Disabling c-Myc in Childhood Medulloblastoma and Atypical Teratoid/Rhabdoid Tumor Cells by the Potent G-Quadruplex Interactive Agent S2T1-6OTD

Tarek Shalaby1, André O. von Bueren1, Marie-Louise Hürlimann1, Giulio Fiaschetti1, Deborah Castelletti1, Tera Masayuki3, Kazuo Nagasawa3, Alexandre Arcaro1, Ilian Jelesarov2, Kazuo Shin-ya4, and Michael Grotzer1

Abstract

We investigated here the effects of S2T1-6OTD, a novel telomestatin derivative that is synthesized to target G-quadruplex-forming DNA sequences, on a representative panel of human medulloblastoma (MB) and atypical teratoid/rhabdoid (AT/RT) childhood brain cancer cell lines. S2T1-6OTD proved to be a potent c-Myc inhibitor through its high-affinity physical interaction with the G-quadruplex structure in the c-Myc promoter. Treatment with S2T1-6OTD reduced the mRNA and protein expressions of c-Myc and hTERT, which is transcriptionally regulated by c-Myc, and decreased the activities of both genes. In remarkable contrast to control cells, short-term (72-hour) treatment with S2T1-6OTD resulted in a dose- and time-dependent antiproliferative effect in all MB and AT/RT brain tumor cell lines tested (IC50, 0.25–0.39 μmol/L). Under conditions where inhibition of both proliferation and c-Myc activity was observed, S2T1-6OTD treatment decreased the protein expression of the cell cycle activator cyclin-dependent kinase 2 and induced cell cycle arrest. Long-term treatment (5 weeks) with nontoxic concentrations of S2T1-6OTD resulted in a time-dependent (mainly c-Myc-dependent) telomere shortening. This was accompanied by cell growth arrest starting on day 28 followed by cell senescence and induction of apoptosis on day 35 in all of the five cell lines investigated. On in vivo animal testing, S2T1-6OTD may well represent a novel therapeutic strategy for childhood brain tumors. Mol Cancer Ther; 9(1); 167–79. ©2010 AACR.

Introduction

Medulloblastomas (MB) are the most common malignant pediatric neoplasms of the central nervous system (CNS) and represent >20% of all pediatric brain tumors (1). With current treatment strategies, nearly half of all patients will eventually die from progressive tumors. CNS atypical teratoid/rhabdoid tumors (AT/RT) are rare but highly malignant embryonal tumors in young children (2–5). Experience to date indicates that infants and children with CNS AT/RT respond very poorly to chemotherapy and radiotherapy (6–9). Accordingly, the identification of novel therapeutic strategies for MB and AT/RT remains a major goal.

Telomestatin is a natural G-quadruplex–intercalating agent isolated from Streptomyces anulatus 3533-SV4 (10). Telomestatin is a potent telomere maintenance-disabling drug in cervical carcinoma, breast cancer, multiple myeloma, leukemia, and neuroblastoma cells (11–14). Telomestatin suppresses cellular proliferation and induces apoptosis within a few days in various cancer cells but not in normal cells (15). Telomestatin has a characteristic macrocyclic ring system that consists of sequential pentaoxazoles, bis-methyloxazoles, and a thiazoline. It has been suggested that these structural features of telomestatin strongly bind to G-quartets by overlapping, and this interaction is believed to promote the formation and stabilization of G-quadruplex structures (16). To increase this interaction, installation of additional functional groups, such as carbonyl, hydroxyl, and/or amino groups, would be required. Based on this concept, telomestatin derivatives were designed, and S2T1-6OTD was synthesized as a derivative bearing a macrocyclic bisamide (15). Based on its three-dimensional structure, it had been speculated that S2T1-6OTD has a high affinity for G-quadruplex–forming sequences, including the nuclease hypersensitivity element III (17). This is a major transcriptional control element in the c-Myc promoter region, and it has been found to control up to 85% of total c-Myc transcription (17–23). c-Myc is a pleiotropic transcription factor that has been linked to diverse cellular functions, such as cell cycle
regulation (24), proliferation (25), and growth (26), and it has an important regulatory effect on telomerase activity (27, 28) that promotes immortalization. Aberrant c-Myc signaling has been observed in human cancers, and c-Myc has been shown to promote cell transformation and tumor progression (29–33). In childhood MB, high c-Myc mRNA expression and c-Myc gene amplification have been suggested as indicators of poor prognosis (33–43). Furthermore, high c-Myc mRNA expression was shown to be significantly associated with tumor anaplasia (44, 45).

In this study, we examined the short- and long-term effects of S2T1-6OTD in a representative set of childhood MB and AT/RT cells.

Materials and Methods

Tumor and Control Cells

DAOY human MB cells, CA46 and Ramos human Burkitt’s lymphoma cells, PC12 rat pheochromocytoma cells, and MRC-5 human untransformed fibroblast cells were purchased from the American Type Culture Collection. D341 and D425 MB cells were the kind gift of Dr. Henry Friedman (Duke University, Durham, NC). DAOY M2 (c-Myc vector-transfected) human MB cells have been described previously (45). BT-12 and BT-16 human CNS AT/RT cells were the kind gift of Dr. Peter Phillips (The Children’s Hospital of Philadelphia, Philadelphia, PA). Rat fibroblast cells HO15.19 [c-Myc knockout (–/–)] and TGR-1 c-Myc wild-type (rat fibroblast) were the kind gift of Prof. B. Amati (European Institute of Oncology, Milan, Italy). DAOY, D341, and D425 cells were cultured in Richter’s zcin option medium/10% fetal bovine serum (FBS); 1% nonessential amino acids were added to the medium for D341 and D425 cells and G418 was added to the medium for DAOY M2 to a concentration of 500 μg/mL). BT-12 and BT-16 cells were cultured in DMEM (with Glutamax)/10% FBS. CA46 and Ramos cells were grown in RPMI 1640 supplemented with 10% FBS. MRC-5 cells were grown in MEM supplemented with 5% FBS. PC12 cells were cultured in complete growth medium with FBS to a final concentration of 2.5% horse serum. HO15.19 and TGR-1 cells were cultured in DMEM with 8% calf serum. BJ foreskin fibroblasts were cultured in DMEM containing 10% iron-supplemented calf serum. All cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂. Cell lines that were not purchased from the American Type Culture Collection in 2009 were tested for their authentication by karyotypic analysis using molecular cytogenetic techniques, such as comparative genomic hybridization.

PCR-Stop Assay

PCR-stop assay was done as previously reported (46). Oligonucleotides ss-telo24 d[T TAGGGTTA GGT AGG TGGGA GAG], ss-Pu22 d[GAGGGTGGG GAGGGTGGGA GAG], mutated mut-ss-Pu22 d[GAGGGTAAGGGTGGGA GAG], and the complementary sequences of telo24 d[TCTCGTCTCCCTA A] (telo24 rev) and ss-Pu22 d[ATCGCTTC GCTTTCCCC CA] (ss-Pu22 rev) were used. The chain extension reaction was done in 1x PCR buffer containing 0.2 mmol/L deoxynucleotide triphosphate, 5 units Taq polymerase, 7.5 pmol oligonucleotides, and various concentrations of S2T1-6OTD and telomestatin. The mixtures were incubated in a thermocycler under the following conditions: 94°C for 2 min followed by 30 cycles of 94°C for 20 s, 47°C for 20 s, and 72°C for 20 s. Amplified PCR products were resolved on 12% native polyacrylamide gels in 0.5x Tris-borate EDTA buffer and stained with ethidium bromide. The IC₅₀ values were calculated based on the fluorescence intensity scanned with a phosphorimager (Typhoon 8600, Molecular Dynamics).

Circular Dichroism (CD) Spectroscopy

Circular dichroism (CD) experiments were carried out with a Jasco J-715 instrument equipped with a computer-controlled water thermostat. Optical path length was 1 mm. The spectra were calculated with J-715 Standard Analysis software (Japan Spectroscopic Co.). Each spectrum was recorded thrice, and the reported spectra represent the average of three scans at 5 nm/min and data pitch 0.2 nm. ss-Pu22 (GAGGGTGGGAGGGTGGGGA GAG) or ss-Telo24 d[T T A G G G C]₄ was diluted by Tris buffer (50 mmol/L, pH 7.0) at a concentration of 10 μmol/L. S2T1-6OTD was dissolved with 10 mmol/L stock solution and titrated into the DNA samples at 5 mol equivalent (the 10 mmol/L stock solution of S2T1-6OTD was made up in 10% DMSO–90% water). The DNA strand concentrations were 10 μmol/L, and the CD data are a representation of three averaged scans taken at 25°C. All CD spectra are baseline corrected for signal contributions due to the buffer and for buffer and DMSO for the samples containing S2T1-6OTD. Thermal melting experiments were done using 10 μmol/L of either ss-Pu22-forming or ss-Telo24-forming sequence in the presence of 50 μmol/L S2T1-6OTD in a Tris-HCl buffer (50 mmol/L, pH 7.4), and the ellipticity at 264 nm was monitored on continuous heating at 2°C/min between 20°C and 95°C.

Real-time Quantitative Reverse Transcription-PCR

Isolation of total RNA, cDNA synthesis, and kinetic real-time PCR quantification of hTERT and c-Myc mRNA were done as previously described (47, 48). Experiments were done in triplicate for each data point. The amount of hTERT and c-Myc, normalized to the endogenous control 18S RNA, was related to the commercially available calibrator human cerebellum (Clontech).

Western Blot Analysis

S2T1-6OTD–treated and control MB and AT/RT cells were washed twice in medium, centrifuged, and lysed in buffer [50 mmol/L Tris (pH 8), 150 mmol/L NaCl, 1% Triton X-100, 1% NP40, 0.1% SDS, 5 mmol/L EDTA] for 20 min on ice followed by centrifugation at 10,000 × g for 15 min. The crude lysates were heated for 5 min at...
95°C in the presence of 3% mercaptoethanol. Equal amounts of protein (30 μg/lane) from the homogenates were separated by 10% (w/v) SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). Nonspecific binding sites were blocked with 10 mmol/L TBS containing 0.1% Tween 20 and 10% nonfat milk. Membranes were then incubated overnight at 4°C with anti-c-Myc monoclonal antibody (Cell Signaling Technology, Inc.), anti-cyclin-dependent kinase 2 (CDK2) monoclonal antibody (Santa Cruz Biotechnology), or anti-β-actin monoclonal antibody (LS2T1-6OTD; Abcam). Membranes were then washed thrice at room temperature, and bound immunoglobulin was detected with anti-isotype monoclonal antibody coupled to horseradish peroxidase (Santa Cruz Biotechnology). The signal was visualized by enhanced chemiluminescence (Amersham Biosciences) and autoradiography. Relative band intensities were determined using Quantity One analysis software (Bio-Rad).

c-Myc Transcription Factor Binding Activation Assay

Nuclear protein extracts were obtained from S2T1-6OTD–treated and S2T1-6OTD–untreated MB and AT/RT cells by using the BD TransFactor Extraction kit (BD Clontech) as described previously (49). The activation of c-Myc was measured by using the Mercury TransFactor assay (BD Clontech), an ELISA-based assay (50), as described previously (49).

Cell Cycle Analysis

MB and AT/RT cells were washed with PBS, fixed in 70% ethanol, and kept at −20°C for at least 24 h. They were then washed in PBS and resuspended in 50 mg/mL propidium iodide and RNase (10 mg/mL) in PBS. The cell suspension was incubated for 30 min at room temperature, and cell cycle distribution was determined by flow cytometry (FACSCalibur, Becton Dickinson), with CellQuest software analysis and quantification using ModFit software as described previously (49).

Cell Growth Assays

For long-term cell growth studies, cells were seeded at 1 × 10³/mL (5 mL) into a 25-cm² tissue culture flask in the presence or absence of subtoxic doses of S2T1-6OTD. Subtoxic dose was defined as the concentration inhibiting a maximum of 10% human MB cells grown on a 72-h drug exposure. Cells were then cultured for 4 d and then trypsinized and counted. At each passage, 1 × 10⁶ cells/mL were replated into a new culture flask with fresh medium containing drug solution. Results were expressed as the cumulated population doublings as a function of the time of culture, as described by Binz et al. (14) and Riou et al. (51). For cytotoxicity assay, cell viability was quantified using a colorimetric MTS assay (Promega) as previously described (47, 52). Each condition was done in triplicate.

Telomerase Activity

Telomerase activity in human MB and AT/RT cell lines was measured by using the TeloTAGGG Telomerase PCR ELISA kit (Roche Diagnostics) as previously described (14, 47). All samples were assayed in triplicate.

Telomere Length

Telomere length in MB and AT/RT cells was determined by using the TeloTAGGG Telomere Length Assay (Roche Diagnostics) for measuring the length of telomere restriction fragments as previously described (47).

Apoptosis Assay

A photometric enzyme immunoassay (Cell Death Detection ELISA, Roche Diagnostics) was used for the
quantitative determination of cytoplasmic histone-associated DNA fragments, as described previously (53).

Statistical Analysis

All data are expressed as mean ± SD. Student’s *t* test was used to test statistical significance. *P* < 0.05 was considered to be significant. GraphPad Prism 4 (GraphPad Software) software was used to calculate IC_{50} values and their 95% confidence intervals and to statistically compare the fitted midpoints (log IC_{50}) of the two curves.

Results

S2T1-6OTD Structure

Supplementary Fig. S1A shows the chemical structure of S2T1-6OTD compared with the well-characterized telomestatin. The structure of S2T1-6OTD was confirmed by ^1^H nuclear magnetic resonance (Supplementary Fig. S1B) and high-resolution mass spectrometry (Supplementary Fig. S1C).

Stabilization of c-Myc G-Quadruplex Structures by S2T1-6OTD

To test whether S2T1-6OTD is able to stabilize the c-Myc G-quadruplex in the guanine-rich sequence of the c-Myc promoter, we used PCR-stop assay (54). In the presence of increasing concentrations of S2T1-6OTD, the 5’ to 3’ DNA extension of the ssDNA of the c-Myc promoter sequence ss-Pu22 oligomer was inhibited in a dose-dependent manner, indicating the stabilization of the G-quadruplex structure by S2T1-6OTD with a low IC_{50} value of 0.70 ± 0.02 μmol/L (Fig. 1A). No significant inhibition was observed when ss-Pu22 mutant (containing a mutation in two guanine repeats into aniline) was used (data not shown).

Because potential G-quadruplex–forming sequences exist not only in the c-Myc promoter region but also in the telomeric ssDNA that proved to be recognized and specifically stabilized by telomestatin, the parental compound of S2T1-6OTD (10), we investigated the selectivity of S2T1-6OTD to stabilize the G-quadruplex in the c-Myc promoter ss-Pu22 over the telomeric ssDNA sequence ss-Telo24 by the PCR-stop assay. The results showed that inhibition of ss-Telo24 fragment extension needed a 35-fold higher concentration of S2T1-6OTD (IC_{50}, 24.9 ± 3.1 μmol/L) compared with ss-Pu22 (IC_{50}, 0.70 ± 0.02 μmol/L; Fig. 1B and C), indicating much weaker binding and stabilization ability to G-quadruplex in ss-Telo24 than in ss-Pu22. This result was supported by CD spectroscopy, which showed that S2T1-6OTD failed to stabilize the conformation of ss-Telo24 to a G-quadruplex (Supplementary Fig. S2). Together, these results show a higher selectivity of S2T1-6OTD for the c-Myc promoter sequence compared with telomere DNA.

The binding affinity of S2T1-6OTD and stabilization of the G-quadruplex in the c-Myc promoter (c-MycQ) were further investigated by CD spectroscopy. Figure 2A shows that, under our experimental conditions, the c-Myc G-quadruplex–forming sequence exhibited the typical spectral signature of a G-quadruplex with a characteristic maximum at 264 nm (55, 56). In the presence of a five-times molar excess of S2T1-6OTD, the peak centered at 264 nm was significantly broader, with lower ellipticity...
Figure 3. A, treatment with S2T1-6OTD results in a reduction of c-Myc mRNA as measured by quantitative real-time PCR (n = 3) and in a reduction of c-Myc protein expression (representative of two independent experiments) in D341, D425, BT-12, and BT-16 cells but not in DAOY cells. Bars, SD. B, treatment with S2T1-6OTD results in a significant reduction of c-Myc activity (measured by c-Myc binding capacity to its consensus sequence “CACG(TG)” of the E-box element) as determined by the ELISA-based TransAM c-Myc activity assay. Columns, mean absorbance (n = 3); bars, SD. *, P < 0.05; ***, P < 0.001. Treatment of MB and AT/RT cells with S2T1-6OTD resulted in a significant decrease of hTERT mRNA expression (C) and telomerase activity (D) as determined by quantitative real-time PCR and the Telomerase PCR ELISA kit. Columns, mean percentage decrease compared with untreated control cells (n = 3); bars, SD.
at the maximum. This indicates a physical interaction between S2T1-6OTD and the ssDNA of the c-Myc promoter sequence. However, the spectral differences were not large enough to permit titration experiments to calculate the binding constant.

Thermodynamic stability profiling provides information about the relative stability of DNA structures on ligand binding (57). Therefore, the stabilization effect of S2T1-6OTD on the G-quadruplex structure in the c-Myc promoter sequence was further studied by CD spectroscopy by measuring the thermodynamic stability profile of the ss-Pu22 oligomer and the change in absorption at 264 nm. The results in Fig. 2B show that the normalized CD intensity of ss-Pu22 at 264 nm with 50 μmol/L S2T1-6OTD in a Tris-HCl buffer (50 mmol/L, pH 7.4) had a T_m value of 46.7°C calculated from the CD melting curves at 264 nm using a sigmoidal fitting. Under the same conditions, the T_m value of S2T1-6OTD-Telo24 complex was

Figure 4. S2T1-6OTD reduces the protein expression of CDK2 and induces cell cycle arrest and apoptotic cell death in human MB and AT/RT cells. A, S2T1-6OTD decreased the cell cycle activator CDK2 protein expression in D341, D425, BT-12, and BT-16 but not in DAOY cells as determined by Western blotting. B, effects of S2T1-6OTD on cell cycle in human brain tumor cells as determined by fluorescence-activated cell sorting analysis. Cells were treated with S2T1-6OTD (0.4 μmol/L) or left untreated for 72 h and then fixed and stained as described in Materials and Methods. At least 20,000 cells were counted. Results are presented as percentages of cells in G_s, G_s-M, and sub-G_s phases in two independent experiments. The subdiploid peak represents the apoptotic fraction. S2T1-6OTD treatment resulted in a decrease in the percentage of cells in the S phase and an increase in the cells in the G_s-G_s-M and sub-G_s phases in all cell lines tested. *, P < 0.05; ***, P < 0.001.
only 30.7°C (Fig. 2C), indicating a strong stabilizing effect of S2T1-6OTD on G-quadruplex in NHE III1 DNA and a weaker effect on G-quadruplex in telomere sequence.

Because indiscriminate binding of a compound to nonspecific DNA can result in significant loss of the compound and may have unintentional effects on the regulation of nontargeted genes, we investigated the binding ability of S2T1-6OTD to duplex DNA. A CD melting curve was done for the duplex ds-Pu22 in the presence or absence of 50 μmol/L S2T1-6OTD. The results showed no detectable change in melting temperature of the ds-Pu22 DNA in the presence or absence of S2T1-6OTD (Supplementary Fig. S3A), indicating the inability of S2T1-6OTD to bind to duplex DNAs. This result was confirmed by the electrophoretic mobility shift assay, which excluded the ability of S2T1-6OTD to interact with dsDNA sequences (Supplementary Fig. S3B).

**S2T1-6OTD Treatment Down-Regulates c-Myc Expression and c-Myc Binding Activity**

To test whether treatment with S2T1-6OTD alters c-Myc expression, we incubated a panel of MB and AT/RT cells with 0.4 μmol/L S2T1-6OTD for 72 hours and measured c-Myc mRNA expression by real-time quantitative reverse transcription-PCR and c-Myc protein by Western blotting. With the exception of DAOY cells (low basal c-Myc expression), treatment with S2T1-6OTD resulted in significant reductions of c-Myc mRNA and protein expression in all other MB and AT/RT cell lines tested (Fig. 3A). To examine whether treatment with S2T1-6OTD alters c-Myc binding activity, we incubated the five cell lines under study with S2T1-6OTD (0.4 μmol/L for 72 hours) and measured binding activity. S2T1-6OTD treatment reduced c-Myc binding activity significantly in all MB and AT/RT cells (Fig. 3B).

**S2T1-6OTD Reduces Telomerase mRNA Expression and Activity**

Because hTERT, the catalytic subunit of telomerase, is transcriptionally under the control of c-Myc (27, 28) and having shown an inhibitory effect of S2T1-6OTD on c-Myc expression in MB and AT/RT cells, we were interested in the effects that this interaction might have on telomerase activity. MB and AT/RT cells were treated with 0.4 μmol/L S2T1-6OTD for 72 hours, total RNA was isolated for reverse transcription-PCR, and total protein was extracted. Using real-time quantitative reverse transcription-PCR to measure hTERT mRNA and the Telomerase PCR ELISA kit to measure telomerase repeat amplification protocol (TRAP) activity, it was found that S2T1-6OTD could indeed reduce hTERT mRNA expression in BT-12, BT-16, DAOY, and D341 cells. No effect was observed on hTERT mRNA in the mainly ALT (Alternative Lengthening of Telomeres)-dependent D425 cell line (Fig. 3C; ref. 47). Moreover, S2T1-6OTD reduced telomerase activity in all cell lines tested, with maximum inhibition in D341 (64%) and a minimum of 17% in D425 (Fig. 3D).

The direct effect of S2T1-6OTD on telomerase activity was examined in a cell-free system. In this experiment, increasing concentrations of S2T1-6OTD (0.005–5 μmol/L) were added to the telomerase reaction mixture containing extract from D341 cells. The results showed that S2T1-6OTD inhibited in vitro the process of telomerase activity; the concentration that resulted in 50% decrease in telomerase inhibition (EC_{50}) was 0.1651 μmol/L when measured by TRAP and was 0.3577 μmol/L when measured by the TRAP-LIG assay (58), which was used to eliminate drug contamination of the PCR reaction. The results of these two experiments not only indicate that S2T1-6OTD by itself has no significant inhibitory effect on the PCR but also confirms that S2T1-6OTD possesses a direct effect on telomerase activity when examined in cell-free system (data not shown).

**NHE III1 Sequence in the Promoter Region of c-Myc Is Needed for S2T1-6OTD to Exert Its Effect on c-Myc**

To further confirm the requirement of the G-quadruplex-forming NHE III1 sequence in the promoter region of c-Myc for the exertion of downregulation of c-Myc expression by S2T1-6OTD, two Burkitt's lymphoma cell lines with different translocation break points within the c-Myc promoter (17, 59, 60) were investigated (Supplementary Fig. S4A). In the CA46 cells where the NHE III1 is deleted, S2T1-6OTD treatment (0.4 and 0.8 μmol/L for 72 hours) had no effect on c-Myc mRNA and protein expression, whereas in the Ramos cells, in which the NHE III1 is present, treatment with S2T1-6OTD significantly reduced c-Myc mRNA and protein expression (Supplementary Fig. S4B and C). In DAOY M2 MB cells where c-Myc is mainly under the control of a cytomegalovirus promoter (45), treatment with S2T1-6OTD did not decrease but rather slightly increased c-Myc expression (Supplementary Fig. S4D), also indicating a high affinity of S2T1-6OTD for NHE III1.

**Effects of S2T1-6OTD on the Cell Cycle of MB and AT/RT Cells**

To determine the possible effects of S2T1-6OTD on cell cycle regulation, we assessed the cellular DNA content by using flow cytometry (fluorescence-activated cell sorting) and determined CDK2 protein expression. With the exception of DAOY cells, S2T1-6OTD (0.4 μmol/L) treatment for 72 hours resulted in a significant decrease in the cell cycle activator CDK2 (Fig. 4A). Moreover, treatment with S2T1-6OTD resulted in a significant decrease in the percentage of cells that were able to enter the S phase and in an increase of the cells in the G0-G1 and sub-G1 phases (Fig. 4B), indicating that S2T1-6OTD mediates cell cycle arrest and induction of apoptotic cell death.

**S2T1-6OTD Treatment Results in Suppression of MB and AT/RT Cell Proliferation**

To test whether treatment with S2T1-6OTD alters cellular proliferation, MB and AT/RT cells were incubated...
Figure 5. A, dose- and time-dependent inhibition of cell viability in MB and AT/RT cells by S2T1-6OTD as determined by MTS assay. B, S2T1-6OTD treatment had no antiproliferative effect on HO15.19 c-Myc knockout (−/−) rat fibroblast cells when compared with the parental TGR-1 c-Myc wild-type (rat fibroblast) cells. C, S2T1-6OTD treatment also had no major antiproliferative effect on PC12 cells (that do not depend on c-Myc in their proliferation) or MRC-5 normal fetal lung fibroblast cells when compared with MB and AT/RT cells. D, S2T1-6OTD exhibits much less cell toxicity than telomestatin on MRC-5.
with various concentrations of S2T1-6OTD for 72 or 96 hours and cell viability was assessed by using the MTS assay (Fig. 5A). Treatment with S2T1-6OTD resulted in a dose- and time-dependent cytotoxic response in all cell lines tested, with IC50 concentrations between 0.14 and 0.33 μmol/L (72-hour treatment time). However, S2T1-6OTD had no antiproliferative effect on c-Myc knockout (-/-) HO15.19 rat fibroblast cells when compared with the parental TGR-1 c-Myc wild-type (rat fibroblast) cells (Fig. 5B; ref. 61). Moreover, S2T1-6OTD also had no antiproliferative effect on PC12 cells. These cells do not depend on c-Myc to proliferate due to the absence of Max, the partner protein to which c-Myc dimerizes to be activated (Fig. 5C; ref. 62). When compared with human tumor cells, S2T1-6OTD showed significantly less cytotoxic effects (IC50: 3.44 μmol/L) on normal (not transformed) fetal lung fibroblast MRC-5 cells (63, 64), indicating some tumor specificity. Interestingly, when compared with the parental compound telomestatin, S2T1-6OTD showed much less cell toxicity on MRC-5 cells (Fig. 5D). Because S2T1-6OTD also causes a decrease in hTERT expression and activity, we investigated whether it was the c-Myc downregulation or the reduction in telomerase that exerted the antiproliferative effect of S2T1-6OTD on MB cell culture. To that end, we tested the effect of S2T1-6OTD on cell viability of telomerase-negative cells (e.g., the human foreskin fibroblasts BJ; ref. 65). Treatment with S2T1-6OTD resulted in a clear concentration- and time-dependent cytotoxic response in the telomerase-negative BJ cells (data not shown), indicating that telomerase is most likely not responsible for the effect of S2T1-6OTD on MB and AT/RT cell growth.

Effects of Long-term Treatment with Nontoxic Concentration of S2T1-6OTD

To examine the long-term effects of S2T1-6OTD on MB and AT/RT cells, cells were treated with nontoxic concentrations of S2T1-6OTD (0.04 or 0.004 μmol/L) for 5 weeks. We then characterized the growth properties of the treated cells during the long-term cultivation experiments. Treatment with S2T1-6OTD at 0.04 μmol/L resulted in telomere shortening in all MB and AT/RT cells tested (Fig. 6A). The shortening observed after 3 weeks of S2T1-6OTD treatment was 2.6 kbp in DAOY cells, 1.5 kbp in D341 cells, 2.6 kbp in BT-12 cells, 2.0 kbp in BT-16 cells, but only 0.8 kbp in D425 cells. The growth kinetics of S2T1-6OTD–treated MB and AT/RT cells did not differ significantly from those of untreated control cells in the first week. After 2 to 3 weeks, cell growth of S2T1-6OTD–treated cells decreased (Fig. 6B). Cells treated with S2T1-6OTD at 0.004 μmol/L were able to grow better as compared with cells treated with S2T1-6OTD at 0.04 μmol/L, but a decrease in cell growth was also observed with the very low concentration. The morphologic examination of the 0.04 μmol/L S2T1-6OTD–treated cells at the plateau phase showed an increased proportion of flat and giant cells with phenotypic characteristics of senescence and positive for senescence-associated β-galactosidase (data not shown). Finally, the cells underwent delayed apoptosis when examined morphologically (data not shown) and were characterized by an increase in apoptotic cell death, as measured by Cell Death Detection ELISA (Fig. 6C).

Discussion

In this study, we investigated the efficiency of S2T1-6OTD to stabilize G-quadruplexes in guanine-rich DNA and examined the strength of its selectivity for the c-Myc over the telomeric sequences. We then examined the S2T1-6OTD–mediated cellular effects in a representative set of childhood MB and AT/RT cells.

We have shown here that S2T1-6OTD binds more selectively with higher affinity to the G-quadruplex–forming sequence in the major transcription control element in the c-Myc promoter than to the telomeric DNA sequence. This high-affinity physical interaction was established by the results obtained from the PCR-stop assay, which showed that a 35-fold higher concentration of S2T1-6OTD is needed to stabilize a G-quadruplex structure in ss-Telo24 compared with ss-Pu22 sequence. This result was further confirmed by the CD spectroscopy experiments, which showed that the G-quadruplex structure stabilization by S2T1-6OTD in the c-Myc promoter tolerated a higher thermal melting point compared with the telomeric DNA sequence.

The preference of a compound for quadruplexes over duplex DNA is of great importance. This is because indiscriminate binding to a duplex DNA can result in a significant loss of the compound and might lead to unexpected cytotoxicity. Our results illustrated that S2T1-6OTD is unable to bind DNA duplexes, as shown by the absence of any changes in the thermal melting curve in the presence of S2T1-6OTD, compared with Tm of naked duplex DNA, rendering it suitable for further studies as a highly selective G-quadruplex–interacting molecule.

Through its ability to recognize and stabilize the quadruplex structure in the c-Myc promoter sequence, S2T1-6OTD was able to suppress both c-Myc mRNA and protein expression and reduce c-Myc activity in MB and AT/RT cell lines after 72-hour incubation; however, the ability to repress c-Myc expression was found to be cell type specific. Interestingly, when we treated MB DAOY M2 cells overexpressing c-Myc under a different promoter, S2T1-6OTD failed to downregulate c-Myc, indicating specific affinity to the transcriptional control element sequence NHE IIII in the c-Myc promoter. In agreement with this was our finding that S2T1-6OTD had no effect on c-Myc expression in Burkitt’s lymphoma cell line CA46 with the deleted NHE IIII element due to its chromosomal aberration. These results therefore provided convincing evidence that the effect of S2T1-6OTD on c-Myc is indeed mediated through the interaction with the c-Myc promoter and further implicated effects on c-Myc–dependent downstream pathways. However, it has to be said that mechanisms regulating c-Myc transcription are multifaceted and involve

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Figure 6. Effects of long-term treatments with nontoxic concentrations of S2T1-6OTD. Every 5 d, the cells were trypsinized and living cells were reseeded at the same number. **A**, effect of 0.04 μmol/L S2T1-6OTD on telomere length as determined by the TeloTAGGG Telomere Length Assay. All cell lines tested showed time-dependent telomere shortening. **B**, effect of 0.04 or 0.004 μmol/L S2T1-6OTD on cell growth as determined by MTS assay. All cell lines tested showed time-dependent decrease in cell growth. **C**, apoptotic cell death was quantified using Cell Death Detection ELISA measuring DNA fragmentation in S2T1-6OTD–treated cells (0.04 μmol/L, for 5 wk). Columns, mean absorbance of cytoplasmic histone-associated DNA fragments (n = 3, representative of two independent experiments); bars, SD. **, P < 0.005; ***, P < 0.001.
not only the regulatory DNA sequence in c-Myc promoter but also several DNA binding proteins that have been shown to preferentially bind to, stabilize, unwind, or cleave the c-Myc G-quadruplex structure (66–68). In addition, recent reports have shown that putative G-quadruplex sequences are widespread in >40% of human gene promoters (66, 67). Hence, it could be speculated that any mutation in the regulatory motifs, differences in DNA binding protein expression patterns, or off-target ligand interaction might be critical for the sensitivity toward G-quadruplex–based c-Myc inhibitor, such as S2T1-6OTD, in some cell lines, and their effect might present a different profile of biological activity than initially expected.

Based on previously published work, it is well established that the suppression of c-Myc expression is closely linked to the specific arrest in the G0-G1 cell cycle phase (69–72) and downregulation of c-Myc inhibits both cyclins and CDK2 expression in rhabdomyosarcoma (73), in T lymphocytes (74), and in colorectal cancer cells (75). Our results show that under the conditions where inhibition of both proliferation and c-Myc activity was observed, S2T1-6OTD treatment resulted in a decrease in the protein expression of the cell cycle activator CDK2 and in a clear G0-G1 cell cycle arrest in all brain tumor cell lines tested and decreased the percentage of the cells that were able to enter the S phase. This provided additional evidence that c-Myc must be a direct target for S2T1-6OTD action. However, it is noteworthy that S2T1-6OTD was able to induce cell cycle arrest despite no measurable decrease in the mRNA/protein level of c-Myc was observed in DAOY cells after 72-hour treatment. These results suggest that inhibition of c-Myc expression by S2T1-6OTD may not be the only mechanism explaining the G0-G1 arrest of the cell cycle and the cell growth inhibition, but the expression level of other oncogenes may be affected with an effect on downstream signaling pathways governing cell proliferation.

Many experimental lines of evidence from different groups have shown the association of c-Myc and cell proliferation (69, 76). We therefore expected that S2T1-6OTD treatments would induce a c-Myc–dependent cell growth arrest. Indeed, short-term treatments with S2T1-6OTD induced a dose- and time-dependent negative effect on cell viability of all the MB- and AT/RT-derived cell lines tested already at concentrations lower than 1 μmol/L. Of considerable interest was the finding that S2T1-6OTD showed a significantly lower antiproliferative effect when applied to normal human fibroblast cells MRC-5 (77). Ideally, a c-Myc–targeted therapy should be active on cells with deregulated c-Myc and should not affect the majority of the normal cells in vivo. Here, we showed a more prominent effect of S2T1-6OTD on cellular growth of MB- and AT/RT-derived cells compared with its weaker effects on normal cells, which would be supportive of a selective activity on neoplastic cells.

Treatment with S2T1-6OTD resulted not only in c-Myc downregulation but also in a decrease in hTERT mRNA and reduction of telomerase activity in most of the cell lines tested. This is of particular interest considering that the catalytic subunit of telomerase hTERT is transcriptionally regulated by c-Myc (27, 78) and that activation or repression of c-Myc can alter hTERT activity in normal or tumor cells both in vitro and in vivo (22, 28, 79–82). However, on the other hand, our finding that S2T1-6OTD binds more potently to the c-Myc promoter sequence did not exclude a weaker binding to telomeric DNA (with 35-fold less efficiency). Together with our discovery that S2T1-6OTD exerted a direct inhibitory effect on TRAP activity in a cell-free system, this result suggests a possible direct inhibition of the telomerase enzyme by S2T1-6OTD and could provide an explanation for the differences between the levels of c-Myc activity inhibition and the degree of hTERT activity reduction we observed in Fig. 4. Nevertheless, one should remember that S2T1-6OTD showed a dose- and time-dependent antiproliferative effect on the telomerase-negative human foreskin fibroblast cells (BJ) and on the ALT-dependent D425 while having no antiproliferative effect on c-Myc knockout (−/−) HO15.19 rat fibroblast cells or PC12 cells, which do not depend on c-Myc in their proliferation. Together, these data confirm that a functional c-Myc pathway, and not telomerase, is needed for S2T1-6OTD to exert its antiproliferative effect. This observation counters the concern about the specificity of S2T1-6OTD toward c-Myc and reveals that it is c-Myc inhibition rather than telomerase downregulation that is responsible for the proliferation arrest exerted by S2T1-6OTD in our experiments.

To extend our findings beyond the outcome of 72-hour treatment, we studied the effects of long-term treatment of MB and AT/RT cells with nontoxic concentrations of S2T1-6OTD, compatible with long-term drug dosage, and observed the effect of telomerase inhibition on the children’s brain cancer cells. The desired effect of telomerase inhibition would be to shorten telomeres to critical lengths, causing replicative senescence and preferably cell death. The data obtained from our experiments showed that prolonged treatment did indeed result in a time-dependent telomere shortening that was preceded by a decrease in telomerase activity and accompanied by cell growth arrest and senescence-associated morphologic changes in MB and AT/RT cells, which also showed positive expression of senescence-associated markers. This finding was followed by apoptosis at day 35 in all cells treated.

In conclusion, we have shown the preferential recognition of the G-rich sequence in the c-Myc promoter over the telomere sequence by a novel small-molecule S2T1-6OTD. Through its ability to recognize and stabilize a G-quadruplex structure in the c-Myc promoter sequence, S2T1-6OTD was able to inhibit c-Myc and decrease telomerase activities, to disrupt telomere maintenance, to force cells into senescence, and to induce growth arrest and apoptosis in childhood brain tumor cell lines. A better understanding about the in vivo pharmacokinetics and efficacy of S2T1-6OTD is mandatory. However, S2T1-6OTD may well represent a potential effective and innovative therapeutic strategy for childhood brain tumors.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Tarek Shalaby, André O. von Bueren, Marie-Louise Hürlimann, et al.

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