Schweinfurthin A Selectively Inhibits Proliferation and Rho Signaling in Glioma and Neurofibromatosis Type 1 Tumor Cells in a NF1-GRD–Dependent Manner

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Abstract

Neurofibromatosis type 1 (NF1) is the most common genetic disease affecting the nervous system. Patients typically develop many tumors over their lifetime, leading to increased morbidity and mortality. The *NF1* gene, mutated in NF1, is also commonly mutated in sporadic glioblastoma multiforme (GBM). Because both NF1 and GBM are currently incurable, new therapeutic approaches are clearly needed. Natural products represent an opportunity to develop new therapies, as they have been evolutionarily selected to play targeted roles in organisms. Schweinfurthin A is a prenylated stilbene natural product that has previously shown specific inhibitory activity against brain and hematopoietic tumor lines. We show that patient-derived GBM and NF1 malignant peripheral nerve sheath tumor (MPNST) lines, as well as tumor lines derived from the *Nf1−/+;Trp53−/+* (*NPcis*) mouse model of astrocytoma and MPNST are highly sensitive to inhibition by schweinfurthin A and its synthetic analogs. In contrast, primary mouse astrocytes are resistant to the growth inhibitory effects of schweinfurthin A, suggesting that schweinfurthin A may act specifically on tumor cells. Stable transfection of the GTPase-activating protein related domain of *Nf1* into *Nf1−/−;Trp53−/−* astrocytoma cells confers resistance to schweinfurthin A. In addition, the profound effect of schweinfurthin A on dynamic reorganization of the actin cytoskeleton led us to discover that schweinfurthin A inhibits growth factor–stimulated Rho signaling. In summary, we have identified a class of small molecules that specifically inhibit growth of cells from both central and peripheral nervous system tumors and seem to act on NF1-deficient cells through cytoskeletal reorganization correlating to changes in Rho signaling. *Mol Cancer Ther; 9(5); 1234–43. ©2010 AACR.*

Introduction

Historically, natural products have provided a wealth of pharmacologically active compounds for the treatment of disease (1). Natural products with small molecular weight seem to function in the producing organism largely as mediators of ecological interactions by targeting conserved cellular pathways and processes in pathogens, niche competitors, or symbiotic organisms (2). By screening natural products against human disease–relevant cellular processes such as proliferation and invasion, one can readily identify bioactive compounds; however, the challenge remains to uncover the molecular mechanisms underlying their modes of action. Here we apply these concepts to the development of therapies for nervous system cancers that are currently incurable.

Schweinfurthin A is a prenylated stilbene natural product that we isolated from a plant native to Cameroon in Africa on the basis of activity of a crude extract in the NCI 60 cell-line assay (Fig. 1A; ref. 3). Schweinfurthin A was identified as part of an effort to collect and characterize natural products from fungi, cyanobacteria, marine invertebrates, and >65,000 terrestrial plants at the National Cancer Institute (NCI) Natural Products Repository. We previously showed that schweinfurthin A produced a unique cell growth inhibition profile with differential activity against the central nervous system (CNS) and leukemia subpanels (3). Schweinfurthin A is highly potent and selective, showing low nanomolar inhibition of the growth of sensitive lines such as the glioblastoma multiforme (GBM)-derived SF-295 line, and 1,000-fold selectivity compared with resistant lines such as the non–small cell lung cancer–derived A549.

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

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doi: 10.1158/1535-7163.MCT-09-0834

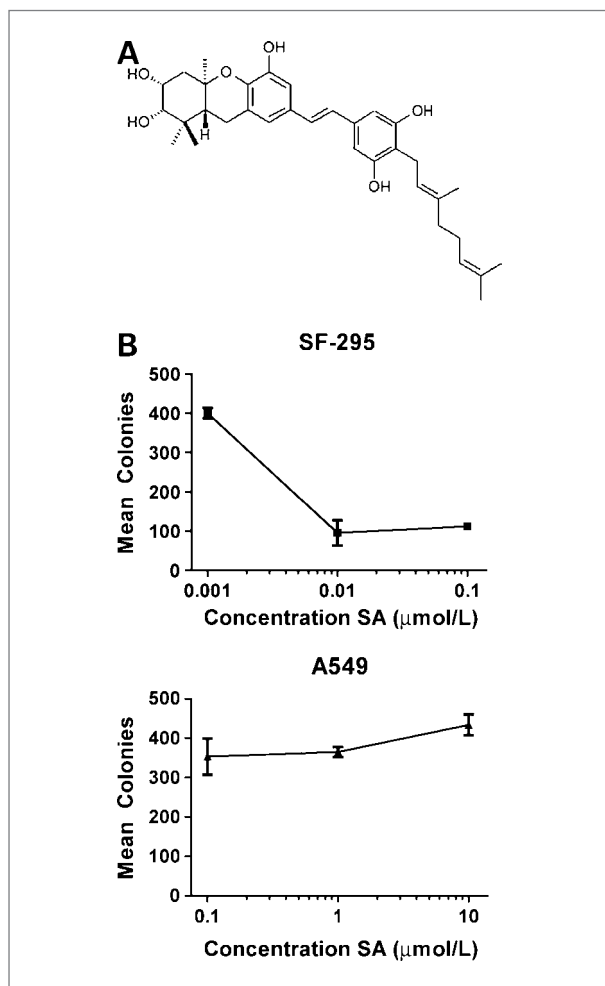*American Association for* **Cancer Research**

Figure 1. Schweinfurthin A (SA) selectively inhibits clonogenicity of glioma cells. A, structure of schweinfurthin A, a geranyl stilbene isolated from the Cameronian plant *Macaranga schweinfurthii*. B, clonal growth of SF-295 human glioma cells is inhibited by schweinfurthin A, whereas growth of A549 human lung carcinoma cells is not inhibited after single administration of varying doses of schweinfurthin A, as evaluated by growth of crystal violet stained colonies. Points, mean number of clones from three dishes; error bars, sample SDs.

GBM is the most common and deadly brain tumor in adults, with an average incidence rate of 3 in 100,000 (4), and a 5-year survival rate of <5%. Lower grades of astrocytoma can progress to GBM, and are similarly incurable. GBMs are highly infiltrative, making surgical resection difficult or impossible, and thus far GBMs are highly resistant to chemotherapy. Current therapeutic advances in the treatment of GBM have focused on targeting molecular pathways upregulated in GBM, with limited success. Specifically, analysis of completed clinical trials using epidermal growth factor receptor (EGFR) inhibitors has shown that these molecularly targeted therapies are highly effective, but only in small molecularly distinct subsets of GBMs (5, 6).

Recent large-scale genomic efforts have been undertaken to comprehensively identify mutations, amplifications, deletions, and gene expression changes in large panels of GBMs. Two independent studies by The Cancer Genome Atlas project and the Kinzler group at Johns Hopkins both identified *NF1* as one of the genes most frequently mutated in sporadic GBM (7, 8). In addition, previous studies in *Nf1* and *Trp53* mutant mice showed that loss of *Nf1* in combination with loss of the gene encoding the p53 protein predisposes mice to astrocytoma and GBM (9, 10), indicating that *NF1* likely can play a causal role in GBM tumorigenesis.

Neurofibromatosis type 1 (NF1) is an autosomal dominant genetic disorder affecting 1 in 3,500 people. NF1 patients carry a mutation in the *NF1* gene (*Nf1* in mice) and the disease is 100% penetrant, but shows variable expressivity, with patients developing a wide variety of developmental, benign, and malignant pathologies (11). The most common tumor types include benign optic pathway gliomas and neurofibromas (12). NF1 patients are at an increased risk of malignant peripheral nerve sheath tumors (MPNST) and also develop deadly malignant astrocytomas/GBMs (13, 14), in keeping with the role of NF1 mutation in sporadic glioblastoma. In addition, NF1 patients develop tumors not associated with the nervous system, such as myeloid leukemia and pheochromocytoma.

NF1 patients have a 5% to 13% lifetime risk of developing MPNSTs, an aggressive soft-tissue sarcoma that is refractory to chemotherapy and the leading cause of mortality in adult NF1 patients (15). MPNSTs are believed to arise from a malignant transformation of plexiform neurofibromas. Neurofibromas have been shown to develop from *NF1*−/− Schwann cells in peripheral nerve tracts in cooperation with other *NF1*−/+ cells in the surrounding stroma (16–18). Many NF1 tumors have now been successfully modeled in genetically engineered mice (9, 10, 18–24). Mice carrying heterozygous mutations in *Nf1* and *Trp53* linked on chromosome 11 (*NPcis* mice) develop MPNSTs and astrocytoma/GBM with high frequency depending on the strain background, as well as pheochromocytomas and hematopoietic tumors (9, 20, 23).

NF1 is a 350 kb gene that encodes a 250 kD protein, neurofibromin (25, 26). To date, its best-characterized function is localized in a 300-amino-acid central domain with homology to RasGAP proteins, known as the RasGAP-related domain (NF1-GRD; ref. 27). The domain negatively regulates RAS by converting RAS from its active GTP bound state to its inactive GDP bound state, which no longer propagates signals from upstream factors to downstream effector molecules. Thus, neurofibromin functionally downregulates RAS activity, and loss or mutation of wild-type (WT) *NF1* leads to constitutive RAS signaling in tumors (28).

The prevailing hypothesis is that loss of NF1 function in cells leads to constitutive RAS signaling in cells of the glial lineage – a critical step in the evolution of both benign and malignant tumor cells (29–32). However, efforts to treat both NF1 and GBM with Ras pathway

inhibitors, such as farnesyl transferase inhibitors, have seen little success (33–35). It is therefore important to find additional therapeutic candidates that inhibit NF1-dependent pathways altered in tumorigenesis.

In addition to its role in regulating RAS effector pathways such as mitogen-activated protein kinase and AKT, neurofibromin also regulates Rho signaling with downstream consequences on the organization of the actin cytoskeleton. When *NF1* expression was knocked down in HeLa and HT1080 cells by small interfering RNA, dramatic changes in actin regulation were observed. It was determined that these changes were regulated via the Rho/Rock pathway (36). Other studies in both Schwann cells and astrocytes have shown that *NF1* heterozygosity and loss of heterozygosity result in abnormal cytoskeletal organization, as well as defects in migration (37–39). Given the invasive nature of GBMs and MPNSTs, it is likely that the effect of *NF1* mutation on cytoskeleton dynamics could have important consequences for tumorigenesis; however, inhibition of Rho signaling pathways has not yet been examined as a therapy for NF1 or GBM.

The purpose of this study was to illuminate the mechanism of action of schweinfurthin A, and therefore we chose to pursue studies in the experimentally tractable system afforded by the *NPcis* mouse model of nervous system tumors (9). Characterization of the mechanisms of action of this compound may lead to new insights into nervous system tumors, therefore schweinfurthin A may serve as a lead molecule in developing novel therapies for a class of tumors for which there are currently no effective therapies.

Materials and Methods

Schweinfurthin A isolation and analog synthesis. The isolation of schweinfurthin A was previously described (3). Synthesis of schweinfurthin analogs (National Service Center numbers 735927, 749942, 749946, and 746620) has been previously reported (40–43).

Cell lines and culture. Human tumor lines SF-295 and A549 were obtained from the Developmental Therapeutics Program (NCI-Frederick) from stocks used in the NCI 60-cell assay (44). Mouse tumor lines were generated from *NPcis* mouse tumors. The isolation of KR158 astrocytoma was described previously (9). K16561 and K14553 tumor lines were isolated from sarcomas in *NPcis* mice, and characterized as MPNSTs by immunocytochemistry for Schwann cell markers (S100 and p75), which were found to be positive, and muscle markers (MyoD1 and Myf4; ref. 45), which were found to be negative, as well as for loss of the WT copy of *Nf1* and *Trp53* by PCR as described previously (ref. 9; Supplementary Data and Methods). Primary astrocytes were prepared as described previously (46) from 1-day-old neonatal brains collected from WT, *Nf1−/+*, and *Nf1−/+; Trp53−/+cis* mice. Human MPNST cell lines (STS26T and T265) were a kind gift from Brigitte Widemann,

Pediatric Oncology Branch, NCI. Human cells were grown in RPMI 1640 and mouse cells were grown in DMEM containing 10% fetal bovine serum supplemented with 2 mmol/L glutamine and incubated in a 37°C humidified atmosphere (5% CO_2).

NF1-GRD retroviral constructs were a kind gift from D.W. Clapp, Indiana University. Cells transduced with the NF1-GRD construct or Murine Stem Cell Virus (PMSCV) empty vector control as previously described (47) were maintained in 1 μg/mL puromycin (Sigma).

All mouse procedures were done according to the guidelines of the NCI-Frederick Animal Care and Use Committee.

Clonogenic assays. Monolayers of cells were treated for 18 hours with schweinfurthin A, and then harvested, counted, and seeded to 35-mm dishes at a density of 1,000 cells/dish. After 1 week, macroscopic colonies were stained with crystal violet (Sigma) and counted.

XTT cell proliferation assay. Cells were seeded into 96-well plates at a density of 2,000 cells/well and allowed to reattach overnight. Cells were treated with schweinfurthin A, synthetic analogs, camptothecin (NCI Chemotherapeutics Repository), or DMSO control at the indicated concentrations continuously for 48 hours followed by assay in the 96-well plate for relative viable cell number using the dye 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (XTT) and a Wallac-Victor 2 plate reader (Perkin-Elmer). Absorbance was determined at 450 nm with 650 nm as a reference reading. Primary astrocytes were additionally assayed at a 96-hour time point.

Cell morphology assays and confocal microscopy. To assay cytoskeleton morphology, KR158 cells, mouse primary astrocytes, KR158 clones expressing the NF1-GRD constructs, and K14553 cells were seeded to cover slips and allowed to reattach overnight. Cells were treated for 18 hours with either the indicated concentrations of schweinfurthin A, or the vehicle control DMSO. The cover slips were fixed in 3.7% paraformaldehyde, permeablized in 0.1% Triton-X100, and stained with Alexa Fluor 488-phalloidin (Invitrogen) to detect actin, and cells were mounted in Prolong Antifade containing 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) to stain nuclei. The cells were imaged on a LSM510 confocal microscope (Carl Zeiss Inc.).

For phospho-myosin light chain 2 (MLC) detection, cells were seeded to cover slips as described above. Cells were serum starved in 0.5% fetal bovine serum and schweinfurthin A or DMSO vehicle control for 18 hours, and then pulsed with 10 ng/mL epidermal growth factor (EGF; Invitrogen) for 5 minutes. Cover slips were fixed and permeablized as described above and immunostained with a Ser19 phospho-specific MLC primary antibody (Cell Signaling Technology) and Alexa Fluor 555 goat anti-rabbit secondary (Invitrogen), and counterstained with Alexa Fluor 488-phalloidin (Invitrogen) to detect actin. Cells were mounted in Prolong Gold Antifade reagent containing DAPI (Invitrogen) to stain nuclei.

KR158 GFP-actin–transfected cells (Supplementary Data and Methods) were seeded to 8-well chamber slides, allowed to reattach, and then treated with indicated concentrations of schweinfurthin A. Actin structures were monitored continuously over a 16-hour period using confocal microscopy equipped with a growth chamber (37°C, humidified atmosphere, and 5% CO_2). To compare schweinfurthin A with Rho/Rock inhibitors, KR158 GFP-actin cells were treated with schweinfurthin A or with the Rock inhibitor Y-27632 (Sigma) and the Rho inhibitor C3 transferase (Cytoskeleton, Inc.) for 16 hours and then examined by confocal microscopy.

Rho GTPase pull-down assay. The Rho pull-down assay was obtained from Upstate Biologicals. KR158 cells were harvested at approximately 80% confluency and seeded at 5×10^5 cells/well in a 6-well plate. After overnight recovery, cells were serum starved in 0.5% fetal bovine serum for 18 hours. Cells were then treated with 10 ng/mL EGF either in the presence or the absence of the indicated concentrations of schweinfurthin A for up to 18 hours. At the indicated time points, the cells were lysed according to the manufacturer's protocols. Proteins pulled down by the Rho-GTP binding beads were eluted by SDS, fractionated on a 10% SDS-PAGE gel, and transferred to a nitrocellulose membrane. Pre-pull-down lysates were run in parallel to determine total levels of Rho in each sample. The membrane was probed by an anti-Rho antibody.

Results

Schweinfurthin A shows differential activity towards SF-295 human glioma and KR158 astrocytoma cells but not primary astrocytes derived from WT, **Nf1 –/+,** *and* **NPcis** *mice.* Previous data in the NCI60 cancer cell assay showed that schweinfurthin A showed cytotoxicity in the brain tumor subpanel (3), but the mechanism of cell inhibition was not understood. To confirm the selectivity of schweinfurthin A seen in the NCI60 assay, we used a cell clonogenicity study in which confluent monolayers of SF-295 GBM cells were treated for 18 hours with schweinfurthin A. A549 lung cancer cells were used as a control for comparison, because they show relative resistance to schweinfurthin A compared with brain tumor lines in the NCI-60 cancer cell assay. Schweinfurthin A–treated cells were then rescued from the drug, harvested, and seeded at low densities. As seen in Fig. 1B, colony growth was dramatically restricted in the sensitive cells, whereas the A549 cells formed colonies up to the highest concentration of schweinfurthin A tested. We further characterized the activity of schweinfurthin A in an aggressive grade III astrocytoma cell line (KR158) derived from the *NPcis* mouse. The human GBM line SF-295 and the mouse astrocytoma line KR158 were compared with the schweinfurthin A–resistant A549 lung cancer cell line in two-day proliferation assays. Schweinfurthin A inhibited both the KR158 and SF-295 cell lines in a dose-dependent manner, with no apparent effect on the A549

cell line (Fig. 2A). The XTT assay used in these experiments measures the metabolic activity of mitochondrial-associated enzymes that are inactivated after cell death, and is a validated end point for measuring inhibition of proliferation. However, because it does not measure cytotoxicity directly, we employed a cell protease assay that measures cell viability and cytotoxicity by detecting two distinct protease activities simultaneously. In this assay, we saw a dose-dependent increase in cell killing in KR158 cells after 48 hours of continuous treatment with schweinfurthin A

Figure 2. Schweinfurthin A selectively inhibits proliferation of SF-295 and KR158 brain tumor cells, but has little effect on A549 lung tumor cells or primary astrocytes. A, viability of KR158, SF-295, and A549 cells in response to schweinfurthin A was evaluated by XTT assay and plotted as a percentage of viable cells compared with cells treated with vehicle only. B, viabilty of A549 cells in response to schweinfurthin A or camptothecin (CPT) as evaluated by XTT assay. C, viability of KR158 and four primary astrocyte cultures established from WT, *Nf1*–/+, or *NPcis* mice in response to schweinfurthin A was measured by XTT assay. Points, mean percentage growth of three wells.

(Supplementary Fig. S1), indicating that schweinfurthin A acts by a cytotoxic rather than cytostatic mechanism.

To show that A549 cells are not generally resistant to small molecule growth inhibition, we tested camptothecin, an inhibitor of topoisomerase 1 that induces apoptosis in proliferating cells, as a positive control. A549 was sensitive to camptothecin, indicating that general upregulated drug metabolism, or increased nonspecific drug efflux, were not responsible for enhancing the schweinfurthin A–insensitive tumor cell survival in the presence of the drug (Fig. 2B).

Furthermore, untransformed astrocytes have the capacity to proliferate, so we tested schweinfurthin A against primary astrocytes from WT, $Nf1-/+$, and $NPcis$ neonates, and found that these cells were resistant to the effects of schweinfurthin A on proliferation (Fig. 2C), even after 96 hours of treatment (Supplementary Fig. S2A). Moreover, at concentrations of schweinfurthin A several log-fold higher than the IC_{50} value for KR158, the inhibitory concentration (IC) at which the growth activity is inhibited by 50%, primary astrocytes were not inhibited more than roughly 40% in their growth, depending on the individual astrocyte line tested (Fig. 2C). Because primary astrocytes grow more slowly than tumor cells, it is possible that the effects of schweinfurthin A are limited to faster proliferating cells. To address this issue we reexamined the response of the NCI60 cell lines to schweinfurthin A (3). The doubling times of the NCI60 cells are well characterized and available online (48). We found that the sensitivity to schweinfurthin A does not correlate to the doubling time of the tumor line (Supplementary Fig. S2B). Indeed, one of the slowest growing CNS lines, SNB-75, with a doubling time of 62.8 hours, is one of the most sensitive lines to schweinfurthin A (log-$GI_{50} = -7.82$), whereas one of the faster growing CNS lines, SF-268, with a doubling time of 33.1 hours, is the most resistant CNS line to schweinfurthin A (log$GI_{50} = -5.62$). These data suggest that schweinfurthin A activity targets the transformed phenotype of astrocytoma cells, and not a general feature of cell division that can be found in proliferating brain cells.

Schweinfurthin A causes morphologic changes reflected in alterations of dynamic actin architecture. Treatment of sensitive cells with schweinfurthin A leads to changes in cell morphology characterized by elongated processes and contraction of the cytoplasm, resulting in an overall spindle shape (Fig. 3A). These changes occur within the first day of treatment with the most dramatic changes occurring after 12 to 18 hours. Hypothesizing that changes in the actin cytoskeleton were responsible for the morphologic changes, we separately treated KR158 cells stably transfected with GFP-actin with the Rock inhibitor Y-27632 or the Rho inhibitor C3 transferase (Supplementary Fig. S3A), both of which are known to disrupt cytoskeletal actin structures, or with schweinfurthin A (Fig. 3B). When compared with untreated controls, there was a dramatic loss of F-actin staining in all treated cells, especially stress fibers. In schweinfurthin
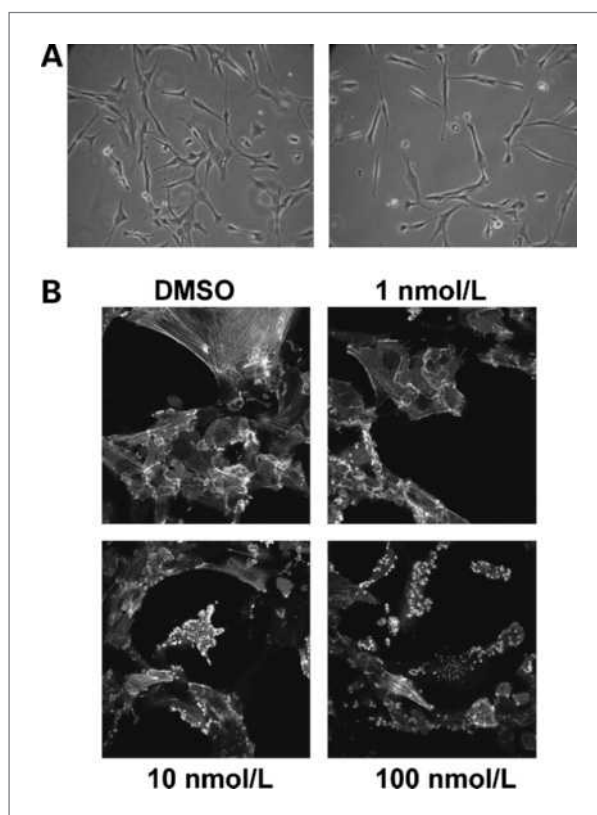
Figure 3. Schweinfurthin A causes changes in cell morphology and reorganization of the actin cytoskeleton. A, phase contrast images of KR158 cells treated overnight with DMSO (left) or schweinfurthin A (right). B, KR158 EGFP-actin cells show a dose-dependent response to schweinfurthin A with loss of stress fibers and an increasingly punctuate appearance of the actin cytoskeleton.

A–treated cells, in particular, we saw dose- (Fig. 3B) and time-dependent changes (Supplementary Fig. S3B) in the architecture of actin including loss of stress fibers, and increased cortical actin at the margins of the cell (Fig. 3B). Distinctively, doxorubicin-treated KR158 cells retained stress fibers when given cytotoxic doses of the DNA intercalating agent (data not shown), suggesting that actin structure reorganization is not a general consequence of cell stress. Finally, schweinfurthin A–resistant primary astrocytes and A549 cells showed no visible changes in actin organization after treatment with schweinfurthin A as visualized by phalloidin staining (Supplementary Figs. S2C and S3C).

Schweinfurthin A inhibits growth factor–induced Rho signaling. The effect of schweinfurthin A on actin cytoskeleton dynamics, the sensitivity of $Nf1-/-;Trp53-/-$ astrocytoma cells to schweinfurthin A, and previously published data which indicate that loss of $NF1$ enhances stress fiber formation and Rho signaling (36) raised the possibility that the Rho signaling pathway may be an important determinant of schweinfurthin A sensitivity. To test whether schweinfurthin A might be targeting the Rho signaling network, KR158 cells were serum starved

and then pulsed with 10 ng/mL of EGF in the presence or absence of schweinfurthin A. Using glutathione S-transferase–fused Rhotekin-Rho binding domain to pull down activated Rho, we observed a marked inhibition of Rho activity at 12 and 18 hours in EGF-pulsed cells treated with schweinfurthin A (Fig. 4A).

As further evidence that the Rho signaling pathway is disrupted, KR158 cells treated with schweinfurthin A and EGF were immunostained for phosphorylation of Ser 19 on myosin light chain 2 (MLC2), which is downstream of Rho/Rock signaling. Ser 19 phosphorylation has been shown to activate the regulatory function of MLC, and is associated with stress fiber polymerization and contractility in the actin cytoskeleton (49). Confocal micrographs of these cells show a dose-dependent
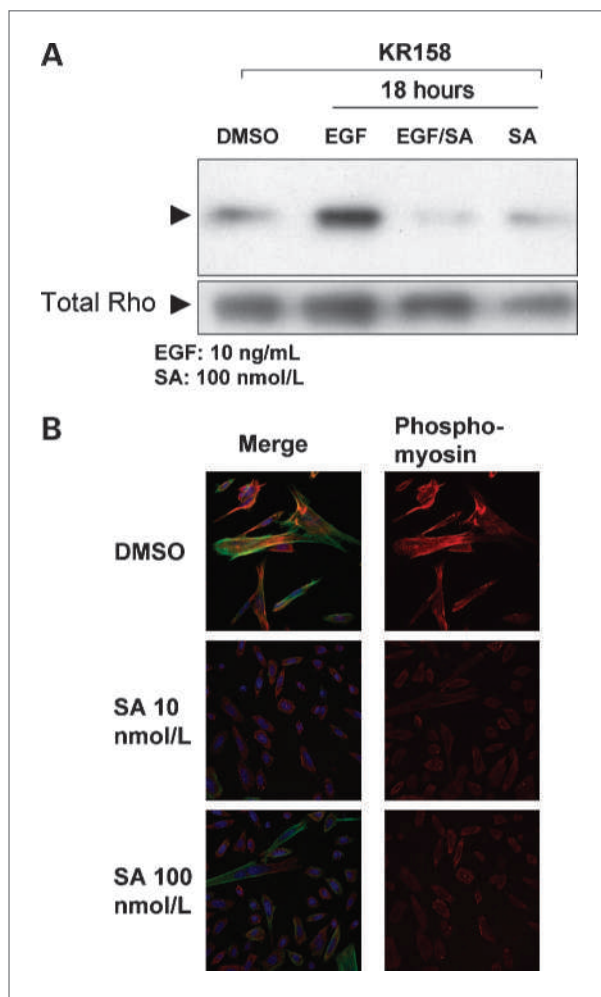
Figure 4. Growth inhibitory cytoskeletal effects of schweinfurthin A are associated with Rho signaling. A, schweinfurthin A inhibits EGF-induced Rho activation in serum-starved KR158 cells assayed by Rho-GTP pull-down. B, schweinfurthin A inhibits phosphorylation of MLC in response to EGF in serum-starved KR158 cells as indicated by immunostaining of phospho-MLC (red). Cells were counterstained with Alexa Fluor 488-phallodin (green) to visualize the actin cytoskeleton and DAPI (blue) to visualize nuclei.
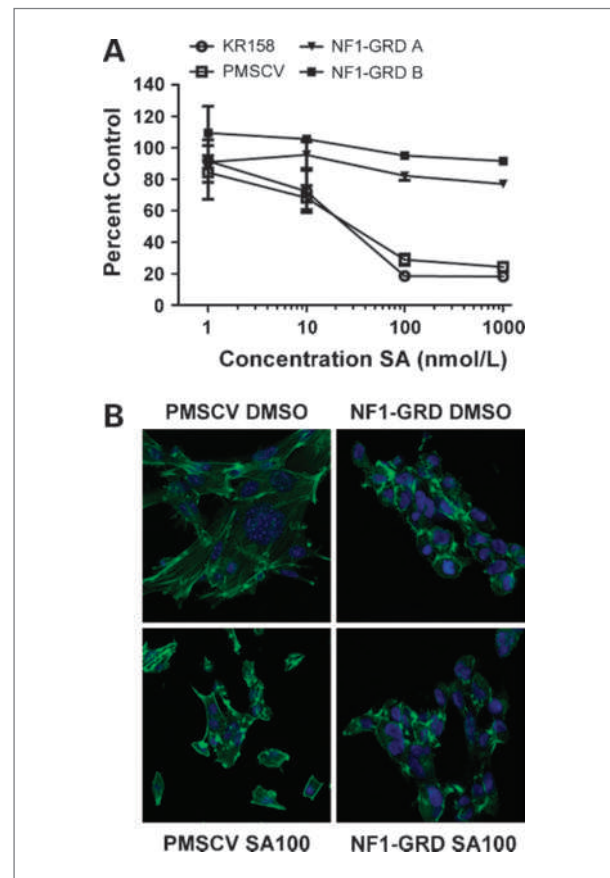
Figure 5. Schweinfurthin A activity is abrogated in NF1-deficient cells by expression of the NF1-GRD domain. A, KR158 cells stably transduced with the NF1-GRD domain are resistant to schweinfurthin A, whereas KR158 cells transduced with the empty vector (PMSCV) are not, as measured by XTT assay. Points, mean percentage growth of three wells compared with DMSO-treated controls. B, NF1-GRD stably transduced KR158 cells do not show loss of stress actin fibers after 18 hours' treatment with schweinfurthin A, in contrast to KR158 cells transduced with PMSCV empty vector. Cells were probed with Alexa Fluor 488-phallodin to visualize the actin cytoskeleton and counterstained with DAPI to visualize the nucleus.

decrease of MLC phosphorylation in schweinfurthin A–treated cells (Fig. 4B). Taken together, these data suggest that the observed cytoskeletal changes in schweinfurthin A–sensitive cells are due to the inhibition of Rho activity, with downstream consequences on Rho effector molecules such as MLC. Because the time course of our experiments show that the effects on the actin cytoskeleton, MLC phosphorylation, and Rho activity require hours of treatment with schweinfurthin A, it is not likely that schweinfurthin A is acting directly within the Rho pathway, but rather it is acting upstream, indirectly leading to downregulation of the pathway. These data also suggest loss of neurofibromin contributes to the upregulation of a signaling network that links the regulation of the actin cytoskeleton and cell survival, and that this upregulated network in NF1-deficient cells is targeted by schweinfurthin A.
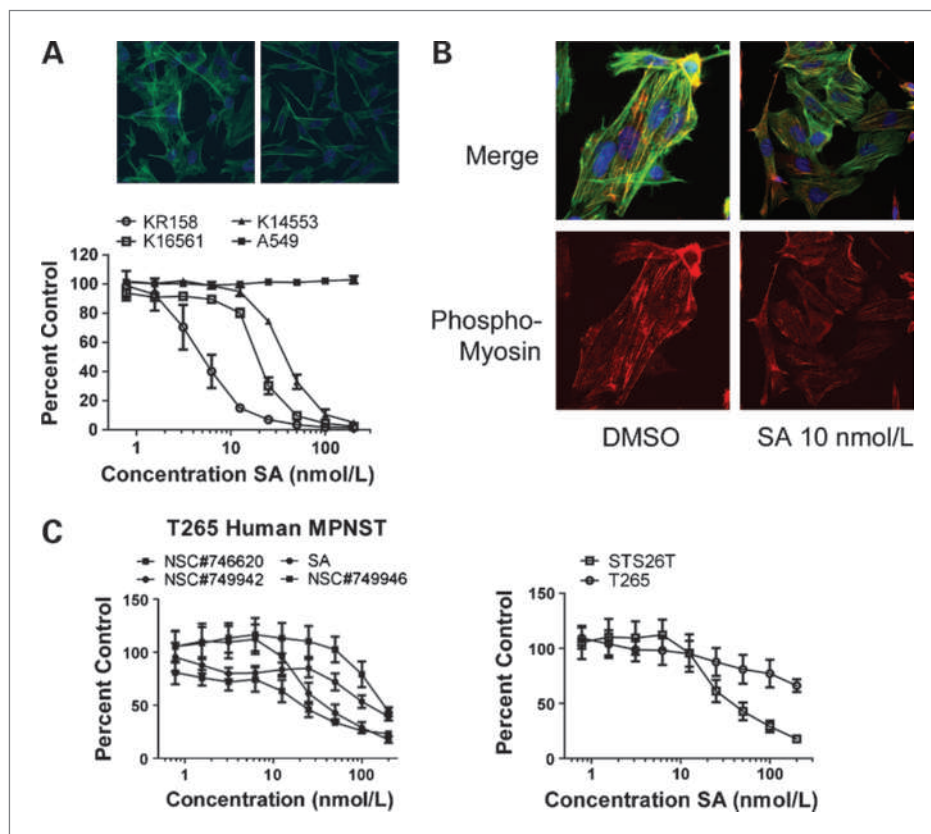
Figure 6. Schweinfurthin A inhibits the growth and MLC phophorylation of human and murine NF1-deficient MPNST cell lines. A, *Nf1*–/–;*Trp53*–/– tumor lines from *NPcis* mice, KR158 (astrocytoma), and K16561 and K14553 (MPNST) are sensitive to inhibition by schweinfurthin A, compared with A549 cells, as evaluated by XTT assay plotted as a percentage of cells treated with vehicle only. Points, mean percentage growth of three wells compared with DMSO-treated controls. Top images, K14553 cells treated for 18 hours with DMSO (left) or schweinfurthin A 100 nmol/L (right) and then stained with Alexa Fluor 488-phallodin (green) to visualize the actin cytoskeleton, and counterstained with DAPI (blue) to visualize the nucleus. B, K14553 cells show loss of phospho-MLC (red) in response to 18-hour treatment with schweinfurthin A, compared with DMSO-treated cells. Cells were counterstained with Alexa Fluor 488-phallodin (green) to visualize the actin cytoskeleton and DAPI (blue) to visualize nuclei. C, the human MPNST cell line T265 from a NF1 patient is sensitive to synthetic analogues of schweinfurthin A as evaluated by XTT assay (left), and the sporadic human MPNST cell line STS26T is resistant compared with T265 (right) as measured by XTT assay.

KR158 cells transfected with the NF1-GRD domain are resistant to schweinfurthin A. Because neurofibromin is a very large protein, stably transfecting cells with the full-length *Nf1* gene was not feasible. Therefore, to test whether loss of *Nf1* was required for cellular sensitivity to schweinfurthin A, we reintroduced the ~300-amino-acid-long NF1-GRD fragment of *Nf1* into KR158 cells. Although neurofibromin may have additional functions not mediated by this domain, this is a key fragment of the larger protein. Cells transduced with this domain, or the empty vector, were tested against schweinfurthin A in a two-day cell proliferation assay. As expected, schweinfurthin A was highly active against both the untransduced and the empty vector–transduced KR158 cells; however, NF1-GRD–expressing cells were resistant to schweinfurthin A inhibition (Fig. 5A). Sequencing of the coding sequence corresponding to the first 1984 amino acids of neurofibromin in SF295 cells revealed one silent mutation in the *N*-term end of the protein (Leu234CTG >

CTA; data not shown), whereas Western blots of SF-295 and A549 for neurofibromin showed a 250 KD reactive band consistent with expression of neurofibromin in these cell lines (Supplementary Fig. S4). We cannot conclude that the neurofibromin expressed in SF295 (or A549) is functional; however, we did not find clear evidence for a mutation of *NF1* in SF295 that would alter protein function. Therefore, our evidence suggests that schweinfurthin A targets a pathway critical for survival of NF1 null cells, but that this pathway could also be important for the survival of other nervous system tumors that may still express neurofibromin, but use similar pathways for tumorigenesis.

Examination of the transduced cells by confocal microscopy showed that the NF1-GRD–expressing clones had a different pattern of F-actin organization from the empty vector–transduced cells, and the actin structures within the NF1-GRD cells did not change in response to schweinfurthin A. The empty vector–transduced cells showed the same reorganization of F-actin structures in response to

schweinfurthin A that was observed in untransduced KR158 cells and other sensitive cells lines tested (Fig. 5B).

NF1–deficient tumor cells are differentially sensitive to the antiproliferative effects of schweinfurthin A and analogs. Given the NF1-GRD dependence of schweinfurthin A sensitivity in astrocytoma cells, we tested two MPNST cell lines derived from the *NPcis* mouse and saw a dose-dependent inhibition of proliferation of these cells (Fig. 6A). Consistent with the effects of schweinfurthin A on *Nf1*-deficent astrocytoma cells, we also saw an effect on the actin cytoskeleton in the MPNST cell lines, including loss of stress fibers (Fig. 6A, top) and reduced MLC phosphorylation (Fig. 6B). When schweinfurthin A was tested against the human MPNST cell line T265, derived from a NF1 patient, we also saw potent inhibition of cell proliferation (Fig. 6C, left). In contrast, when we measured the effect of schweinfurthin A on a sporadic MPNST cell line, STS-26T (50), which has been shown to express WT NF1 (51), we observed much weaker inhibition of proliferation (Fig. 6C, right). Four synthetic analogs of schweinfurthin A (Supplementary Fig. S5) also selectively inhibited the growth of NF1-deficient cells (Fig. 6C and Supplementary Fig. S6). These findings further support the model that schweinfurthin A targets a signaling network that is essential for tumor cell survival in NF1-deficient cells.

Discussion

We found that cytoskeletal rearrangements are a hallmark of sensitivity to schweinfurthin A, and are accompanied by inhibition of Rho signaling. Furthermore, expression of NF1-GRD imparts resistance to schweinfurthin A. These findings suggest that *Nf1*-deficient tumor cells are differentially sensitive to schweinfurthin A because of alterations in Rho signaling due to loss of regulation by neurofibromin. This signaling network regulates the actin cytoskeleton, but also contributes significantly to cell proliferation and tumorigenicity. This may explain in part the specificity of schweinfurthin A for brain tumors and leukemia in the NCI60 cell assay, because both astrocytoma and myeloid leukemia have been associated with NF1 alterations.

Neurofibromin is primarily described as an inhibitor of Ras signaling, and thus it is perhaps surprising that *Nf1*−/− tumor cells show differential susceptibility to schweinfurthin A, compared with A549 cells that carry an activating mutation in *K-ras*. The specificity of schweinfurthin A for a subset of NCI60 tumor cell lines further supports the idea that schweinfurthin A is not acting at the level of Ras inhibition. Our observations that schweinfurthin A inhibits Rho signaling and causes changes in actin dynamics in a tumor type–specific manner indicates that the signaling pathways downstream of Ras may be different in nervous system tumors than in other tumor types. This may help to explain the highly invasive nature of astrocytomas/GBMs and

MPNSTs. Recent work by Ozawa et al. (36) showed that in addition to its role in Ras regulation, NF1 also regulates Rho signaling. Understanding how signaling pathways in nervous system tumors differ from other tumor types may provide keys to designing therapy to target brain tumors, as well as other NF1-related conditions.

NF1 patients carry a mutation of the *NF1* gene in the normal cells throughout their body. Many studies on *NF1*−/+ mutant cells have shown altered signaling and a haploinsufficient phenotype in the presence of one WT copy of *NF1* (52–54). This raises the possibility that NF1 patients may respond to therapies differently from the general population. Furthermore, due to the nature of the disease, NF1 patients develop tumors at a very young age, and thus might require therapy for the condition over extended periods of time, perhaps their entire lifetime. Therapies developed for the treatment of NF1 will need to be highly specific to provide a therapeutic index between the pathologic *NF1*−/− cells in tumors and the *NF1*−/+ cells of the patient. It is therefore encouraging that schweinfurthin A sensitivity shows dependence on *Nf1* loss. *Nf1* heterozygous primary astrocytes are resistant to schweinfurthin A, whereas multiple different *Nf1* nullizygous tumors of the central and peripheral nervous system are sensitive to schweinfurthin A.

Future studies will determine whether schweinfurthin A or any of its synthetic analogs maintain potency and specificity *in vivo*. For schweinfurthin A to be an effective treatment for GBM it should be able to cross the blood brain barrier (BBB); the ability of schweinfurthin A to cross the BBB is unknown at this time. In contrast, peripheral tumors associated with NF1, such as MPNSTs, develop outside the BBB, and may be accessible to schweinfurthin A or its analogs regardless of whether these compounds cross the BBB. Because analogs of this natural product can now be synthesized, it will be important to determine which analogs are most metabolically stable, pharmacologically active, and able to cross the BBB. Insights into the mechanism of action of schweinfurthin A and its analogs will help determine whether activity is maintained *in vivo*.

In conclusion, we have identified a natural product, schweinfurthin A, that phenocopies neurofibromin effects on cell proliferation and on actin cytoskeletal regulation. The study of the mechanism of tumor inhibition by schweinfurthin A may lead to a better understanding of how brain tumors differ from other more treatable tumor types, and may lead to a more detailed understanding of how neurofibromin functions as a tumor suppressor.

Disclosure of Potential Conflicts of Interest

Acknowledgments

Grant Support

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