK9, thereby decreasing global transcription of essential maintenance genes in the cell.²⁹ As a result, further inhibition of CDK9 with small-molecule inhibitors in cells that overexpress cyclin D1 could potentially induce cellular death through synthetic lethality. Due to these associations between CDK9 and cyclin D1, CDK9 inhibition has become an emerging target of molecular therapy for cancers associated with cyclin D1 overexpression.

Traditionally, downregulation of CDKs was considered necessary for cell cycle arrest to allow for DNA repair mechanisms. A growing body of evidence now supports a secondary role of CDKs as inducers of DNA repair. In particular, CDK1 and CDK2, considered the master regulators

of the cell cycle, have been associated with the recruitment of proteins involved in repairing double-stranded DNA breaks.³⁰ Furthermore, these kinases have been shown to compensate for each other when one is inhibited, suggesting that inhibition of both CDK1 and CDK2 is necessary to effectively impair cellular response to DNA damage.³¹ Since RT primarily acts through DNA damage, CDK inhibitors have unsurprisingly demonstrated promising radiosensitizing effects in various solid tumors.^{32–34} Comitant CDK inhibition with RT therefore has the potential to decrease the dose of radiation needed for effective therapeutic management. This is of particular importance for patients with ATRTs, most of whom are diagnosed at less than 3 years of age and are poor candidates for RT.

Key Results and Interpretation

Based on previous work evaluating TG02 in GBMs,¹⁹ we chose to evaluate the effect of TG02 on ATRT cell lines using a similar range of concentrations. While proliferation assays with TG02 determined a broad range of concentrations that were effective for each cell line, our CCK-8 assays subsequently examined the effect of TG02 at a more granular concentration range. These results together show strong evidence that TG02 significantly impairs proliferation of ATRT cells in a dose-dependent fashion at nanomolar concentrations, which corroborate other studies that have measured TG02 EC₅₀ values ranging from 36 nM to 287 nM.^{19,35,36} Given the clear need for a multimodal approach to ATRT treatment, our group was particularly interested in examining the interaction between TG02 and other therapeutics that have been used clinically against ATRTs, including radiation and cisplatin.^{37–39}

RT poses a dilemma for the treatment of ATRTs, as it has been associated with prolonged survival but can cause devastating neurocognitive sequelae in young patients.³⁹ CDK inhibitors have previously been shown to sensitize tumor cells to RT through disruption of DNA damage repair and therefore decrease doses necessary for adequate antitumor effects.^{32,34} Based on our clonogenic assays, while RT alone decreased the clonogenic capacity of ATRT cells in a dose-dependent fashion, the addition of 25 nM TG02 was enough to further inhibit clonogenicity by more than 50% in BT12 cells. These findings suggest that the effective dose of radiation necessary to achieve antitumor effects can be significantly decreased with the addition of TG02, thereby decreasing potential adverse effects associated with radiation.

A major challenge in ATRTs is the development of treatment resistance, often to cisplatin, which has been linked to CDK-dependent mechanisms involving Akt and cyclin D1.⁴⁰ We hypothesized that TG02 would potentiate cisplatin toxicity by modulating these resistance pathways. Indeed, the addition of TG02 reliably and significantly decreased the EC₅₀ of cisplatin compared with monotherapy. While effective cytotoxic doses of cisplatin are in the micromolar range, TG02 achieves an effective response at nanomolar concentrations. Given the discrepancy of effective doses between these two compounds, a cisplatin-to-TG02 ratio of 50:1 was sufficient to confer synergy between the two compounds. Synergy calculations were performed using the Chou-Talalay method, a widely used

method for quantifying synergistic interactions in compounds that are combined at fixed ratios.²¹ The major quantitative indices from this analysis are the CI and the DRI, both of which are derived from the unified theory of the median-effect equation. For a given effect level, CI < 1 indicates that the combination of compounds resulted in synergism, or a stronger effect than the additive effects of each compound alone. DRI, on the other hand, represents the fold reduction of the dose of each compound for a given effect level when used in a synergistic combination, compared with the doses of each compound alone. Our CI analysis has shown that at all effective concentrations, TG02 and cisplatin exhibit synergy in combination therapy. Furthermore, our DRI calculations have shown that a cisplatin-to-TG02 ratio of 50:1 caused a significant reduction in the dose of cisplatin needed for effective therapy. This combination could therefore potentially reduce serious adverse effects of cisplatin in pediatric patients.

ATRT Molecular Subgroups

The genetic heterogeneity of ATRTs has been extensively studied, with the most recent consensus dividing ATRTs into three distinct molecular subgroups: ATRT-SHH, ATRT-TYR, and ATRT-MYC.⁴¹ Since these subgroups exhibit different molecular expression profiles (ATRT-SHH overexpresses SHH pathway genes, ATRT-TYR overexpresses melanosomal genes, and ATRT-MYC overexpresses MYC and HOX cluster genes), their responses to therapy are subject to variation. With these distinctions in mind, the ATRT cell lines evaluated in this study represent all three molecular subgroups of ATRTs. Based on molecular signatures, BT12 and CHLA06 have been classified as ATRT-MYC cell lines, while BT37 and CHLA05 have been categorized as ATRT-TYR and ATRT-SHH cell lines, respectively.⁴¹ Given that TG02 significantly decreased proliferation in all four of these cell lines at nanomolar concentrations and that it synergized with cisplatin in BT12, BT37, and CHLA05, we have provided evidence for TG02 as an effective therapy for all molecular subgroups of ATRTs.

Prior CDK Inhibition in ATRTs

Previous studies involving CDK inhibition in ATRTs focused primarily on targeting the CDK4/6 axis. One pre-clinical study demonstrated that CDK4/6 inhibition with palbociclib effectively decreased cellular proliferation in vitro, prolonged survival in murine models, and sensitized tumor cells to RT for both ATRT and GBM cell lines.²⁶ CDK4/6 inhibition was subsequently studied in a clinical trial involving the selective CDK4/6 inhibitor LEE011 (identifier no. NCT01747876, ClinicalTrials.gov). However, enrollment in the study was stopped due to lack of efficacy. In contrast to LEE011 and palbociclib, TG02 not only inhibited CDK4/6 but also inhibited other CDKs associated with cyclin D1 expression and activity, namely CDK1, CDK2, and CDK9.³⁵ Based on prior literature, we posit that these alternative pathways may be partially responsible for the lack of efficacy observed with selective CDK4/6 inhibition alone.

Multi-CDK inhibition with flavopiridol has also been studied in malignant rhabdoid tumors, with an EC₅₀ of approximately 200 nM. However, resistance to the drug was

correlated with overexpression of cyclin D1. In the context of intracranial ATRTs, TG02 has a number of characteristics that may provide more clinical benefit than flavopiridol. In particular, unlike flavopiridol, TG02 is not rapidly cleared from systemic circulation.⁴² Additionally, TG02 has EC₅₀ values for its molecular targets that are significantly lower than those of flavopiridol and has been shown to achieve concentrations in the brain that are 2.4 times that measured in plasma, making it a promising therapeutic with effective blood-brain barrier penetrance.^{35,43,44} Taken together, TG02 may represent a more promising drug than prior CDK inhibitors that is effective at low concentrations and capable of achieving these therapeutic concentrations intracranially.

Clinical Relevance

As mentioned, two appealing aspects of TG02 for ATRT treatment are its potent antitumor effects at low therapeutic concentrations and its ability to cross the blood-brain barrier. Both characteristics are especially important for intracranial tumors, which typically require increasing doses of systemically administered therapies in order to achieve effective intracranial concentrations. Furthermore, TG02 is capable of augmenting existing therapies currently employed for ATRTs. Although early craniospinal RT has been shown to increase survival in patients with ATRTs, its use is largely limited to patients older than 3 years of age. We have shown that TG02 effectively radiosensitizes ATRT cells and can therefore decrease the effective dose needed to achieve an adequate antitumor response. As a result, TG02 could potentially close the gap for patients currently ineligible for RT. Additionally, TG02 potentiates antitumor response to cisplatin, a mainstay treatment in adjuvant chemotherapy regimens for ATRTs. By consequence, the addition of TG02 may reduce the doses of cisplatin needed to achieve adequate antitumor effects and has the potential to dampen drug-related toxicity.

Importantly, previous clinical studies have reported that TG02 has a favorable safety profile. The adverse events associated with TG02 are mild diarrhea, neutropenia, fatigue, and elevated transaminases, all of which were well tolerated at doses ≤ 250 mg per day. At these doses, TG02 monotherapy was also able to achieve an objective antitumor response. Moreover, pharmacokinetic studies in a phase I trial with TG02 showed that a plasma concentration of 3 μM TG02 is sustained with daily 50-mg oral dosing.⁴² Given its strong blood-brain barrier penetrance, oral administration of TG02 is expected to achieve intracranial concentrations that are significantly higher than the reported EC₅₀ values in this study. Based on these results and its suitability for intracranial malignancies, TG02 is currently under clinical investigation in combination with temozolamide and/or RT in adults with anaplastic astrocytomas and GBMs (identifier nos. NCT02942264, NCT03224104, ClinicalTrials.gov). To our knowledge, there are currently no studies or clinical trials investigating TG02 in pediatric patients, although we suspect that this may be a promising future direction of this drug.

Limitations and Future Directions

We recognize several limitations of our study. First,

although we investigated TG02 in four ATRT cell lines that represent all three ATRT molecular subgroups, the efficacy of TG02 should be verified in additional ATRT cell lines for further confirmation of its general use in ATRTs. Second, investigation of TG02 in ATRTs has been limited to *in vitro* studies. Further work is necessary to evaluate the efficacy of TG02 *in vivo*, ideally with intracranially implanted patient-derived xenograft models. In prior pre-clinical studies, TG02 exhibited a favorable oral bioavailability of 24% in mice with extensive tissue distribution.⁴⁵ Because of this, we believe the concentrations of TG02 listed in this study are achievable for *in vivo* work. Third, while the molecular characteristics of ATRTs are well known, the precise mechanism of action for TG02 was not evaluated in the present work and should be pursued in future studies. Previous studies of TG02 in GBMs have shown that TG02 decreases CDK9 activity but not expression. We expect similar results in ATRTs; mechanistic studies should include quantitative and activity analyses of CDK9 and cyclin D1, as well as cell cycle analysis to confirm this hypothesis and inform treatment planning with combination therapy regimens. We also note that since TG02 is a novel therapeutic, there is currently a lack of clinical data pertaining to its safety and efficacy in children. Future preclinical and clinical work is necessary to determine the full potential and safety profile of TG02 in children. Ultimately, we postulate that TG02 may represent a promising addition to combination treatments for ATRTs, although future studies will be needed to better understand and characterize these potential effects.

Conclusions

There is a strong clinical need for therapies that target multiple aspects of ATRT biology. We investigated the preclinical efficacy of the multikinase inhibitor TG02 as a targeted therapeutic in ATRTs based on the known associations between multiple CDKs, SMARCB1, cyclin D1, and EZH2. We demonstrated TG02 efficacy in multiple *in vitro* studies using cell lines that represent all three molecular subgroups of ATRTs. Through these studies, we have demonstrated the ability of TG02 to potentiate therapeutic responses for both RT and cisplatin. TG02 is an appealing candidate for further preclinical and clinical studies in ATRTs given its ability to cross the blood-brain barrier, low doses necessary for effect, radiosensitizing effects, and mild safety profile in humans. Given the results herein, and demonstrated efficacy of TG02 in other CNS malignancies, TG02 is a promising therapeutic agent that merits further preclinical and clinical evaluation in ATRTs.

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Disclosures

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Supplemental Information

Previous Presentations

An abstract regarding this body of work was presented as an e-poster for AANS Virtual 2020.

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