Molecular Cancer Therapeutics

ST-11: A New Brain-Penetrant Microtubule-Destabilizing Agent with Therapeutic Potential for Glioblastoma Multiforme

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Abstract

Glioblastoma multiforme is a devastating and intractable type of cancer. Current antineoplastic drugs do not improve the median survival of patients diagnosed with glioblastoma multiforme beyond 14 to 15 months, in part because the blood–brain barrier is generally impermeable to many therapeutic agents. Drugs that target microtubules (MT) have shown remarkable efficacy in a variety of cancers, yet their use as glioblastoma multiforme treatments has also been hindered by the scarcity of brain-penetrant MT-targeting compounds. We have discovered a new alkylindole compound, ST-11, that acts directly on MTs and rapidly attenuates their rate of assembly. Accordingly, ST-11 arrests glioblastoma multiforme cells in prometaphase and triggers apoptosis. *In vivo* analyses reveal that unlike current antitubulin agents, ST-11 readily crosses the blood–brain barrier. Further investigation in a syngeneic orthotopic mouse model of glioblastoma multiforme shows that ST-11 activates caspase-3 in tumors to reduce tumor volume without overt toxicity. Thus, ST-11 represents the first member of a new class of brain-penetrant antitubulin therapeutic agents. *Mol Cancer Ther; 15(9); 2018–29. ©2016 AACR.*

Introduction

Gliomas comprise 80% of primary tumors in the central nervous system (1) and the most common subtype is glioblastoma multiforme (WHO grade IV astrocytoma). Patients diagnosed with glioblastoma multiforme have a median overall survival of 14 to 15 months when treated with a standard regimen of surgery, radiation, and the DNA intercalating agent temozolomide (Temodar; ref. 2). Nonselective chemotherapeutics remain the sole drug treatment option for this patient population in large part because recent targeted therapies, such as gefitinib and bevacizumab that block EGFR and VEGF signaling, respectively, have fallen short of expectations (3–6). This realization has fueled a need for novel therapeutics to combat glioblastoma multiforme through a different mechanism of action; however, the development of new glioblastoma multiforme therapeutics is complicated by the necessity for efficient brain penetrance.

A recent study demonstrated that glioblastoma multiforme cells are particularly sensitive to mitotic disruption when compared with matched nonmalignant cells (7). This has led to the notion that therapies that perturb microtubule (MT) assembly may represent a promising strategy to manage this type of cancer. For example, recently approved antimitotic devices that generate electromagnetic fields known as tumor treatment fields (TTFields) and function by disrupting MTs show significant efficacy as novel glioblastoma multiforme treatments (8, 9). This result indicates that antitubulin agents are likely to show antineoplastic efficacy in patients diagnosed with glioblastoma multiforme; however, the majority of antitubulin agents do not readily cross the blood–brain barrier (reviewed in ref. 10). In fact, of the few brain-penetrant antitubulin agents, only 2, sagopilone (ZK-EPO) and the nonanalgesic opioid noscapine, have been evaluated for glioma indications (11, 12). However, sagopilone suffered from a lack of efficacy in clinical trials (13), and noscapine required high doses to elicit an effect (12). Irrespective of the limited success of currently available compounds, the susceptibility of glioblastoma multiforme cells to mitotic perturbation holds considerable promise for the treatment of this type of cancer (7). To this end, it is necessary to discover and develop novel brain-penetrant antitubulin agents with high therapeutic potential.

Our laboratory has recently developed a library of alkylindole (AI) compounds with the capacity to kill glioma cells *in vitro* (14, 15). These compounds belong to the broader group

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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of 2-aroylindole agents that inhibit tubulin polymerization by interacting with the colchicine-binding site and are insensitive to multidrug resistance efflux pumps that can hinder brain penetrance (16–18). Here, we used a combination of biochemical and cell biology approaches to demonstrate that the AI compound ST-11 arrests the cell cycle and kills glioma cells by directly acting on MTs. To determine the pharmacokinetics and *in vivo* efficacy of ST-11, we established a stable formulation of this compound in liposomes and developed a liquid chromatography-mass spectrometry (LC-MS) method to quantify ST-11 in blood and brain. Using these tools, we determined that ST-11 readily penetrates mouse brain after intraperitoneal injection and dose-dependently reduces tumor volume in a syngeneic glioblastoma multiforme mouse model. Importantly, ST-11 does not produce overt toxicity in mice. Our study highlights the therapeutic potential of AI compounds as brain-penetrant antitubulin agents with antineoplastic activity in glioblastoma multiforme.

Materials and Methods

See Supplementary Data for detailed methods.

Chemicals

All chemicals and drugs were purchased from Sigma unless otherwise noted. JWH-015, and JWH-200 were from Cayman Chemical. JWH-120, JWH-148, and JWH-042 were a kind gift from John W. Huffman (Clemson University, Clemson, SC). ST compounds were synthesized in house as described previously (14, 15). Z-DEVD-FMK was from Santa Cruz Biotechnology. L-α-Phosphatidylcholine (EPC) was from Avanti Polar Lipids. 1,2-Dimyristoyl-*sn*-glycero-3-phosphoglycerol, sodium salt (DMPG), and N-(Carbonyl-methosypolyethyleneglycol-2000)-1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolzmine, sodium salt (DMPE-mPEG2000) were from Corden Pharma. Cell culture materials were purchased from Life Technologies (Thermo Fisher Scientific).

Cell culture

Cells were cultured in DMEM or RPMI containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2 humidified atmosphere. DBT, T98G, U251, A172, and U87MG cells (ATCC) were authenticated by the ATCC when purchased using human short tandem repeat analysis and maintained in culture for less than 6 months. BT74, MGG4 and MGG8 cells were a generous gift from Samuel Rabkin and Hiroaki Wakimoto (Massachusetts General Hospital and Harvard University, Boston, MA) and maintained as floating spheroid cultures in Neurobasal-A medium (19). All spheroid cultures were maintained in *ex vivo* culture for less than 6 months and not authenticated [BT74 as glioblastoma multiforme6 in ref. 20, MGG8/8 in ref. 19]. Primary mouse neuron and astrocyte cultures were prepared as described previously (21, 22) in accordance with the Institutional Animal Care and Use Committee of the University of Washington.

Viability and proliferation

[^{3}H]-thymidine (5 μCi/mL; PerkinElmer) was added to cells 30 minutes after drug treatments and measured at the indicated times by adding 1 mol/L NaOH and quantifying radioactivity. WST-1 (Roche) was used to evaluate cell viability and was measured according to the manufacturer's protocol. For proliferation and cell death using Trypan blue exclusion, cells were trypsinized and counted in the presence of Trypan blue (1:10; Sigma).

Lysates and Western blotting

Western blotting was performed as described previously (23) with the following primary antibodies: cleaved PARP (1:1,000; Asp214), cyclin B1 (1:1,000; Val152), phospho-histone H3 (1:1,000; Ser10), β-tubulin (1:1,000), and activated caspase-3 (1:500) were all from Cell Signaling Technology; GAPDH (1:1,000) was from Sigma.

MT assembly

MTs were polymerized and sheared in BRB80 (80 mmol/L PIPES-KOH pH 6.85, 1 mmol/L $MgCl_2$, 1 mmol/L EGTA) supplemented with 4% DMSO, 2 mmol/L GTP, 1 mmol/L DTT, 2.5 mmol/L $MgCl_2$ and 60 μmol/L bovine brain tubulin and added to paclitaxel (Taxol), nocodazole, or ST-11 to a 1% final DMSO concentration. Details can be found in the Supplementary Methods. Free tubulin from supernatants and pellets was separated on 4% to 12% polyacrylamide gels (Thermo Fisher Scientific) and stained with Coomassie G-250. Peak intensities were quantified using ImageJ. Tubulin was purified in-house (24).

Live cell imaging

Cells were transfected with EB3-GFP and RFP-CenpB to label assembling MTs and centromeres, respectively (25). Movies of MT assembly were collected over 30 seconds at 500 ms intervals 10 minutes after drug application (0.5 μmol/L ST-11, 3 μmol/L ST-11, or 1 μmol/L nocodazole) on a Deltavision microscope system (Applied Precision). Images were deconvolved using SoftWorx 5.0 (Applied Precision). MT assembly rates were scored in interphase cells using Fiji TrackMate (26).

Immunocytochemistry

Cells were treated with ST-11 as indicated. Activated caspase-3, MTs, and centromeres were visualized using anti-activated caspase-3 (1:200, Abcam), anti-DM1 alpha (1:500; Sigma), and human anti-centromere (1:100; ACA, Antibodies Inc.) primary antibodies, respectively. Representative images are presented as flat Z-projections (using ImageJ). Spindle profiles and multipolar spindles were scored by hand using a Nikon FX-A microscope.

Flow cytometry analysis

Cells were lysed in a DAPI-containing lysis solution (146 mmol/L NaCl, 10 mmol/L Tris-base pH 7.4, 2 mmol/L $CaCl_2$, 22 mmol/L $MgCl_2$, 0.05% BSA, 0.1% nonidet P-40, 10 μg/mL DAPI, 10% DMSO) or stained for PI and Annexin V using the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Ten thousand events were counted for each condition using an LSRII flow cytometer (BD Biosciences). Data were analyzed using FCS Express (v4 De Novo Software) equipped with the MultiCycle plugin. See Supplementary Methods for details.

ST-11 nanoparticle formulation

The chemical parameters of ST-11 were determined using Chemicalize Beta (www.chemicalize.org). The likelihood of ST-11 brain penetration was estimated using the induction tree models outlined by (27). EPC:DMPG:DMPE-mPEG2000 (9:1:0.5 m/m/m) nanoparticles containing ST-11 for *in vivo* studies were

produced as described previously (28). Details can be found in the Supplementary Methods.

Pilot safety studies

All animal experiments were conducted in accordance with the American Association for Accreditation of Laboratory Animal Care, and all procedures were approved by the University of Washington Institutional Animal Care and Use Committee. A 5-day dose-range finding study was performed following screening for MTD. CD1 mice received once daily intraperitoneal dosing with 15, 40, 80, 160, or 240 mg/kg ST-11, or the corresponding volume of liposome vehicle. Mice were monitored for signs of general distress, including ataxia, squinting, piloerection, hunching, bradykinesia, and dyspnea. In addition, mice were monitored for organ dysfunction and/or moribund state. Mice were then euthanized and organs were harvested for histopathologic analysis by a board-certified veterinary pathologist (D. Liggitt) who was blinded to group assignment.

Tissue extraction and ST-11 quantification by LC-MS

Lipids were isolated from CD1 mouse blood and whole brain, and JWH-015 was used as an internal standard for quantification. Lipid composition was analyzed using a Waters Micromass Quattro Premier XE equipped with a C18 LC column (AQUITY UPLC BEH C18 1.7 μm 2.1 × 100 mm, Waters). Compounds were distinguished by their ES+ daughter ions with ST-11 at 169 m/z and JWH-015 at 155 m/z. ST-11 was quantified using a ratio of the AUC of ST-11/AUC of JWH-015 and extrapolating from the linear standard curve. Details can be found in Supplementary Methods.

Orthotopic DBT tumors and treatment regimen

A total of 2×10^4 DBT cells were implanted in 8-week-old mail BALB-c mice at 2 mm cranial from bregma and 1.5 mm left lateral. Details can be found in Supplementary Methods. Tumors exhibited features of aggressive progression within 3 weeks, including frequent mitotic figures, multifocal necrosis, edema, and densely arranged neoplastic cells with enhanced cellular atypia, anisocytosis, and anisokaryosis (Supplementary Fig. S1). Mice were distributed into 4 arms 1 week after implantation: vehicle (liposome-only), 5 mg/kg, 15 mg/kg and 40 mg/kg ST-11, and they received daily intraperitoneal injections for 2 weeks. Mice were euthanized and perfused with 4% paraformaldehyde, and whole brains were harvested.

H&E and IHC

Whole brains were stained as described previously (29), with the following primary antibodies: anti-activated caspase-3 (1:200, Abcam) and anti–Iba-1 (1:1,000, Abcam). For hematoxylin and eosin (H&E) staining, fixed brains were either sent to Histology Consultation Services, or processed in-house using a standard method (5 seconds in concentrated Mayer hematoxylin and 60 seconds in concentrated Eosin Y).

Tumor volume

Tumor volume was estimated by slab approximation (30). Details can be found in Supplementary Methods. Large numbers of microglia invade glioblastoma multiforme tumor masses (reviewed in ref. 31; Supplementary Fig. S2). We calculated the percentage of infiltrating microglia in each tumor using semi-quantitative immunohistochemistry (IHC) as described previously (29) and subtracted this value from the calculated tumor volume to obtain the adjusted tumor volume.

Statistical analysis

GraphPad Prism (v5.01) was used for statistical analysis. Data are presented as the mean ± SEM, and statistical significance was determined using a Student *t* test, a one-way ANOVA followed by a Dunnett or Tukey *post hoc* test, or a two-way ANOVA followed by a Bonferroni *post hoc* test.

Results

ST-11 inhibits proliferation and kills glioblastoma multiforme cells *in vitro*

A library of 10 AI analogs was screened for activity on the human glioblastoma multiforme cell line T98G (Supplementary Table S1). One compound, named ST-11, exhibited a combination of properties requisite for further mechanistic and *in vivo* analyses: it reduced T98G cell number with the highest potency and efficacy, and it encompasses the basic chemical scaffold of the series (colloquially termed the ST compound library; Fig. 1A; Supplementary Table S1). Thus, ST-11 was selected for further screening on additional human glioblastoma multiforme cell lines, including 3 adherent cell lines (U251, A172, and U87MG cells) and 3 stem cell lines (BT74, MGG4, and MGG8; ref. 32). ST-11 reduced cell number in all of the glioblastoma multiforme lines tested when applied at micromolar concentrations (EC_{50} from 2.4–8.6 μmol/L, maximal efficacy from 51%–96%; Table 1). A vital and desired feature of any new antineoplastic drug is a favorable therapeutic index, which is related to the preferential activity of the compound for tumor cells. Thus, we tested the antineoplastic activity of ST-11 on malignant and non-malignant mouse cells. ST-11 killed mouse DBT glioma cells with a potency and efficacy similar to T98G cells (EC_{50} = 2.5 μmol/L, maximal efficacy = 60.7%). Notably, this compound did not affect the viability of primary mouse astrocytes or neurons (Fig. 1B). Therefore, ST-11 preferentially targets a variety of glioblastoma multiforme cell lines, including glioma stem cells, which tend to be refractive to therapy (33, 34).

A loss of cell number can result from reductions in cell proliferation and/or increases in cell death. To delineate between both outcomes, we initially determined whether ST-11 reduces cell viability using a trypan blue exclusion assay. ST-11 dose-dependently increased the percentage of Trypan blue–positive dead cells with a potency and efficacy similar to what was detected using WST-1 (WST-1 EC_{50} = 2.5 μmol/L, maximal efficacy = 66%; trypan blue EC_{50} = 4.3 μmol/L, maximal efficacy = 68%, WST-1 EC_{50} = 2.5 μmol/L, maximal efficacy = 66%; Supplementary Table S1, Fig. 1C). We ascertained that ST-11 reduces cell proliferation using two independent approaches. First, we measured the dose-dependent effects of ST-11 on [^{3}H]-thymidine incorporation in T98G and DBT cells and found that this compound reduced [^{3}H]-thymidine incorporation in both T98G and DBT cells within 24 hours of treatment (Fig. 1D and Supplementary Fig. S3A). Second, we monitored the time-dependent effects of ST-11 action on the accumulation of 2 mitotic markers: cyclin B1 and phospho-histone H3. Application of ST-11 led to an increase in both cyclin B1 and phospho-histone H3 levels (Fig. 1E and F). Thus, we propose that ST-11 reduces glioblastoma multiforme cell number by arresting cells in mitosis and subsequently killing them.

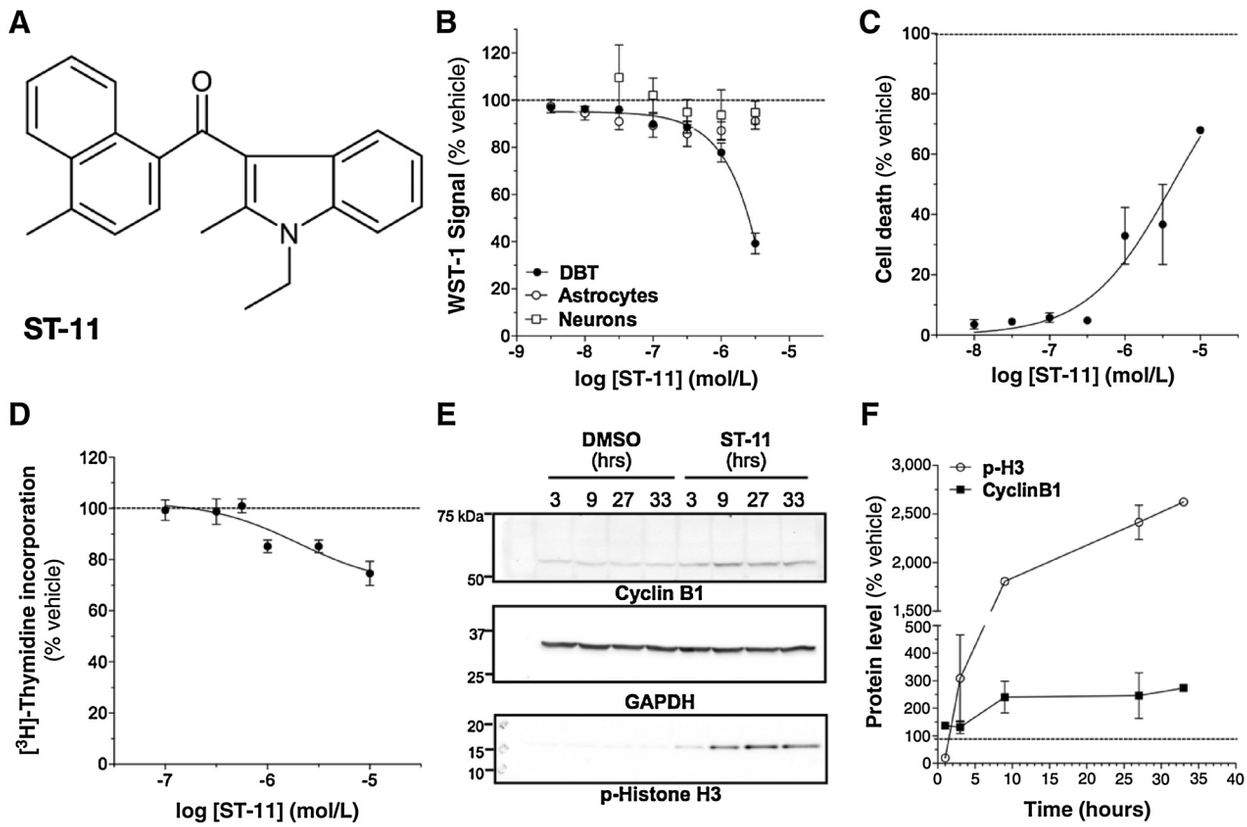

Figure 1.

ST-11 reduces proliferation and kills glioblastoma multiforme (GBM) cells. **A,** chemical structure of ST-11. **B,** viability of cultured mouse DBT, astrocytes, and neurons measured with WST-1 72 hours after ST-11 treatment. **C,** T98G cell death measured with trypan blue 72 hours after ST-11 treatment. **D,** [^{3}H]-thymidine incorporation in T98G cells 24 hours after ST-11 treatment. **E** and **F,** the time-dependent accumulation of cyclin B1 and phospho-histone H3 after ST-11 treatment (3 μmol/L) shown by a representative Western blot analysis (**E**) and quantified using GAPDH as a loading control (**F**). Data are the mean ± SEM of at least three independent experiments.

ST-11 arrests cells in prometaphase by directly targeting MTs

To further confirm our hypothesis that ST-11 arrests glioblastoma multiforme cells in mitosis, we used flow cytometry to analyze the proportion of cells in each cell-cycle phase. Treatment with ST-11 increased the percentage of T98G and DBT cells in G_2–M phase and decreased the number of cells in G_1 phase (Fig. 2A and Supplementary Fig. S3B). To determine whether this effect resulted from cell-cycle arrest in mitosis, we quantified the proportion of cells in each stage of mitosis after application of ST-11. Our compound increased the percentage of prometaphase T98G and DBT cells in a dose-dependent manner (Fig. 2B and Supplementary Fig. S3C), suggesting that ST-11 arrests cells in mitosis by triggering the spindle assembly checkpoint (reviewed in refs. 35, 36). In line with this observation, we also noted that ST-11 both dose-dependently increased the proportion of cells with multipolar spindles (Fig. 2C and Supplementary Fig. S3D) and induced severe defects in chromosomal biorientation (Fig. 2D).

Table 1. ST-11 kills cultured glioblastoma multiforme cells

Cells	Potency (μmol/L)	Efficacy (% dead)
MGG8	4.6	96.0
BT74	2.4	89.9
A172	5.8	68.2
U251	4.3	67.0
T98G	2.5	65.7
U87	5.5	63.2
MGG4	8.6	51.3

NOTE: Glioblastoma multiforme cell lines were treated with ST-11, and cell number was measured after 72 hours using WST-1. Dose–response curves were analyzed and data shown are the mean ± SEM from at least three independent experiments.

Improper spindle assembly can be attributed to defects in MT dynamics and polymerization, and 2-aroylindole agents are known to directly depolymerize MTs (16, 17, 37, 38). Therefore, we used three approaches to determine whether ST-11 was acting directly on MTs. First, we used a cell-free assay with purified tubulin to assess the partitioning of MT dimers and polymer. As expected, the MT destabilizing agent nocodazole triggered an increase the free tubulin (Fig. 3A). Conversely, a decrease in free tubulin levels was observed upon addition of the MT stabilizing agent paclitaxel (Fig. 3A). Similar to nocodazole, ST-11 dose-dependently increased the level of free tubulin, which corresponds to MT disassembly (Fig. 3A). Thus, ST-11 destabilizes MTs in a cell-free system containing purified MTs, implying that it directly acts on tubulin. The EC_{50} of ST-11 on MT destabilization (EC_{50} = 3.7 μmol/L) is similar to its EC_{50} on T98G and DBT cell viability, indicating MTs may be the direct molecular target of

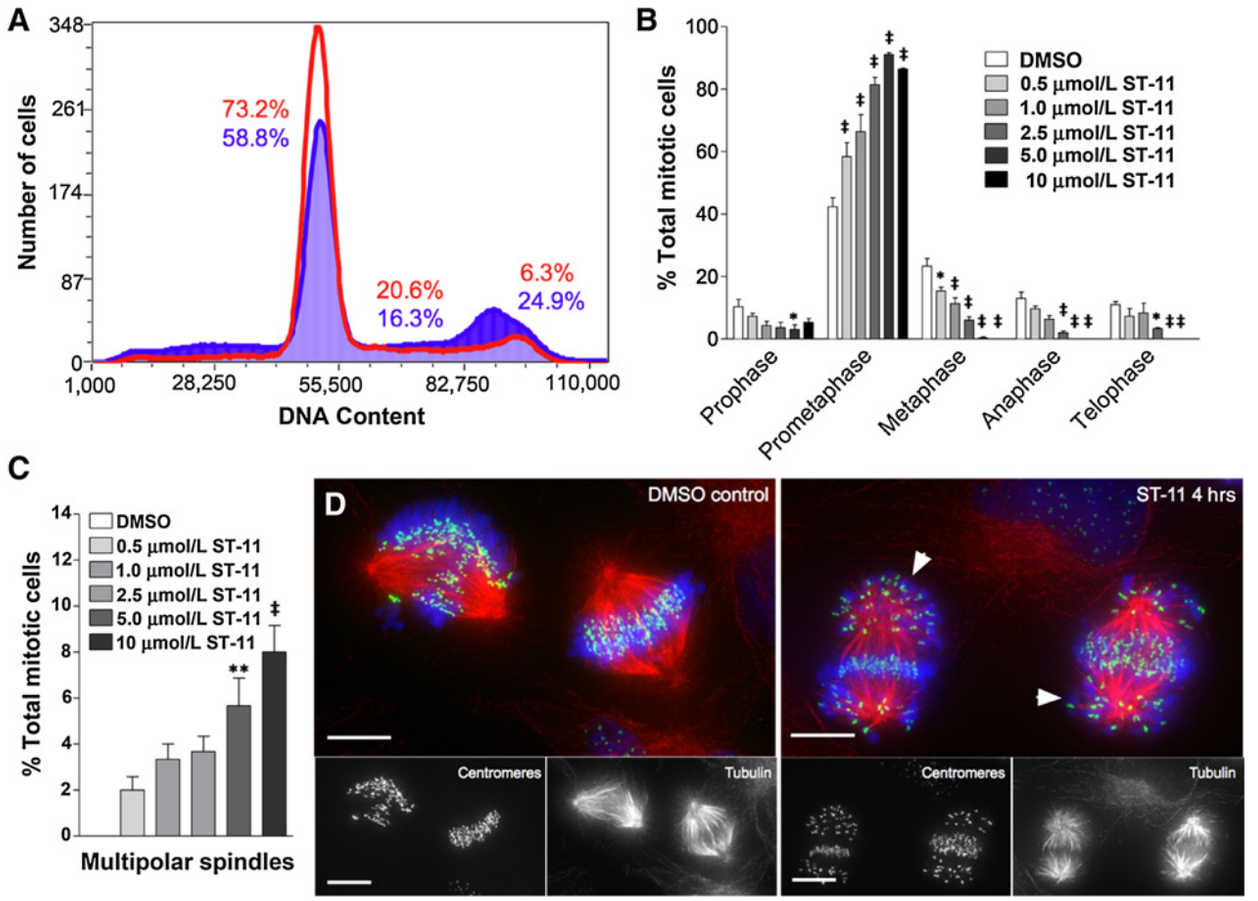

Figure 2.

ST-11 arrests cultured T98G cells in prometaphase. **A,** cell-cycle analysis of T98G cells treated with ST-11 (blue) or vehicle (red) for 24 hours using flow cytometry. The percentages of cells in G_1, S, and G_2–M phases are indicated. **B,** the percentage of cells in each stage of mitosis after treatment with ST-11. *, $P < 0.05$; ‡, $P < 0.001$ compared with vehicle, two-way ANOVA with the Bonferroni *post hoc* test. **C,** ST-11 dose-dependently increases the formation of multipolar spindles. **, $P < 0.01$; ‡, $P < 0.001$ compared with vehicle, one-way ANOVA with the Tukey *post hoc* test. Data are the mean ± SEM of at least three independent experiments. **D,** representative images of ST-11–induced (5 µmol/L) abnormalities in chromosomal alignment (arrowheads). Tubulin is shown in red, centromeres in green, and DNA in blue; scale bars, 10 µm.

ST-11 involved in its antineoplastic effect. Second, we assessed whether ST-11 affected MT polymer levels in T98G cells *in vitro*. MTs were significantly less abundant in T98G cells treated with ST-11, demonstrating that this compound directly acts on MTs in cells (Fig. 3B). Finally, using live cell imaging of EB3-GFP on assembling MTs in T98G cells, we found that submicromolar concentrations of ST-11 (0.5 µmol/L) decreased the MT assembly rate by 32% within 10 minutes of treatment (Fig. 3C and D, Supplementary Movies S1 and S2). Importantly, the effects of ST-11 on MT assembly were reversible by 24 hours after drug removal (Supplementary Fig. S4A). Nocodazole and ST-11 both exhibit robust MT depolymerizing activity on purified tubulin (Fig. 3A). To compare the activity of these compounds on live T98G cells, we selected concentrations of ST-11 (3 µmol/L) and nocodazole (1 µmol/L) that exhibit comparable MT-destabilizing activity on purified tubulin (Fig. 3A). We noted that nocodazole abolished all assembling MTs in live T98G cells by 15 minutes (Supplementary Fig. S4B). In contrast, MTs continued to assemble after ST-11 treatment (3 µmol/L; Supplementary Fig. S4B) albeit more slowly. Thus, although ST-11 has a significant effect on cell-cycle arrest at this concentration (Fig. 2B), it does not abolish all dynamic MTs in interphase cells compared with nocodazole. Together, these results demonstrate that ST-11 directly acts on MTs to reduce MT assembly, leading to mitotic spindle defects and prometaphase arrest in glioblastoma multiforme cells.

ST-11 activates caspase-3–dependent apoptosis

Antitubulin agents often promote caspase-dependent apoptosis (39). Therefore, we sought to determine whether ST-11 activated apoptosis in glioblastoma multiforme cells *in vitro*. ST-11 induced caspase-3 activation in both T98G and DBT cells as early as 2 to 6 hours after treatment (Fig. 4A and Supplementary Fig. S3E).

This result was confirmed by western blot analysis 24 hours after treatment with ST-11 (Fig. 4B). Accordingly, this compound induced time-dependent cleavage of PARP (Fig. 4C). Moreover, cells exhibited nuclear condensation and membrane blebbing within 24 and 48 hours of ST-11 treatment, respectively (Fig. 4A).

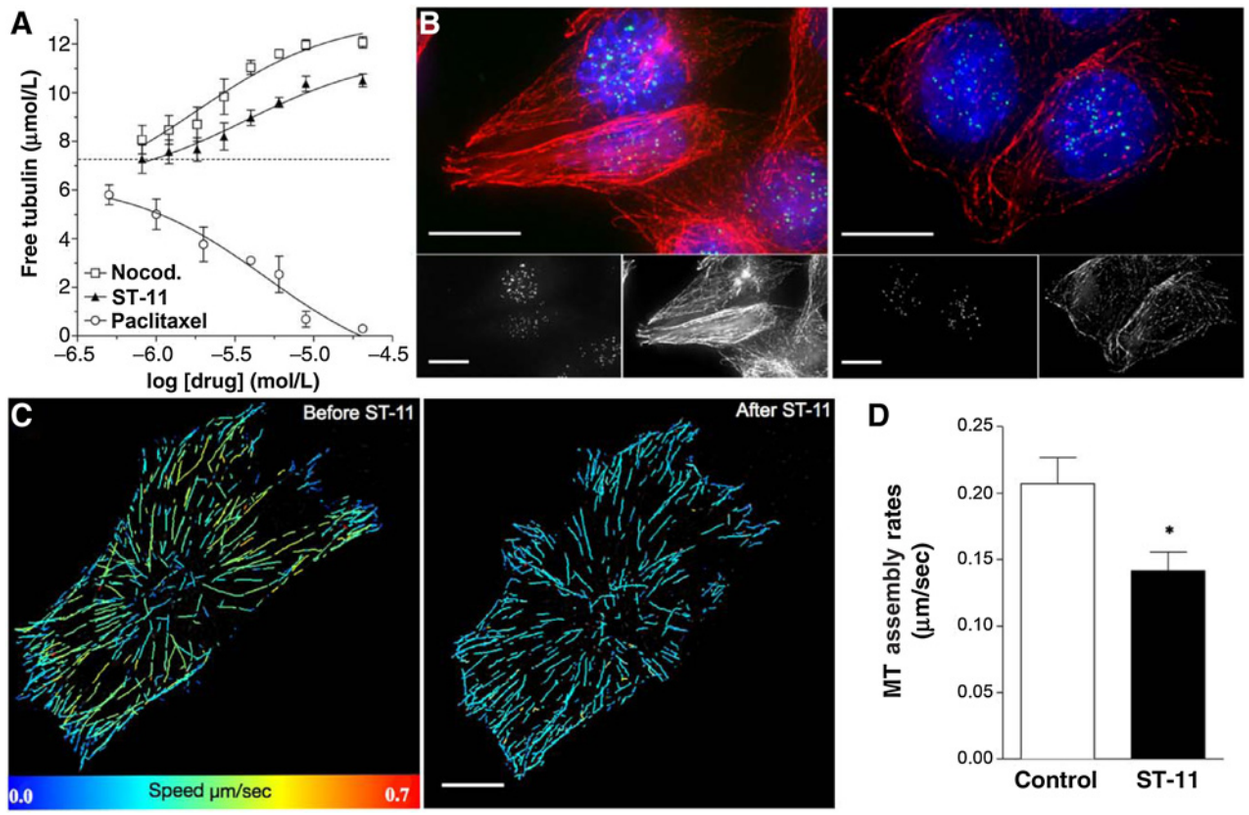

Figure 3.

ST-11 directly destabilizes MTs and decreases their assembly in T98G cells. **A,** incubation of ST-11 with purified tubulin induced a dose-dependent increase in free tubulin. Paclitaxel and nocodazole were used as positive controls to decrease and increase free tubulin, respectively. The dashed line indicates the steady-state level of free tubulin established prior to the addition of antitubulin agents (7.8 μmol/L). Results are the mean ± SEM of at least three independent experiments. **B,** representative images of ST-11 (5 μmol/L) disrupting MTs in T98G cells. Tubulin is shown in red, centromeres in green, and DNA in blue; scale bars, 10 μm. **C** and **D,** ST-11 (0.5 μmol/L) slows MT dynamics in live T98G cells within 10 minutes of treatment (**C,** quantified in **D**). *, $P < 0.05$, Student *t* test; scale bars, 10 μm. Data are the mean ± SEM of at least three independent experiments.

Control experiments confirmed that inhibition of caspase-3 with Z-DEVD-FMK blocked the antineoplastic action of ST-11 (Fig. 4D). ST-11 treatment for 24 hours increased both propidium iodide (PI) and Annexin V staining quantified with flow cytometry (Supplementary Fig. S5). Therefore, ST-11 activates caspase-3–dependent apoptosis in glioblastoma multiforme cells.

To compare the *in vitro* efficacy of ST-11 with other antitubulin agents, we measured the time-dependent effects of ST-11, paclitaxel, and nocodazole on T98G cell number using WST-1. By 12 hours, all 3 compounds had reduced cell number by 10% to 15%. However, by 72 hours, paclitaxel was inactive, whereas cell number was reduced by 34% with nocodazole and by 48% with ST-11 (Fig. 4E). Notably, paclitaxel is known to have reduced activity in T98G cells due to their high expression of multidrug resistance (MDR) efflux pumps, such as MDR-1 (40). In contrast, paclitaxel reduced the viability of MDA-MB-231 breast cancer cells used as a positive control by 72 hours (Supplementary Fig. S6). This finding underscores the ability of ST-11 to retain efficacy in a cell line that expresses high levels of MDR pumps, a feature maintained by other 2-aroylindole compounds (17, 41). ST-11 was therefore more effective at reducing cell number than two antitubulin compounds, one of which (paclitaxel) is widely used in the clinic as an antineoplastic drug. These findings suggest that further research into the *in vivo* safety and efficacy of ST-11 is warranted.

Solubility and safety of ST-11 *in vivo*

Because ST-11 does not adversely affect the viability of nonmalignant cells *in vitro*, we sought to study its safety and efficacy *in vivo*. We first assessed the MTD and pharmacokinetic profile of ST-11 in healthy mice. The lipophilic nature of ST-11 necessitated the development of a stable formulation of this compound. Standard solvents such as 30% mouse serum, 10% ethanol, 1% DMSO, 1% Tween-80, 2% Tween-80, 1:1:18 ethanol:Cremophor RH40:saline, or 1% Tween-80 + 10% FBS did not solubilize ST-11 (Supplementary Fig. S7). Thus, we explored liposome formulations, which are composed of combinations of lipids that form semisolid nanospheres (42). Our analyses revealed that ST-11 was soluble and stable for at least 14 days in nanospheres composed of 9:1:0.5 (m/m/m) EPC:DMPG:DMPE-mPEG2000 at a final lipid-to-drug ratio of 6:1 (m/m; experimental details in Materials and Methods and Supplementary Methods). This formulation was used to administer ST-11 in all subsequent *in vivo* experiments.

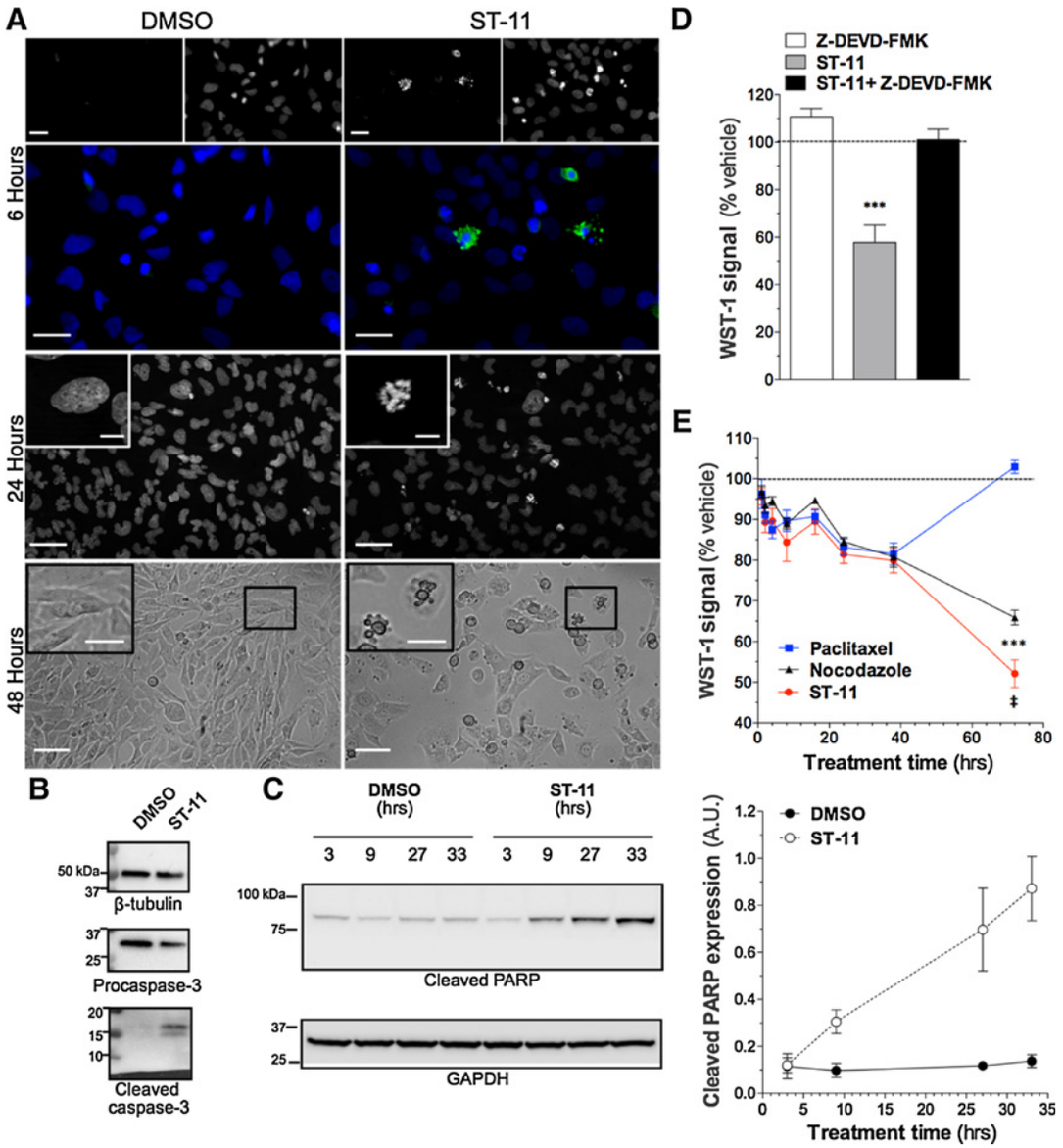

Figure 4.

ST-11 triggers caspase-3–dependent apoptosis in cultured T98G cells. **A,** caspase-3 activation (green) correlating with condensed nuclei (blue; top), overt nuclear condensation (middle), and plasma membrane blebbing (bottom) after treatment with ST-11 (10 μmol/L) at the indicated times. Scale bars: top and middle, 50 and 10 μm (inset); bottom, 200 and 50 μm (inset). **B** and **C,** representative Western blots showing activation of caspase-3 24 hours after ST-11 treatment (3 μmol/L; **B**) and an increase in cleaved PARP (**C,** left) after ST-11 treatment (3 μmol/L) for the indicated times. PARP cleavage was quantified using GAPDH as a loading control (**C,** right). **D,** the antineoplastic effect of ST-11 (10 μmol/L) was ablated by a caspase-3 inhibitor (Z-DEVD-FMK, 10 μmol/L, 15 minutes preincubation); ***, $P < 0.001$ compared with vehicle, one-way ANOVA with the Tukey *post-hoc* test. Data are the mean ± SEM of at least three independent experiments. **E,** cell number over a 72 hours period measured with WST-1 after treatment with ST-11 (10 μmol/L), nocodazole (10 μmol/L), or paclitaxel (10 μmol/L). ***, $P < 0.001$ compared with paclitaxel; ‡, $P < 0.001$ compared with nocodazole, two-way ANOVA with the Bonferroni *post hoc* test. Data are the mean ± SEM of at least three independent experiments.

Initially, we assessed the MTD of ST-11 in a 5-day pilot dose-range finding study. Mice were treated with ST-11 (15, 40, 80, 160, and 240 mg/kg, i.p.) and monitored for signs of distress (see Materials and Methods). No adverse effects were observed when the compound was administered at doses up to 240 mg/kg daily over 5 days, indicating that ST-11 appears to be well tolerated. In addition, tissue damage was assessed using H&E staining of 6 principal organs (heart, liver, kidney, lungs, spleen, and brain). No signs of tissue injury were observed in any organ harvested from any ST-11 dosage group. However, we noted a prominence of slightly foamy macrophages in the marginal zone of the spleen of mice treated with both 240 mg/kg ST-11 and the matched liposome-only control. This finding indicates that the large amount of liposomes injected in this condition induced a high

rate of liposome phagocytosis in a manner that was independent of ST-11 (data not shown). This pilot safety study indicates that ST-11 is well tolerated at high doses and does not produce overt toxic effects when administered daily over a wide dose range.

Pharmacokinetic profile of ST-11

A limiting factor of the use of many antitubulin agents to treat glioblastoma multiforme is their inability to cross the blood–brain barrier (reviewed in ref. 10). Therefore, we used predictive models to determine the likelihood that ST-11 is brain-penetrant. On the basis of two decision tree induction models described in ref. 27, ST-11 is predicted to cross the blood-brain barrier (LogP = 5.81, rotatable bonds = 3, and polar surface area = 22). To confirm our predictions, we established the pharmacokinetic profile of ST-11 in healthy mice to evaluate whether this compound reaches the brain at concentrations that could produce antineoplastic activity (i.e., above its EC_{50} for killing glioblastoma multiforme cells in culture). We developed an LC-MS method to quantify ST-11 in biological matrices using a related compound, JWH-015, as an internal standard (see chemical structure in Supplementary Table S1). Both compounds eluted at approximately 2.8 minutes (Fig. 5A) and were independently detected with base peaks of 169.0 (ST-11) and 155.0 m/z (JWH-015; Fig. 5B and C). The quantification of ST-11 was linear from 30 fg to 1 ng ($r^2 > 0.99$) upon analyzing the compound when spiked into either blood or brain matrices (Fig. 5D). The limit of detection was 100 fg and the limit of quantitation was 3 pg (error < 10%). Thus, our newly developed method allows for the reliable and precise quantification of ST-11 from 0.1 to 1 ng in samples from mouse blood serum and brain tissue.

To establish the pharmacokinetic profile of ST-11, mice were treated with ST-11 (40 mg/kg, i.p.), and blood and brain tissue were harvested 10, 30, 60, 90, and 480 minutes after injections. Serum levels of ST-11 peaked after 10 minutes ($C_{max} = 15.3 \pm 3.6$ μmol/L), declined steadily thereafter and reached undetectable levels within 8 hours after injection (Fig. 5E). In contrast, brain levels of ST-11 peaked after 60 minutes ($C_{max} = 8.7 \pm 0.9$ μmol/L) and remained above its average antineoplastic EC_{50} (i.e., 1.8 ± 0.2 μmol/L) for up to 8 hours. In a follow-up experiment, mice were treated with 5, 15, or 40 mg/kg ST-11 (i.p.), and blood and brain tissue were harvested 60 minutes after injection. Both 15 and 40 mg/kg injections led to brain levels of ST-11 that were greater than the EC_{50} for killing glioblastoma multiforme cells (Fig. 5F). Hence, liposomes deliver micromolar concentrations of ST-11 to the brain within 60 minutes of intraperitoneal injection that remain above its efficacious concentration for killing glioblastoma multiforme cells for 4 to 8 hours.

ST-11 induces apoptosis in orthotopic DBT tumors and reduces tumor volume

Finally, to determine whether ST-11 affects glioblastoma multiforme growth *in vivo*, we used a syngeneic mouse model in which DBT cells were orthotopically implanted into BALB/c mice (Fig. 6A and Supplementary Fig. S1 and S2). One week after DBT cell implantation, mice were treated daily with 5, 15, or 40 mg/kg ST-11 or the corresponding liposome vehicle for 2 weeks. The total volume of each tumor after 2 weeks was estimated by slab approximation and adjusted for the abundant invasion of microglia using semi-quantitative IHC analysis of Iba-1 (Supplementary Fig. S2). ST-11 treatment promoted a dose-dependent reduction in tumor volume (Fig. 6B). Importantly, we detected a dose-dependent increase in caspase-3 activation, nuclear condensation, and cellular loss within the tumor mass (Fig. 6C). Collectively, these results indicate that intraperitoneal administration of ST-11 dose-dependently activates apoptosis and reduces the volume of orthotopically implanted DBT tumors.

Discussion

In this study, we describe the pharmacology and mechanism of action of ST-11, a new AI compound that destabilizes MTs. This compound displays three properties that are requisite for treating glioblastoma multiforme: (i) the ability to cross the blood–brain barrier, (ii) preferential selectivity for killing glioblastoma multiforme cells, and iii) a favorable safety profile when administered at doses above its therapeutic efficacy. Our study not only provides a solid foundation for further chemical optimization of this compound but also launches the pharmacologic and biological characterization of this new class of brain-penetrant antitubulin agents. Accordingly, ST-11 and future derivatives may prove to be efficacious for the treatment of brain cancers such as glioblastoma multiforme, which remain both one of the most devastating and therapeutically intractable forms of cancer.

ST-11 reduces glioblastoma multiforme cell number by reducing both proliferation and viability. Mechanistically, we have discovered that, similar to other 2-aroylindoles, this compound acts directly on MTs to reduce their assembly. The EC_{50}s of the effects of ST-11 on MT destabilization in a cell-free assay and glioblastoma multiforme cell viability are comparable. Taken together, these findings infer that tubulin is likely the primary target of this compound. Consistent with this result, ST-11 reduced the level of MT polymer and, at lower concentrations, the rate of assembling MTs. In living cells, MTs rapidly interconvert between assembly and disassembly. This dynamic action is essential for the proper spindle assembly, chromosome attachment and segregation during mitosis (43). Accordingly, we concluded that ST-11 suppressed MT dynamics in a manner that leads to aberrant spindle formation, improper alignment of chromosomes, and cell-cycle arrest in a prometaphase-like configuration. This effect is consistent with mitotic checkpoint arrest produced by the MT destabilizing activity of this compound.

Several important features distinguish ST-11 from nocodazole, which, similarly to 2-aroylindoles, directly interacts with the colchicine-binding site. ST-11 and nocodazole exhibit different effects on MT dynamics in live T98G cells at concentrations that promote similar levels of MT depolymerization in a cell-free system. Nocodazole rapidly abolishes dynamic MTs whereas ST-11 leaves them intact. Like nocodazole, the effects of ST-11 on suppression of MT dynamics are fully reversible within 24 hours. These findings are significant for two reasons. First, the reversibility of this compound might limit toxicity, which is notable from a therapeutic standpoint. Irreversible MT destabilizing compounds, such as colchicine, often have a challenging therapeutic index and their clinical use can be hindered by toxicity (36). Second, although ST-11 potently arrests cells in mitosis, its effects on interphase MT dynamics appear to be less severe. Likewise, this property may limit the clinical toxicity of this compound toward nondividing cells, especially neuronal cells.

Drugs that disrupt MT dynamics are commonly prescribed therapeutics as they both reduce tumor cell proliferation and trigger apoptosis (36, 39). Our finding that ST-11 kills

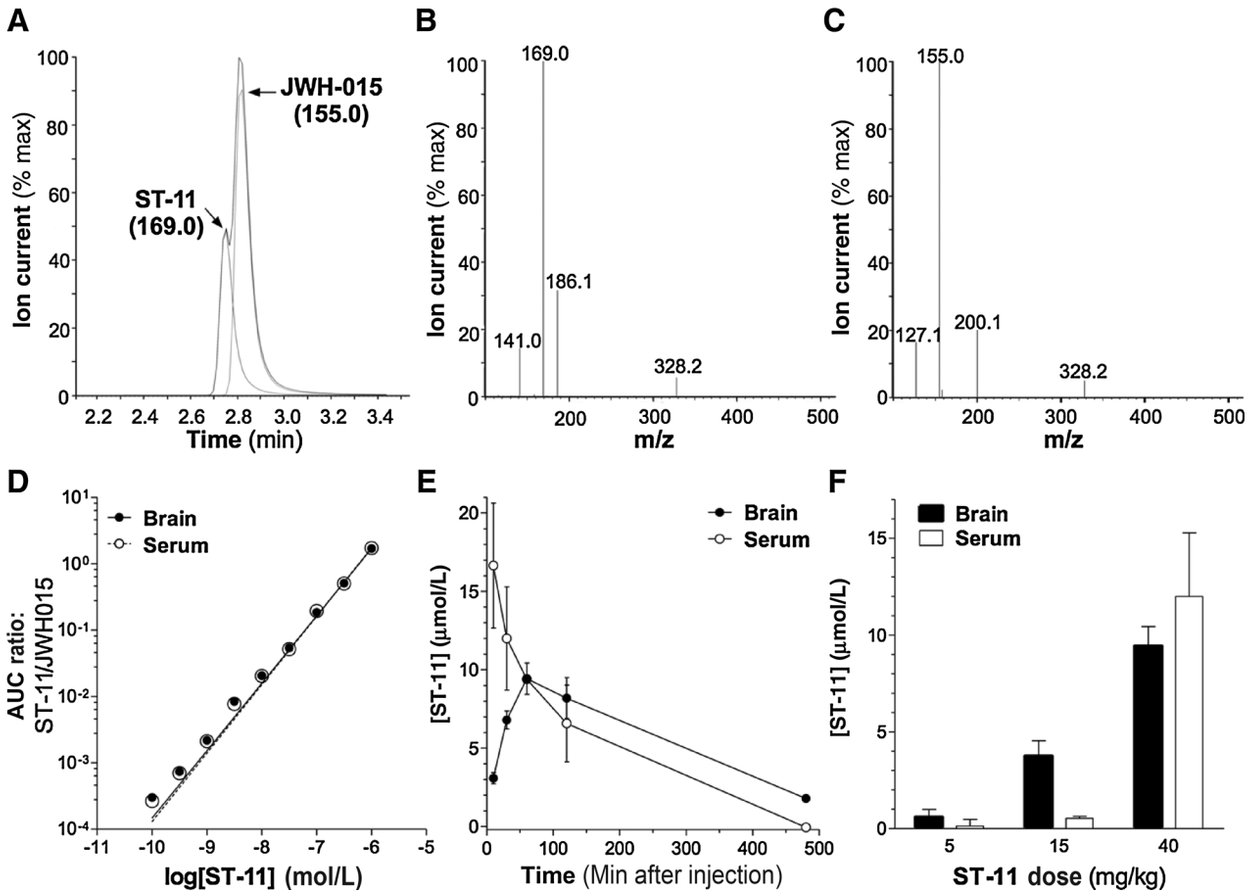

Figure 5.

LC-MS and pharmacokinetic analysis of ST-11 in mouse serum and brain. **A,** elution time of ST-11 and JWH-015 showing total current (black line) and individual currents from ST-11 and JWH-015. **B** and **C,** resolution of ST-11 (**B**) and JWH-015 (**C**) daughter ions. **D,** quantification of ST-11 extracted from serum and brain matrices shows a linear range of detection from 30 fg–1 ng. **E,** time course of ST-11 levels in serum and brain after intraperitoneal injection of 40 mg/kg ST-11. **F,** brain and serum levels of ST-11 60 minutes after intraperitoneal injection of the indicated doses. Results are presented as the mean ± SEM of three independent experiments.

glioblastoma multiforme cells through the activation of caspase-3–dependent apoptosis is evidenced by caspase-3 and PARP cleavage, morphologic changes, and increases in Annexin V/PI staining. Further support for this postulate is provided from our investigation of ST-11 on T98G cells where induction of apoptosis was ablated upon pretreatment with the caspase-3 inhibitor Z-DEVD-FMK. Therefore, we conclude that the principle mechanism of action of ST-11 is to directly suppress MT assembly, which leads to defects in mitotic spindle formation and chromosome attachment. This response is known to trigger mitotic checkpoint arrest and ultimately render glioma cells susceptible to apoptotic cell death (reviewed in ref. 36).

A recent study designed to identify novel therapeutic targets for the treatment of glioblastoma multiforme that do not adversely affect healthy brain cells revealed that this type of cancer is particularly sensitive to mitotic disruption (7). Other investigators have examined the efficacy and potency of indole-containing compounds on MT disruption and tumor reduction using different cancer models (16, 17, 37). For example, the antitubulin agent BPR0L075 effectively reduces tumor size in flank xenograft models of gastric and cervical carcinomas (17). Importantly, our studies suggest that ST-11 has increased therapeutic potential over other classical antitubulin compounds. Specifically, it has a higher efficacy and more lasting effect than either paclitaxel or nocodazole on the viability of glioblastoma multiforme cells *in vitro*. Thus, as a novel brain-penetrant compound, ST-11 underscores the therapeutic potential of antitubulin agents for the treatment of glioblastoma multiforme.

Another feature of our study was the establishment of ST-11 brain permeability. We observed that single injections of 15 and 40 mg/kg resulted in micromolar concentrations of ST-11 in brain that exceed its EC_{50} for killing glioblastoma multiforme cells in culture for 4 to 8 hours. In addition, the brain concentration of ST-11 was higher than that found in plasma by 2 hours post-administration with a brain:serum ratio of 1.2. Importantly, although possible and limited, liposomes are not known to favor passage across the blood–brain barrier without the aid of other factors such as convection-enhanced delivery (CED; ref. 44), ultrasound focused ultrasound (45) or receptor-mediated processes. Accordingly, it is likely that ability of formulated ST-11 to permeate the brain and provide significant efficacy against orthotopic gliomas represents an intrinsic property of ST-11. The

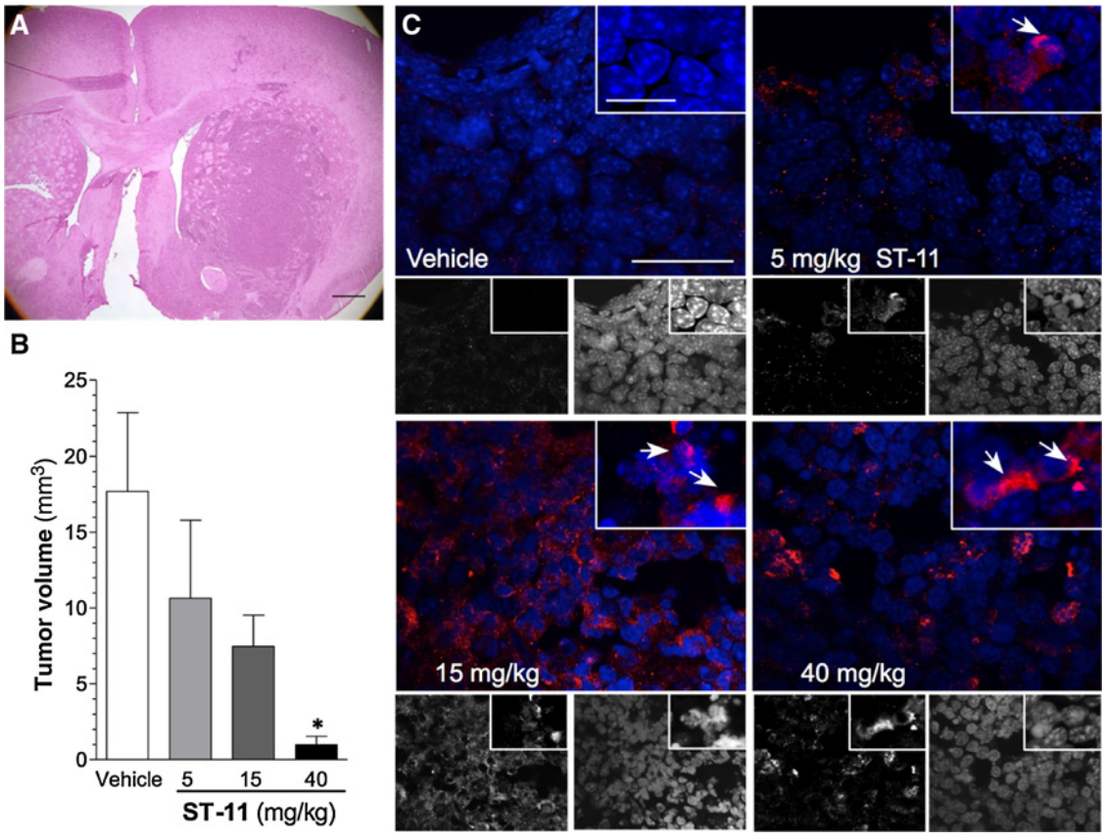

Figure 6.

ST-11 activates caspase-3 and reduces glioblastoma multiforme tumor burden in a syngeneic mouse model of glioblastoma multiforme. **A,** representative image of a BALB/c mouse brain containing a DBT tumor 3 weeks after implantation and stained with H&E; scale bar, 500 μm. Additional H&E features are presented in Supplementary Fig. S1. **B,** daily intraperitoneal treatments of ST-11 for 2 weeks dose-dependently decreased tumor volume *, $P < 0.05$, one-way ANOVA with the Dunnett *post hoc* test. Results are mean ± SEM of at least 5 mice. **C,** representative images of activated caspase-3 (red) and DAPI (blue) staining in DBT tumor slices obtained from mice given the indicated treatments; scale bars, 25 and 10 μm (inset). Arrows, DBT cells with high levels of activated caspase-3 neighboring condensed nuclei.

structure-based predictions suggesting the brain-penetrating qualities of ST-11 provide additional support to this possibility. Hence, ST-11 dose-dependently activated caspase-3 and reduced intracranial tumor volume when delivered intraperitoneal daily for 2 weeks. Notably, we did not observe overt signs of toxicity in these mice during the treatment period (data not shown). Moreover, we monitored the MTD of ST-11 in a 5-day dose-range finding study that incorporated clinical and histopathologic assessments. These rudimentary analyses of ST-11 toxicity indicate that this compound does not produce overt side effects when administered chronically at 40 mg/kg or acutely up to 240 mg/kg *in vivo*. This result agrees with our *in vitro* finding that ST-11 is nontoxic to nonmalignant cells. Thus, we have derived a new delivery method that achieves therapeutic brain levels of ST-11 and exhibits an encouraging safety profile when assessed in mice.

To conclude, our study introduces ST-11 as a new brain-penetrant antineoplastic agent with preferential selectivity for glioblastoma multiforme cells and no discernible toxicities in mice. The use of antitubulin agents for the treatment of glioblastoma multiforme is all the more convincing when one considers that they act in a manner that has been anticipated to be effective for the treatment of this type of cancer (7). We therefore propose that ST-11 belongs to a promising new series of brain-penetrant antineoplastic agents that slow MT assembly, promote cell-cycle arrest, and trigger apoptosis in glioblastoma multiforme. The future development and characterization of ST-11 analogs may ultimately lead to the advent of a treatment of this intractable and debilitating form of brain cancer.

Disclosure of Potential Conflicts of Interest

B.R. Haas is Senior Medical Writer at the Vaniam Group. E.A. Horne is Lead Scientist/Program Manager at Stella Therapeutics Inc.; reports receiving a commercial research grant from NIH/NCI SBIR Phase I and Life Science Discovery Fund; and has ownership interest (including patents) in Stella Therapeutics Inc. N. Stella is consulting at Stella Therapeutics, Inc.; has ownership interest (including patents) in as founder of Stella Therapeutics, which has exclusive right to ST-11, and is a consultant and Scientific Advisory Board member. No potential conflicts of interest were disclosed by the other authors.

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