

## SC68896, a Novel Small Molecule Proteasome Inhibitor, Exerts Antiglioma Activity *In vitro* and *In vivo*

Patrick Roth,<sup>1,2</sup> Maria Kissel,<sup>3</sup> Caroline Herrmann,<sup>2</sup> Günter Eisele,<sup>1,2</sup> Johann Leban,<sup>3</sup> Michael Weller,<sup>1,2</sup> and Friederike Schmidt<sup>2,4</sup>

**Abstract Purpose:** Glioblastomas are among the most lethal neoplasms, with a median survival of <1 year. Modulation of the proteasome function has emerged as a novel approach to cancer pharmacotherapy. Here, we characterized the antitumor properties of SC68896, a novel small molecule proteasome inhibitor.

**Experimental Design:** Different tumor cell lines were tested by crystal violet staining for sensitivity to SC68896, given alone or in combination with death ligands. The molecular mechanisms mediating SC68896-induced cell death and changes in cell cycle progression were assessed by immunoblot and flow cytometry. An orthotopic human glioma xenograft model in nude mice was used to examine the *in vivo* activity of SC68896.

**Results:** SC68896 inhibits the proliferation of cell lines of different types of cancer, including malignant glioma. Exposure of LNT-229 glioma cells to SC68896 results in a concentration- and time-dependent inhibition of the proteasome, with a consequent accumulation of p21 and p27 proteins, cell cycle arrest, caspase cleavage, and induction of apoptosis. Using RNA interference, we show that the effect of SC68896 on glioma cells is facilitated by wild-type p53. SC68896 sensitizes glioma cells to tumor necrosis factor-related apoptosis-inducing ligand and CD95 ligand and up-regulates the cell surface expression of the tumor necrosis factor-related apoptosis-inducing ligand receptor cell death receptors 4 and 5, which may contribute to this sensitization. Intracerebral glioma-bearing nude mice treated either *i.p.* or intratumorally with SC68896 experience prolonged survival.

**Conclusions:** SC68896 is the first proteasome inhibitor that exerts antiglioma activity *in vivo*. It may represent a novel prototype agent for the treatment of malignant gliomas and warrants clinical evaluation. (Clin Cancer Res 2009;15(21):6609-18)

Gliomas are intrinsic tumors of the brain that are characterized by resistance to apoptotic stimuli, local immunosuppression, and diffuse infiltration of the surrounding healthy tissue. The currently available treatment options, including surgery, radiotherapy, and chemotherapy, aim at stopping the local destruc-

tive tumor growth. Despite all efforts, the current standard treatment of care prolongs the median survival of glioblastoma patients only to 12 to 15 months in selected patient populations (1). Because of the limited activity of the conventional chemotherapeutic agents, new therapeutic approaches are urgently needed to improve the prognosis of the affected patients. One of the potential new targets is the proteasome, which is a protease complex responsible for the degradation of damaged or misfolded cellular proteins. It also removes proteins from the cell that influence cell cycle and growth (2). The 26S proteasome consists of two 19S subunits, and the 20S proteasome core and has trypsin-, chymotrypsin-, and caspase-like protease activities. The complex balance between synthesis and degradation of proteins is regulated by their ubiquitination status. Cells use long polyubiquitin chains to target proteins that are processed by the 26S proteasome, the site for ATP-dependent degradation of ubiquitin-tagged proteins. The ubiquitin-proteasome protein degradation pathway has thereby a central role within the regulation of cellular proliferation, differentiation, and apoptosis (3). Targeting the proteasome with specific inhibitors represents a novel and logical approach to induce cell death. The clinical relevance of this pathway has been exemplified by the proteasome inhibitor bortezomib, which shows beneficial effects in patients suffering from multiple myeloma and non-Hodgkin's

**Authors' Affiliations:** <sup>1</sup>Laboratory of Molecular Neuro-Oncology, Department of Neurology, University Hospital Zurich, Zurich, Switzerland; <sup>2</sup>Department of General Neurology, University of Tübingen, Tübingen, Germany; <sup>3</sup>4SC AG, Planegg-Martinsried, Germany; and <sup>4</sup>Department of Neurology, Klinikum Rechts der Isar, Technische Universität München, Munich, Germany

Received 3/3/09; revised 7/14/09; accepted 7/21/09; published OnlineFirst 10/13/09.

**Grant support:** Else-Uebelmesser Foundation (P. Roth) and the Jacqueline Seroussi Memorial Foundation (M. Weller).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

**Requests for reprints:** Patrick Roth, Department of Neurology, University Hospital Zurich, Frauenklinikstrasse 26, 8091 Zurich, Switzerland. Phone: 41-0-44-255-5540; Fax: 41-0-44-255-4380; E-mail: patrick.roth@usz.ch.

© 2009 American Association for Cancer Research.  
doi:10.1158/1078-0432.CCR-09-0548

## Translational Relevance

Despite recent advances in the treatment of malignant gliomas, these tumors remain a dominant challenge in the field of neurologic oncology. Because of the poor prognosis of the affected patients, there is an urgent need to develop novel therapeutic strategies. Inhibition of the proteasome has attracted much attention as a promising approach for cancer therapy. However, bortezomib, the only drug that has been clinically approved for other tumor entities, failed to inhibit the growth of experimental gliomas *in vivo*. Here, we characterize the activity of SC68896, a novel proteasome inhibitor, against malignant gliomas. SC68896 induced apoptosis in glioma cells and sensitized them to death ligands. Most importantly, the survival of glioma-bearing mice was increased after *i.p.* or intratumoral administration of SC68896. Based on these findings, our study provides a strong basis for a clinical evaluation of SC68896 as a novel therapeutic agent for glioma patients.

lymphoma (4, 5). Bortezomib has also been shown to inhibit the growth of malignant glioma cells *in vitro*; however, it was not effective in the treatment of experimental glioma xenografts *in vivo* (6). In addition, there is also a lack of clinical data proving its activity in patients with malignant gliomas. Therefore, the aim of the present study was to characterize the effects of a novel proteasome inhibitor, SC68896, on glioma cell survival *in vitro* and *in vivo*. Because gliomas are paradigmatic for resistance to therapy, a multitude of mechanisms has been explored to induce apoptosis in these cells. Theoretically, the induction of apoptosis by systemic or local application of death ligands, such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; alternative name, Apo2 ligand) and CD95 ligand, offers the possibility to target tumor cells. TRAIL mediates proapoptotic effects in the treatment of experimental gliomas through cell death receptors 4 and 5, which are expressed on the surface of these cells (7). By contrast, binding of TRAIL to the decoy receptors 1 and 2 fails to induce apoptosis. As a result of these antiapoptotic mechanisms, glioma cells exhibit a significant resistance toward TRAIL. Accordingly, we explored a direct effect of SC68896 on glioma cell proliferation *in vitro* and its effects on cell cycle progression. In addition, we assessed a possible sensitization of glioma cells to TRAIL- and CD95 ligand-induced apoptosis by this novel compound. The *in vivo* activity was evaluated in an orthotopic glioma nude mouse model by intratumoral or systemic *i.p.* application of SC68896.

## Materials and Methods

**Materials and cell lines.** SC68896 belongs to a new family of proteasome inhibitors. It was discovered by 4SC AG, a company that develops novel drug candidates based on the results of high-throughput screenings. The chemical synthesis of SC68896 has been described before (8). A stock solution was prepared in DMSO and stored at -20°C. TRAIL was purchased from Peprotech. CD95 ligand-containing super-

natant was harvested from CD95 ligand-transfected N2A neuroblastoma cells (9). Propidium iodide and all other reagents, unless indicated otherwise, were purchased from Sigma. Antibodies against human p21, p27, and poly(ADP-ribose) polymerase (PARP) were from BD Bioscience, antibody to p53 was purchased from Santa Cruz. Antibody against cleaved caspase-3 was from Cell Signalling. Antibody against caspase-3 was obtained from Merck, and that against caspase-8 was from Alexis. Glyceraldehyde-3-phosphate dehydrogenase antibody was purchased from Chemicon. DAOY medulloblastoma, the MCF-7 breast adenocarcinoma, and K-562 chronic myelogenous leukemia cell lines were obtained from the American Type Culture Collection. HCT-116 colorectal carcinoma cells were a kind gift of B. Vogelstein (Baltimore, MD). All other glioma cell lines were kindly provided by Dr. N. de Tribolet (Lausanne, Switzerland). K-562 cells were maintained in RPMI-1640 supplemented with 1 mmol/L sodium pyruvate (Gibco Life Technologies), penicillin (100 IU/mL)/streptomycin (100 µg/mL; Gibco), and 10% fetal calf serum (Biocrom KG). All other cell lines were cultured in DMEM containing 10% fetal calf serum, 2 mmol/L glutamine, and penicillin (100 IU/mL)/streptomycin (100 µg/mL). Primary glioblastoma cells were established from freshly resected tumors, cultured in monolayers, and used between passages 4 and 9 (10). The generation of stable LNT-229-p53si cells by RNA interference has been described (11).

**Viability assays.** The cells were seeded at  $5 \times 10^3$  cells per well in 96-well plates, allowed to attach for 24 h, and subsequently exposed to SC68896, as indicated. Cell density was assessed by crystal violet staining for adherent cells. The MTT assay was used to determine the viability of nonadherent cells (12).

**Immunoblot analysis.** The cells were treated with SC68896 or solvent control containing DMSO and lysed. Cellular soluble proteins (20 µg/lane) were harvested and separated on 10% acrylamide gels (Biorad). After transfer to nitrocellulose (Biorad), the blots were blocked in PBS containing 5% skim milk and 0.05% Tween 20 and incubated overnight at 4°C with antibodies to p21, p27, p53, PARP, β-actin, or glyceraldehyde-3-phosphate dehydrogenase. Visualization of protein bands was accomplished using horseradish peroxidase-coupled secondary antibodies (Santa Cruz) and enhanced chemiluminescence (Amersham).

**Flow cytometry.** For cell cycle analysis, glioma cells were treated with SC68896, as indicated, harvested, fixed, and permeabilized overnight in ice-cold 70% ethanol (Merck). The cells were washed twice with PBS. RNA was digested with RNase A (Gibco). The DNA was stained with propidium iodide (50 µg/mL). Fluorescence was recorded in a CyAn ADP S2536 analyzer (Dako Cytomation). Cells that seemed left of the G<sub>0</sub>/G<sub>1</sub> peak were considered to have a DNA content below 2*n*, indicative of cell death. Aggregated cells were gated out. For analysis of cell death, LNT-229 cells were grown in six-well plates, incubated with SC68896 at different concentrations for 24 h, washed in PBS, and resuspended in 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/NaOH (pH 7.4), 140 mmol/L NaCl, and 2.5 mmol/L CaCl<sub>2</sub>. Annexin V-fluorescein isothiocyanate (1:100) and propidium iodide (50 µg/mL) were added, and fluorescence in a total of 10,000 events per condition was recorded in a CyAn flow cytometer. Annexin V-fluorescein isothiocyanate- or propidium iodide-positive cells were counted as dead cells (either apoptotic or necrotic), and the remaining cells were designated the surviving cell fraction.

To analyze the cell surface expression of TRAIL receptors and CD95, the cells were incubated with or without SC68896, as indicated, harvested using Accutase (PAA), and washed with PBS. The following steps were carried out at 4°C in flow cytometry buffer (PBS; 1% bovine serum albumin; 1% fetal calf serum). The cells were incubated for 1 h with 10 µg/mL mouse isotype control antibody or mouse anti-human cell death receptor 4 clone 69036, cell death receptor 5 clone 71908, decoy receptor 1 clone 90906, decoy receptor 2 clone 104918 (all R&D Systems), or mouse anti-human CD95 clone DX2 (Santa Cruz). After two further washes, 4 µg/mL biotinylated rabbit anti-mouse F(ab')<sub>2</sub> (Dako Cytomation) were added for 20 min. Following two more

washes, the cells were stained for 20 min in 2 µg/mL streptavidin-allophycocyanin (BD Bioscience). Unbound staining reagent was removed by washing, propidium iodide (50 µg/mL) was added, and receptor expression was analyzed by flow cytometry. propidium iodide-positive cells, that is, dead cells, were gated out. The median fluorescence intensities of the samples achieved with specific antibody were divided by those achieved with isotype control antibody to obtain specific fluorescence indexes.

**Proteasome activity assay.** The activity of the three enzymatic sites of the proteasome was separately determined using commercially available kits (Proteasome-Glo, Promega). These are luminescent assays based on the measurement of the chymotrypsin-, trypsin-, and caspase-like activity associated with the proteasome complex in the investigated cells. Briefly,  $10^4$  cells per well were seeded in 96-well plates, allowed to adhere for 24 h, treated with SC68896, as indicated, and assessed for the enzymatic activities of the proteasome, according to the manufacturer's protocol. To assess the activity of the proteasome in whole blood, 10 µL of blood was taken from the tail vein of mice treated with SC68896 or vehicle and assessed by Proteasome-Glo.

**Mice and animal experiments.** Athymic CD1 nude mice were purchased from Charles River Laboratories. Mice of 6 to 12 wk of age were used in all experiments. The experiments were done according to NIH guidelines and Guide for the Care and Use of Laboratory Animals. Before all intracranial procedures mice were anesthetized by an i.p. injection of 7% chloral hydrate. For intracranial implantation, the mice were placed in a stereotactic fixation device (Stoelting), and a burr hole was drilled in the skull, 2 mm lateral to the bregma. The needle of a Hamilton syringe was introduced to a depth of 3 mm. LNT-229 glioma cells ( $7.5 \times 10^4$ ) in a volume of 2 µL PBS were injected into the right striatum. For the local application of SC68896, 80 µg of SC68896 or 2 µL of DMSO were injected intratumorally thrice in eight mice per group. For the i.p. administration, a SC68896 solution containing 5% of DMSO, 10% of cremophor, and NaCl 0.9% was produced daily and administered 5 days per week. To assess the inhibition of the proteasome *in vivo*, 10 µL of blood were taken from six mice of each group after 15 d of therapy, and the activity of the proteasome was assessed with the Proteasome-Glo kit. Both groups of 14 mice were observed daily, and the animals were sacrificed at the onset of neurologic symptoms.

For the assessment of tumor growth and histology, two animals per group were killed 2 d after the second intratumoral injection, and cryostat brain sections (8 µm) were stained with hematoxylin and eosin. For immunohistochemical stainings, the following antibodies were used: rabbit anti-von Willebrand factor (DAKO), mouse anti-p21 (Calbiochem), and mouse anti-Ki-67 (Zymed). Subsequently, the slices were stained with secondary antibody and developed with *Dolichos biflorus* agglutinin (Vectastain, Vector Laboratories). Tumor-containing sections of each brain were used for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining (Roche).

**Data analysis.** Data are representative of experiments done thrice with similar results. Viability and proliferation studies were done using triplicate wells. Where indicated, analysis of significance was done using the Mann-Whitney-Wilcoxon or the two-tailed Student's *t* test (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). Synergy of SC68896 and death ligands was evaluated using the fractional product method. Here, "predicted values" correspond to an additive action of two compounds that can be compared with the observed effect (13). In addition, the combination index for each of the combinations was evaluated using Calcsyn software (Biosoft). The program uses the multiple drug-effect equation of Chou-Talalay. When testing a drug combination, the software calculates for each fractional effect point a specific parameter, the combination index, which indicates the interactive effect of the drugs. Synergy levels were classified according to the Calcsyn manual: combination index  $< 0.9$  indicates synergy, combination index = 0.9 to 1.1 is additive, and combination index  $> 1.1$  means antagonism.

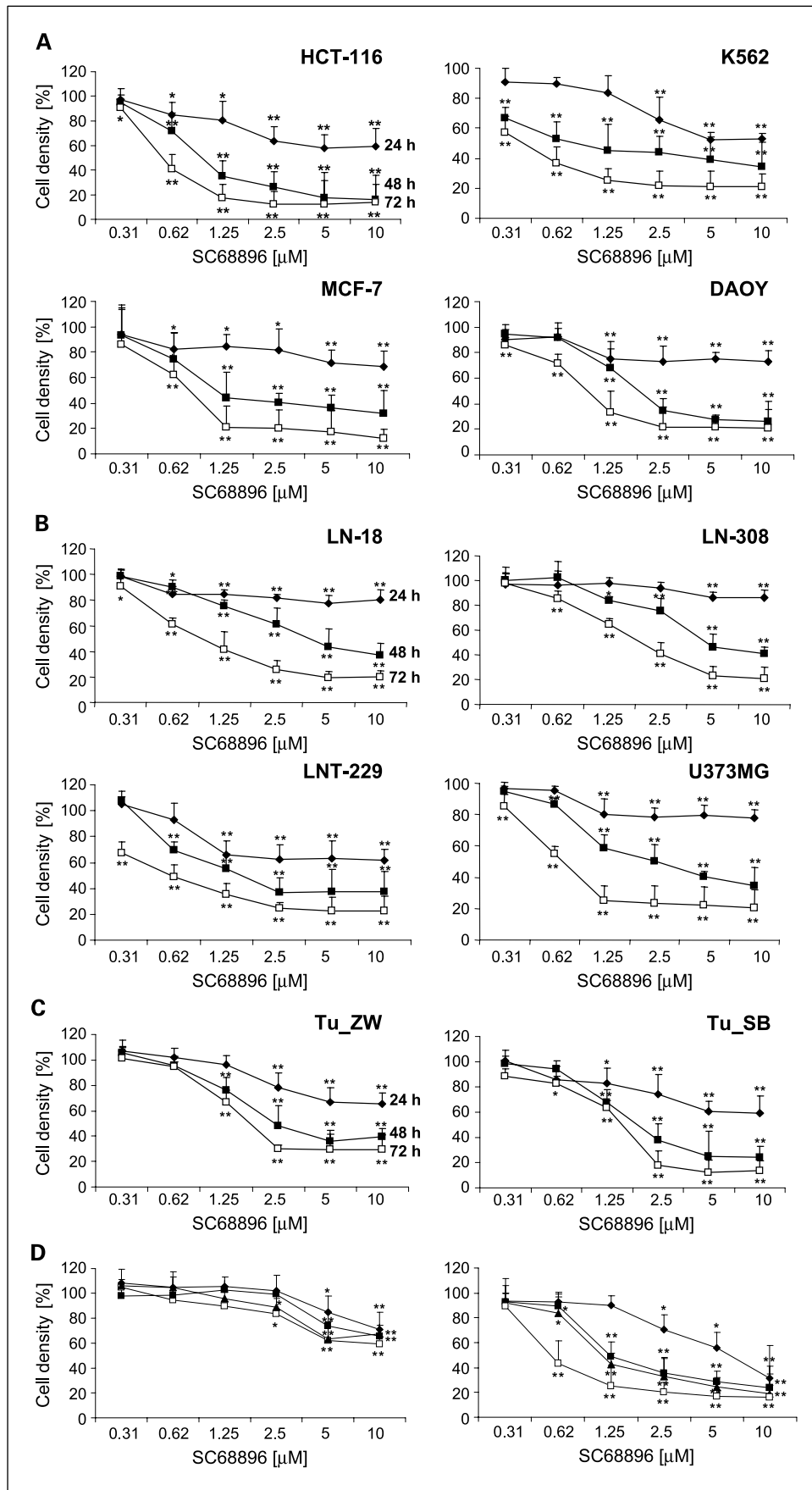
## Results

**SC68896 inhibits the proliferation of different tumor cell lines.** To assess its effect on tumor cell proliferation, we administered different concentrations of SC68896 to tumor cell lines from different origin. Exposure to SC68896 led to a time- and concentration-dependent inhibition of cell density, showing highest activity against HCT-116 colon carcinoma cells. When compared with cells that were grown in medium alone, the proliferation was massively diminished after exposure to SC68896 for 72 h (Fig. 1A). The same observation was made when SC68896 was added to glioma cell cultures in different concentrations, with highest efficacy against LNT-229 and U373MG cells (Fig. 1B). Exposure to SC68896 of primary glioma cells showed modest effects after 24 h but a considerable decrease of cell density after 48 and 72 h (Fig. 1C). To assess the effect of short-time exposure of SC68896 on glioma cells, we exposed LNT-229 glioma cells for 1, 3, or 6 h to SC68896 and evaluated the cell density after 24 h (Fig. 1D, *left*) or 72 h (*right*). Compared with a continuous exposure for 24 or 72 h, we noticed smaller but still considerable effects on the proliferation of these cells.

**SC68896 acts as an inhibitor of the proteasome in glioma cells and induces cell cycle arrest.** Because SC68896 was designed as an inhibitor of the cellular proteasome (8), we used commercially available kits to confirm its effect on the three enzymatic activities of the proteasome of glioma cells. Intriguingly, the chymotryptic site was reduced to  $<20\%$  after exposure to 1 or 10 µmol/L SC68896 for 3 h. Because no effect on cell density was observed at this point, this is not the result of a reduced number of tumor cells investigated. We then aimed at assessing the potential recovery of proteasomal function and measured the activity of the enzymatic sites at 3, 9, or 21 h after removal of the compound. The chymotryptic site remained completely inhibited after exposure to 10 µmol/L SC68896. In contrast, we noticed a slow recovery in cells that had been treated with  $\leq 1$  µmol/L of the compound. When SC68896 was continuously administered for 24 h, the proteasomal activity remained inhibited at concentrations of  $\geq 1$  µmol/L. The trypsin- and caspase-like sites of the proteasome were only slightly affected by SC68896, indicating a high specificity for the chymotryptic site (Fig. 2A).

To allow for a better understanding of the mechanisms underlying the inhibition of glioma cell proliferation, we next investigated whether exposure to SC68896 influences cell cycle and cell cycle-related proteins. Using immunoblot, we found a slight increase of p27 levels and a massive induction of p21 expression in LNT-229 cells after treatment with SC68896 for 24 h. Accumulation was p53-independent because it was maintained when the wild-type p53 gene was silenced by RNA interference (Fig. 2B). To investigate the mechanism of growth inhibition in more detail, we analyzed the effects of SC68896 on cell cycle distribution by flow cytometry. The antiproliferative effect of SC68896 was accompanied by a massive G<sub>2</sub>/M arrest and an increase in cells with sub-G<sub>1</sub> DNA content (Fig. 2C).

**Glioma cell sensitivity toward SC68896 is modulated by p53.** Because p53 plays an important role in cell cycle control and in the induction of apoptosis in response to various cellular stress signals, we asked whether SC68896 exerts p53-dependent antiproliferative or cytotoxic effects. To obtain a cell-based

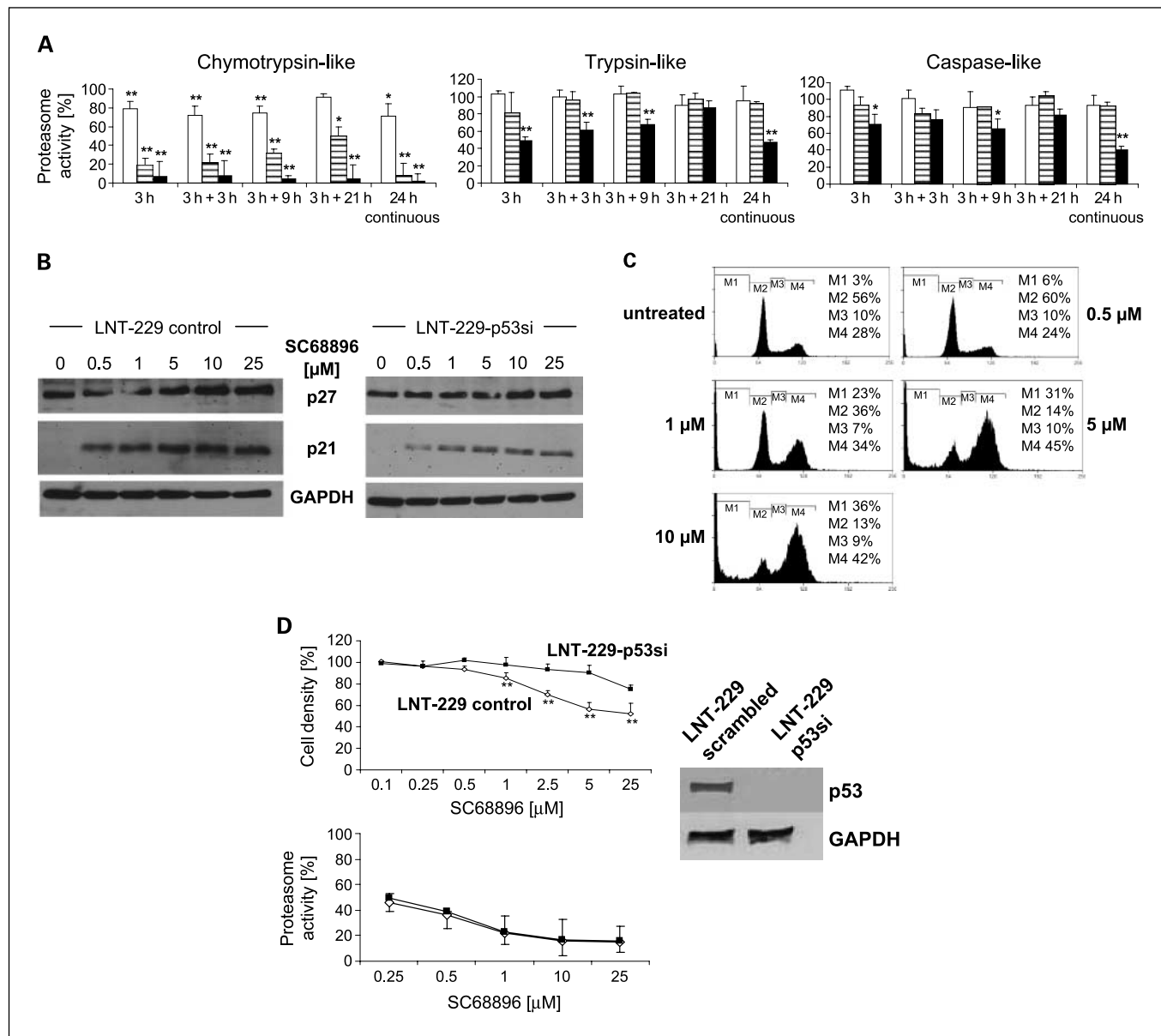


**Fig. 1.** SC68896 inhibits the proliferation of cancer cells. **A**, tumor cell lines from different origin were treated with increasing concentrations of SC68896 for 24 h (filled diamonds), 48 h (filled squares), or 72 h (open squares). Cell viability was assessed by the MTT assay for K-562 cells or cell density by crystal violet staining for all other cell lines. The growth of four different glioma cell lines (**B**) and freshly isolated glioma cells (**C**) after exposure to SC68896 was assessed by crystal violet staining. The proteasome inhibitor was administered for 24, 48, or 72 h. **D**, LNT-229 glioma cells were exposed to SC68896 for 1 h (filled diamonds), 3 h (filled squares), 6 h (triangles), or continuously (open squares) and examined after 24 h (left). The same experiment with a read out after 72 h is on the right. Statistically significant changes compared with untreated cells are marked with asterisks. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , two-sided Student's  $t$  test.

experimental system that allows distinguishing between p53-dependent and -independent effects, we used LNT-229 cells with a siRNA-mediated stable p53 knockdown. Compared with cells transfected with a scrambled control plasmid, hardly any p53 expression was detectable in LNT-229-p53si cells (Fig. 2D, inset). SC68896 inhibited the proliferation of the LNT-229 sublines partially in a p53-dependent manner because the

p53-deleted cells were more resistant (Fig. 2D, top). Irrespective of p53 status, administration of SC68896 led to a similar inhibition of the chymotryptic activity of the proteasome (bottom), indicating that p53 protects the cells downstream of proteasome inhibition.

**Administration of SC68896 induces apoptosis and activation of caspases in glioma cells.** Administration of SC68896 for 24 h



**Fig. 2.** SC68896 inhibits the proteasomal activity of glioma cells and induces cell cycle arrest in glioma cells. **A**, LNT-229 glioma cells were exposed to different concentrations of SC68896 for 3 h (open bars, 0.1 μmol/L; striped bars, 1 μmol/L; filled bars, 10 μmol/L). Activities of three enzymatic sites were separately determined at this point and at 3, 9, or 21 h after removal of the compound or continuous exposure for 24 h. Statistically significant changes compared with untreated cells are marked with asterisks. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , two-sided Student's  $t$  test. **B**, after exposure to SC68896 for 24 h, whole cell lysates of LNT-229 control (left) or p53si (right) glioma cells were assessed by immunoblot for the expression of p21 and p27, using glyceraldehyde-3-phosphate dehydrogenase as a reference. **C**, LNT-229 cells were treated with various concentrations of SC68896 for 24 h, fixed, permeabilized, stained with propidium iodide, and analyzed by flow cytometry. The figure shows representative examples of flow cytometry profiles and the distribution of the cells to the indicated histogram regions (M1, sub-G<sub>1</sub>; M2, G<sub>1</sub>; M3, S; M4, G<sub>2</sub>/M). **D**, the expression of p53 was assessed by immunoblot in LNT-229 control or p53si cells (inset). LNT-229 control (filled squares) or p53si cells (open diamonds) were exposed to SC68896 with the indicated concentrations for 24 h. Cell density was assessed by crystal violet staining (top). The chymotryptic proteasomal activity of LNT-229 control or p53si cells was measured after treatment with SC68896 for 3 h (bottom). Statistically significant differences between LNT-229 control and p53si cells are marked with asterisks. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , two-sided Student's  $t$  test.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/15/21/6609/1986391/6609.pdf by guest on 16 May 2022

induced apoptosis in LNT-229 glioma cells, as assessed by Annexin V/propidium iodide staining. This effect was concentration dependent, with the highest percentage of apoptotic cells at 25  $\mu\text{mol/L}$  (Fig. 3A). These findings are in accordance with the effects of SC68896 on glioma cell proliferation after exposure for 24 h (combined results in Fig. 3A, *bottom*). We next asked whether activation of caspases occurs after application of SC68896. To this end, whole cell lysates were generated after treatment of LNT-229 cells for 24 h with different concentrations of SC68896. Immunoblot revealed the proteolytic processing of caspase-3 and caspase-8 (Fig. 3B) and cleavage of PARP (Fig. 3C).

**SC68896 sensitizes human malignant glioma cell lines to TRAIL- and CD95 ligand-induced cell death.** Resistance of glioma cells to apoptotic signaling mediated by death ligands is a major obstacle for their therapeutic use. We therefore asked whether SC68896 sensitized human glioma cells to TRAIL- and CD95 ligand-induced cell death. LNT-229 and LN-308 cells were treated with SC68896 at different concentrations and stimulated with TRAIL or CD95 ligand for 20 h. Both glioma cell lines were strongly sensitized by SC68896 to TRAIL- and CD95 ligand-induced cell death at conditions wherein either agent alone had little or no effect (Fig. 4A and B, *top*). Selected survival data were analyzed by the fractional product method, revealing a considerable synergistic activity of SC68896 with both death receptor ligands (Fig. 4A and B, *bottom*). The synergistic action of SC68896 and death ligands was also confirmed by a statistical analysis based on the Chou-Talalay method (Supplementary Fig. S2). Light microscopic analysis confirmed that the observed loss in cell density was due to decreased survival because the cells rounded off and detached (data not shown).

We next assessed whether the cell surface expression of the TRAIL and CD95 receptors was induced by the inhibition of the proteasome through SC68896 as one possible explanation for this sensitization. To this end, we exposed LNT-229 and LN-308 human glioma cell lines to SC68896 and monitored the surface expression of cell death receptor 4, cell death receptor 5, decoy receptor 1, decoy receptor 2, and CD95 by flow cytometry. Cell death receptor 4 cell surface levels were moderately upregulated in LNT-229 but not in LN-308 cells (Fig. 4C). In contrast, the surface expression of cell death receptor 5 was strongly induced in both cell lines (Fig. 4D). The surface expression of the decoy receptors decoy receptors 1 and 2 remained unchanged. Similarly, CD95 levels on the cell surface were not induced by SC68896 (data not shown).

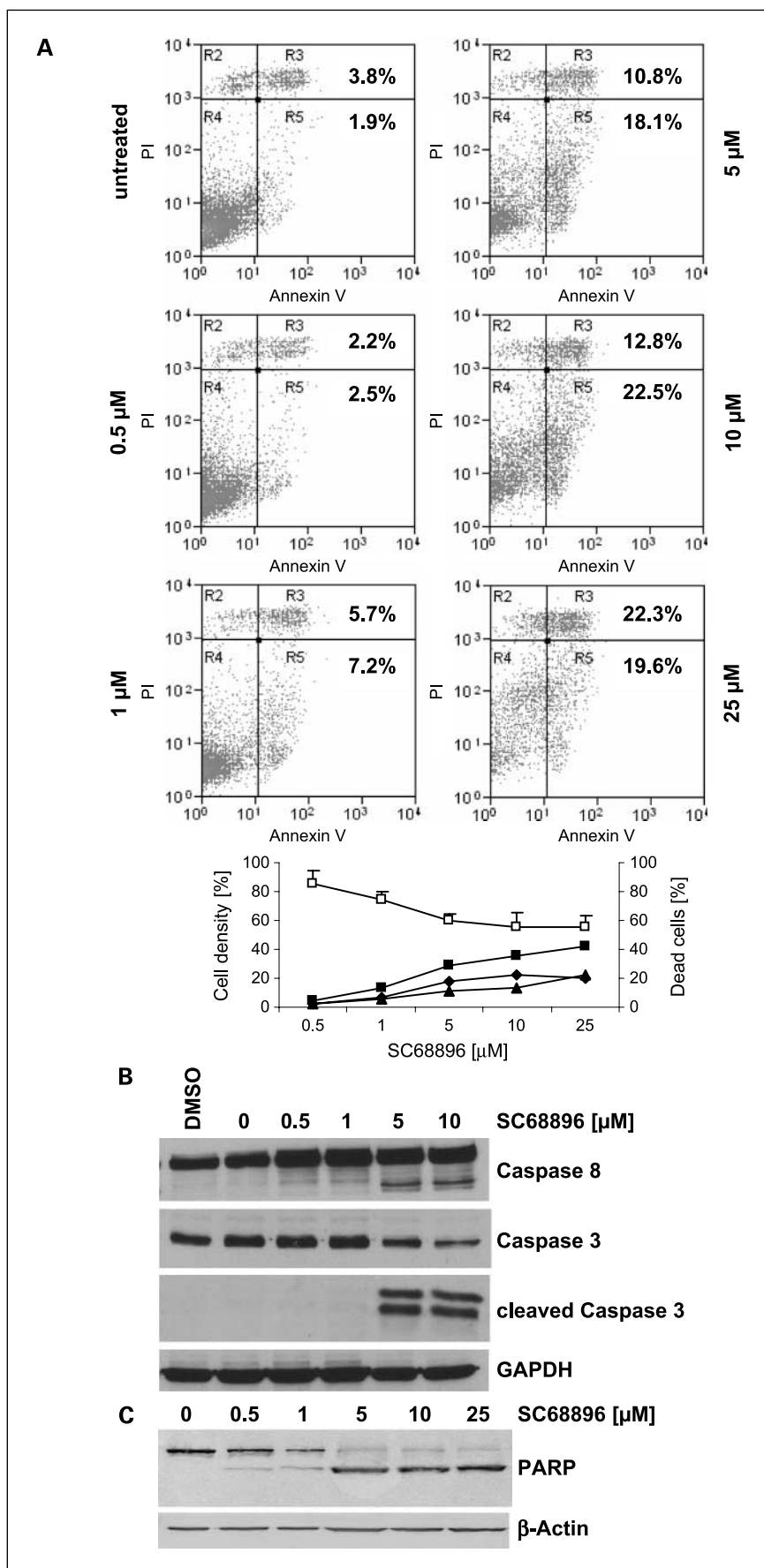
**Systemic and local application of SC68896 to glioma-bearing mice prolongs their survival.** The efficacy of SC68896 as a proteasome inhibitor that delays the growth of malignant glioma cells was subsequently assessed *in vivo* in an intracerebral glioma xenograft mouse model. Starting 8 days after stereotactic implantation of LNT-229 tumors, the mice were treated *i.p.* on 5 days per week with 150 mg/kg SC68896 or vehicle as a control, until they developed neurologic symptoms and had to be sacrificed. The median survival was prolonged from 50.5 days in vehicle-treated mice to 60 days for the SC68896 group (Fig. 5A;  $P < 0.01$  by Mann-Whitney-Wilcoxon test). To allow for an examination of the *in vivo* efficacy of SC68896 to target the proteasome, blood samples were taken from six mice of each group after 15 days of therapy. The activ-

ity of the proteasome was significantly reduced in the samples of SC68896-treated animals (Fig. 5B). Because local application of antitumor compounds is also a feasible delivery method, we additionally checked whether an intratumoral administration of SC68896 also inhibited the growth of intracerebral glioma xenografts. To this end, 80  $\mu\text{g}$  of SC68896 or 2  $\mu\text{L}$  of DMSO were injected intratumorally on days 7, 14, and 21 after tumor implantation. Animals that were treated with DMSO developed neurologic symptoms and had to be sacrificed earlier than animals that received SC68896. Survival at 55 days was 0% with vehicle treatment but 83% with SC68896 administration ( $P < 0.01$ , Fig. 5C). Two mice per group were sacrificed after the second administration of DMSO or the proteasome inhibitor for histologic analyses. Treatment with SC68896 led to smaller tumors with larger necrotic areas (Fig. 5D, *top*). The proliferation of glioma cells, assessed by Ki-67 staining, was reduced in mice that had received the proteasome inhibitor. We also noticed modest differences in p21 staining and apoptotic cells, that is, positive in the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay, with a preference to SC68896-treated tumors (Fig. 5D). No differences were detected on the level of angiogenesis, as assessed by von Willebrand factor staining (data not shown).

## Discussion

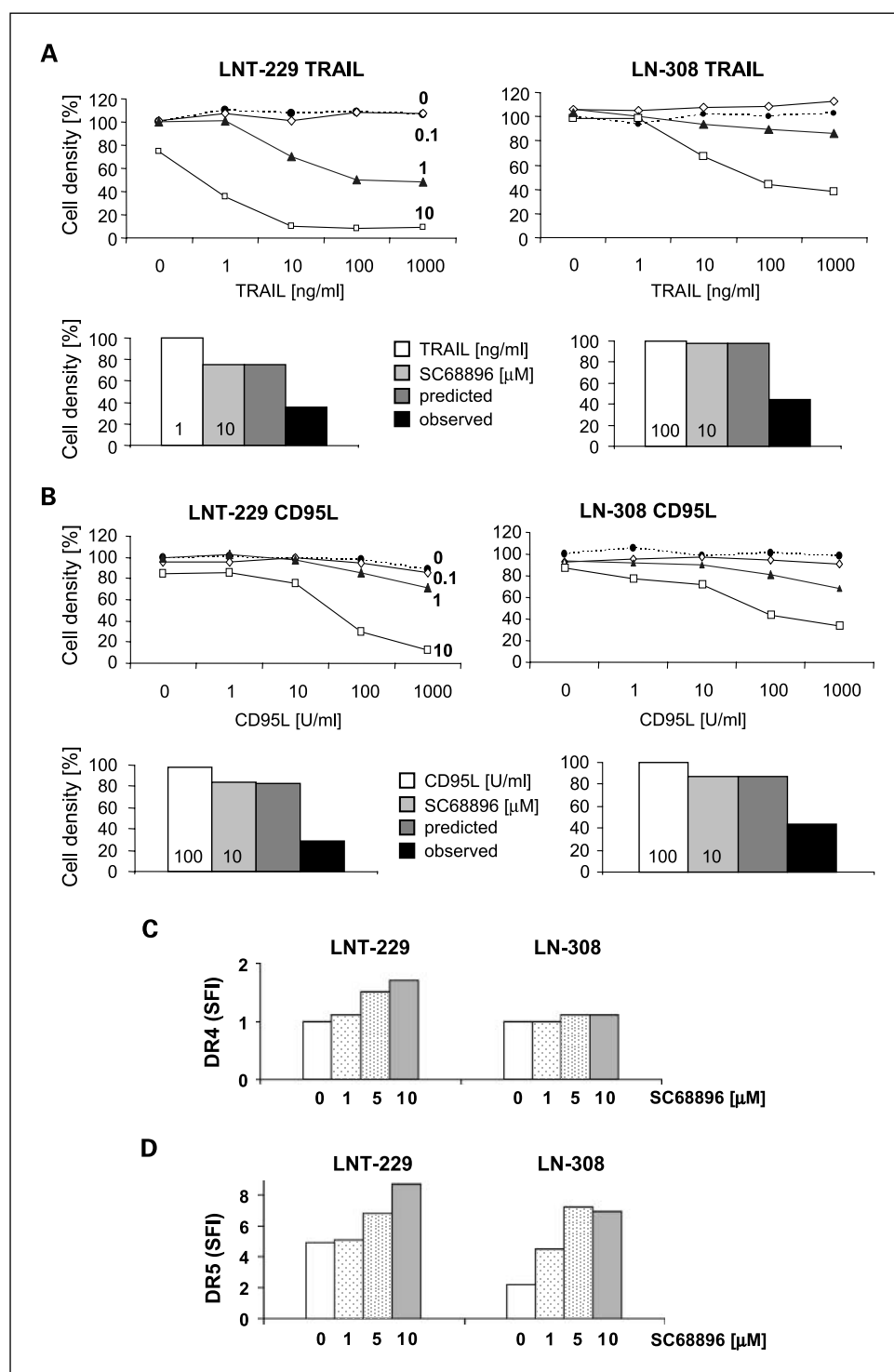
The survival of patients affected by malignant gliomas is still poor. The major obstacle to overcome is the resistance of glioma cells to undergo therapeutically induced cell death. The proteasome represents a potential target for novel therapeutic approaches because cancer cells are believed to be more dependent on proteasomal activity because of the accumulation of more misfolded and mutated proteins (14). Proteasome inhibitors have already entered the clinic and have proven their efficacy against hematologic malignancies and solid tumors. Here, we report the characterization of a novel small molecule, SC68896, which inhibits the proteasome of glioma cells with the subsequent induction of cell cycle arrest and apoptosis. Of note, short-time exposure of SC68896 also led to significant effects on glioma cells, which might have important ramifications for its clinical use (Fig. 1D). As described for other proteasome inhibitors, SC68896 arrested glioma cells in G<sub>2</sub>/M and induced the accumulation of p21 and p27 in a p53-independent manner (Fig. 2B; ref. 15). RNA interference-mediated silencing of wild-type p53 in LNT-229 glioma cells also did not affect the inhibition of proteasomal activity by SC68896. This is not unexpected because p53-dependent effects act downstream of the proteasome. Specific silencing of p53 also allowed clarifying its role for the susceptibility of LNT-229 glioma cells to proteasome inhibition. Interestingly, p53-depleted cells were less vulnerable to the deleterious effects of proteasome inhibition (Fig. 2D).

Exposure of glioma cells to SC68896 was accompanied by the induction of cell death and the cleavage of caspases and PARP (Fig. 3). Other compounds that target the proteasome act in a similar way on glioma and other neoplastic cell lines (16, 17). At equimolar concentrations, SC68896 is less potent than the proteasome inhibitor bortezomib (Supplementary Fig. S1). However, the clinical perspectives for proteasome inhibitors are determined by the doses that are tolerated *in vivo*.



**Fig. 3.** SC68896 induces apoptosis in glioma cells. **A**, LNT-229 glioma cells were exposed to SC68896 at different concentrations for 24 h and analyzed for Annexin V–fluorescein isothiocyanate/propidium iodide staining by flow cytometry. Data are expressed as mean percentages of either Annexin V–positive and propidium iodide–negative (apoptotic) or Annexin V– and propidium iodide–positive (necrotic) cells (representative experiment,  $n = 3$ ). The data are summarized (bottom) with early apoptotic cells (diamonds), necrotic cells (triangles), and their sum as the net cytotoxicity (closed squares). Cell density after exposure of LNT-229 glioma cells to SC68896 for 24 h was assessed by crystal violet staining (open squares). **B** and **C**, LNT-229 cells were treated with SC68896 for 24 h. Immunoblot analysis for caspase-8, caspase-3, and cleaved caspase-3 (**B**) or PARP (**C**) was done as described. Glyceraldehyde-3-phosphate dehydrogenase or  $\beta$ -actin was used as reference.

Downloaded from <http://aacrjournals.org/clincancerres/article-pdf/15/21/6609/1986391/6609.pdf> by guest on 16 May 2022



**Fig. 4.** SC68896 sensitizes human glioma cell lines to TRAIL- and CD95 ligand-induced cell death. *A* and *B*, the cells were treated with the indicated concentrations of SC68896 and TRAIL (*A*) or CD95 ligand (*B*). Cell density was assessed by crystal violet staining after 20 h (*top*). Selected data are displayed according to the fractional product method (*bottom*). The expected combined effects of SC68896 and TRAIL in case of independent mechanisms of action are designated as predicted. A synergy between both compounds was assumed when the observed growth inhibition of the combined treatment (black bars) exceeded the predicted growth inhibition (dark gray bars) by >30%. *C* and *D*, LNT-229 or LN-308 cells were treated with different concentrations of SC68896 for 20 h. Cell surface expression of cell death receptors 4 (*C*) and 5 (*D*) was analyzed by flow cytometry. Specific fluorescence index values for both receptors are displayed.

Downloaded from <http://aacrjournals.org/clincancerres/article-pdf/15/21/6609/1986391/6609.pdf> by guest on 16 May 2022

In that regard, it is noteworthy that SC68896 is the first proteasome inhibitor that exerts anti glioma activity in an experimental glioma model *in vivo* (Fig. 5). Because these compounds may not only influence glioma cell survival directly but also through indirect implications on their susceptibility toward apoptotic stimuli (18), we were further interested in the potential of SC68896 to sensitize glioma cells to death ligands. Although

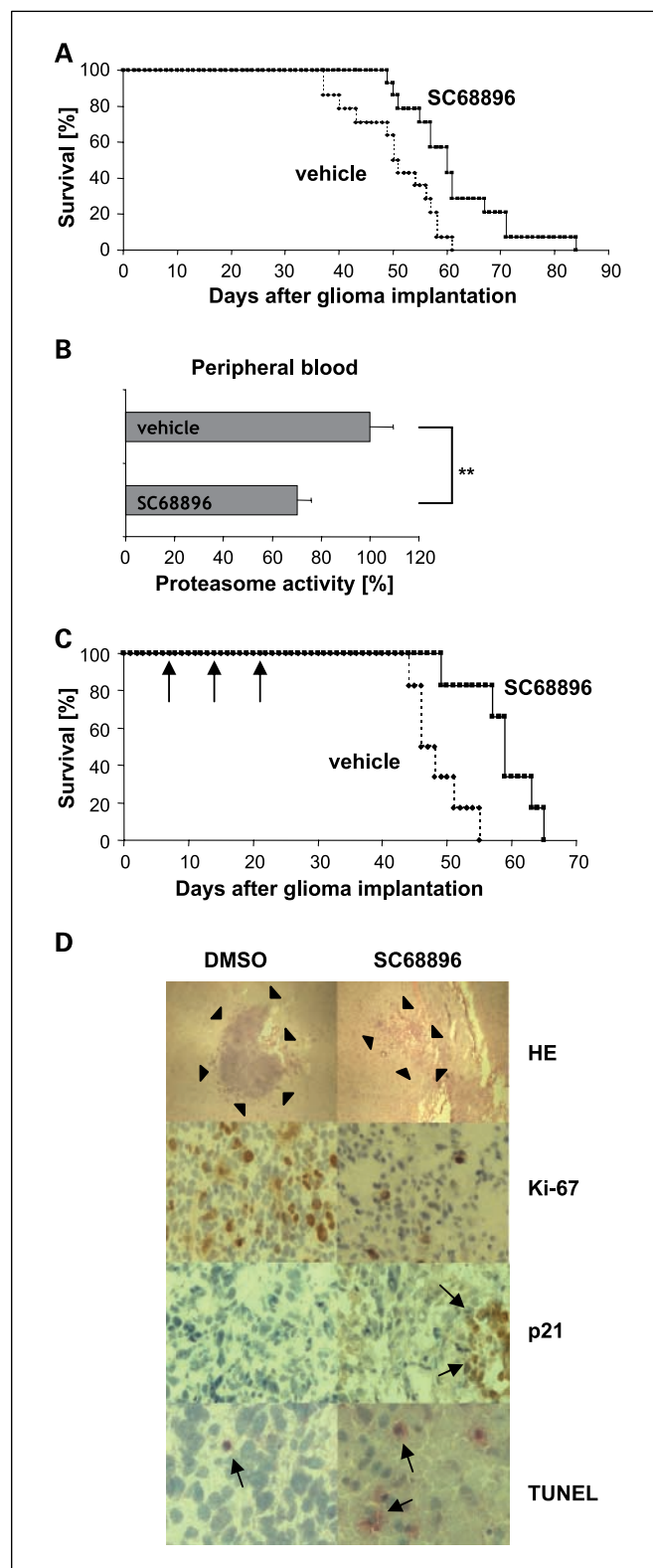
the proapoptotic TRAIL receptor cell death receptors 4 and 5 are expressed on human glioma cell lines (19), only a minority of glioma cells is sensitive to TRAIL. Smac agonists, which antagonize the antiapoptotic effector protein X-linked inhibitor of apoptosis protein, are one option to overcome TRAIL resistance in glioma cells *in vivo* (20). Bortezomib has also been described to sensitize glioma cell to different apoptotic stimuli (21, 22).



We used LNT-229 and LN-308 glioma cells, which are resistant to TRAIL when applied alone, for this study. SC68896 efficiently sensitized resistant glioma cells to TRAIL and CD95 ligand (Fig. 4A and B). We observed a pronounced synergy between SC68896 and both death receptor ligands (Fig. 4; Supplementary Fig. S2). This effect was independent of p53 because LN-308 cells do not express p53. The TRAIL receptor cell death receptors 4 and 5 are damage-inducible genes, and the subsequent upregulation of their expression on the cell surface on neoplastic cells leads to a sensitization for TRAIL-induced apoptosis (23, 24). We found that SC68896 induces the expression of cell death receptors 4 and 5 on the cell surface, whereas the expression of decoy receptors 1 and 2 remains unaffected (Fig. 4C and D). The enhanced receptor levels at the cell surface were paralleled by a sensitization to TRAIL-induced apoptosis (Fig. 4). However, we cannot exclude other mechanisms that contribute to the SC68896-mediated sensitization to TRAIL. Accordingly, the fact that a sensitization to CD95 ligand occurs without an upregulation of cell surface-bound CD95 may also easily be explained by the huge variety of antiapoptotic mechanisms present in glioma cells and affected by proteasome inhibition. Based on these results, a combined administration of SC68896 with a death receptor ligand may be a promising clinical approach that warrants further exploration.

Most importantly, we also provide *in vivo* data showing the efficacy of SC68896 against established experimental gliomas. In contrast, no such activity was shown for bortezomib against U87MG and TCG3 glioma xenografts in nude mice and for ritonavir in the rat 9L glioma model (6, 25). Systemic or local administration of SC68896 led to increased survival of these mice (Fig. 5A, C). As assessed by immunohistochemistry, the proliferation rate of glioma cells was markedly decreased after intratumoral administration of SC68896. These findings are in line with reduced Ki-67 levels after exposure of myeloma cells to a proteasome inhibitor (26). The failure of SC68896 to establish long-lasting control of tumor growth may result from insufficient levels in blood and tumor after *i.p.* administration (Fig. 5B). The lack of a complete eradication of the tumor by the intratumoral injection of SC68896 may be explained by the limited number of only three applications and an uncertain depth of penetration. Although central parts of these tumors were necrotic (Fig. 5D), the inhibitor presum-

ably did not reach cells at the tumor border, leading to a lack of sustained tumor growth control. This is consistent with the relatively low number of apoptotic or p21-positive cells. Continuous administration, as used in clinical trials with



**Fig. 5.** Local or systemic administration of SC68896 prolongs the survival of glioma-bearing mice. **A** and **B**, LNT-229 cells were inoculated intracerebrally in athymic CD1 nude mice. The animals were treated *i.p.* with SC68896 (150 mg/kg body weight; *solid lines*) or vehicle (*dotted lines*). The mice were observed in daily intervals and killed at the onset of neurologic symptoms. Survival data for 14 animals per group are presented as a Kaplan-Meier plot (**A**). Blood samples were taken from six mice of each group after 15 d of treatment. The enzymatic activity of the proteasome was measured with the Proteasome-Glo kit (**B**). **C**, LNT-229 cells were implanted into the right striatum of nude mice. Eighty micrograms of SC68896 (*solid lines*) or 2  $\mu$ L DMSO (*dotted lines*) were injected intratumorally on days 7, 14, and 21 after tumor implantation (*arrows*). Survival data for six animals per group are presented as a Kaplan-Meier plot. **D**, after two intratumoral applications of DMSO or SC68896 (day 15 after tumor inoculation), the brains of the animals were removed, shock frozen, and stained as indicated. Tumor borders are marked with arrows (*top*); original magnifications,  $\times 20$  for hematoxylin and eosin,  $\times 100$  for Ki-67 and p21, and  $\times 200$  for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling).

convection-enhanced delivery, may allow for a more pronounced antitumor activity. Summarized, these data set shows the *in vitro* and *in vivo* activity of SC68896 against malignant glioma cells. The fact that SC68896 exerts antiglioma effects *in vivo* and prolongs the survival of glioma-bearing mice delineates SC68896 as a novel promising compound that warrants evaluation in a clinical phase I trial for patients with malignant glioma.

## References

- Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 2005;352:987–96.
- Thompson SJ, Loftus LT, Ashley MD, Meller R. Ubiquitin-proteasome system as a modulator of cell fate. *Curr Opin Pharmacol* 2008;8:90–5.
- Mani A, Gelmann EP. The ubiquitin-proteasome pathway and its role in cancer. *J Clin Oncol* 2005;23:4776–89.
- Richardson PG, Sonneveld P, Schuster MW, et al. Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. *N Engl J Med* 2005;352:2487–98.
- Goy A, Younes A, McLaughlin P, et al. Phase II study of proteasome inhibitor bortezomib in relapsed or refractory B-cell non-Hodgkin's lymphoma. *J Clin Oncol* 2005;23:667–75.
- Labussiere M, Pinel S, Delfortrie S, Plenat F, Chastagner P. Proteasome inhibition by bortezomib does not translate into efficacy on two malignant glioma xenografts. *Oncol Rep* 2008;20:1283–7.
- Rieger J, Naumann U, Glaser T, Ashkenazi A, Weller M. APO2 ligand: a novel lethal weapon against malignant glioma? *FEBS Lett* 1998;427:124–8.
- Leban J, Blisse M, Krauss B, Rath S, Baumgartner R, Seifert MH. Proteasome inhibition by peptide-semicarbazones. *Bioorg Med Chem* 2008;16:4579–88.
- Rensing-Ehl A, Frei K, Flury R, et al. Local Fas/APO-1 (CD95) ligand-mediated tumor cell killing *in vivo*. *Eur J Immunol* 1995;25:2253–8.
- Bähr O, Rieger J, Duffner F, Meyermann R, Weller M, Wick W. P-glycoprotein and multidrug resistance-associated protein mediate specific patterns of multidrug resistance in malignant glioma cell lines, but not in primary glioma cells. *Brain Pathol* 2003;13:482–94.
- Wischhusen J, Naumann U, Ohgaki H, Rastinejad F, Weller M. CP-31398, a novel p53-stabilizing agent, induces p53-dependent and p53-independent glioma cell death. *Oncogene* 2003;22:8233–45.
- Wagenknecht B, Glaser T, Naumann U, et al. Expression and biological activity of X-linked inhibitor of apoptosis (XIAP) in human malignant glioma. *Cell Death Differ* 1999;6:370–6.
- Webb JL. Enzyme and metabolic inhibitors. 1st ed New York: Academic Press; 1963.
- Adams J. The proteasome: a suitable antineoplastic target. *Nat Rev Cancer* 2004;4:349–60.
- Wagenknecht B, Hermisson M, Eitel K, Weller M. Proteasome inhibitors induce p53/p21-independent apoptosis in human glioma cells. *Cell Physiol Biochem* 1999;9:117–25.
- Liu X, Yue P, Chen S, et al. The proteasome inhibitor PS-341 (bortezomib) up-regulates DR5 expression leading to induction of apoptosis and enhancement of TRAIL-induced apoptosis despite up-regulation of c-FLIP and survivin expression in human NSCLC cells. *Cancer Res* 2007;67:4981–8.
- Wagenknecht B, Hermisson M, Groscurth P, Liston P, Krammer PH, Weller M. Proteasome inhibitor-induced apoptosis of glioma cells involves the processing of multiple caspases and cytochrome *c* release. *J Neurochem* 2000;75:2288–97.
- Koschny R, Holland H, Sykora J, et al. Bortezomib sensitizes primary human astrocytoma cells of WHO grades I to IV for tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. *Clin Cancer Res* 2007;13:3403–12.
- Röhn TA, Wagenknecht B, Roth W, et al. CCNU-dependent potentiation of TRAIL/Apo2L-induced apoptosis in human glioma cells is p53-independent but may involve enhanced cytochrome *c* release. *Oncogene* 2001;20:4128–37.
- Fulda S, Wick W, Weller M, Debatin KM. Smac agonists sensitize for Apo2L/TRAIL- or anti-cancer drug-induced apoptosis and induce regression of malignant glioma *in vivo*. *Nat Med* 2002;8:808–15.
- La Ferla-Bruhl K, Westhoff MA, Karl S, et al. NF- $\kappa$ B-independent sensitization of glioblastoma cells for TRAIL-induced apoptosis by proteasome inhibition. *Oncogene* 2007;26:571–82.
- Yin D, Zhou H, Kumagai T, et al. Proteasome inhibitor PS-341 causes cell growth arrest and apoptosis in human glioblastoma multiforme (GBM). *Oncogene* 2005;24:344–54.
- Guan B, Yue P, Clayman GL, Sun SY. Evidence that the death receptor DR4 is a DNA damage-inducible, p53-regulated gene. *J Cell Physiol* 2001;188:98–105.
- Wu GS, Burns TF, McDonald ER III, et al. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nat Genet* 1997;17:141–3.
- Laurent N, de Bouard S, Guillamo JS, et al. Effects of the proteasome inhibitor ritonavir on glioma growth *in vitro* and *in vivo*. *Mol Cancer Ther* 2004;3:129–36.
- Zavrski I, Naujokat C, Niemoller K, et al. Proteasome inhibitors induce growth inhibition and apoptosis in myeloma cell lines and in human bone marrow myeloma cells irrespective of chromosome 13 deletion. *J Cancer Res Clin Oncol* 2003;129:383–91.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

We thank Gabriele von Kürthy, Petra Wolint, and Manuela Silgner for the excellent technical assistance.