Narciclasine, a plant growth modulator, activates Rho and stress fibers in glioblastoma cells

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Abstract

Cell motility and resistance to apoptosis characterize glioblastoma multiforme growth and malignancy. Narciclasine, a plant growth modulator, could represent a powerful new weapon targeting the Achilles’ heel of glioblastoma multiforme and may offer the potential to better combat these devastating malignancies. The in vitro effects of narciclasine on cell proliferation, morphology, actin cytoskeleton organization, and the Rho/Rho kinase/LIM kinase/cofilin pathway and its antitumor activity in vivo have been determined in models of human glioblastoma multiforme. Narciclasine impairs glioblastoma multiforme growth by markedly decreasing mitotic rates without inducing apoptosis. The compound also modulates the Rho/Rho kinase/LIM kinase/cofilin signaling pathway, greatly increasing GTPase RhoA activity as well as inducing actin stress fiber formation in a RhoA-dependent manner. Lastly, the treatment of human glioblastoma multiforme orthotopic xenograft-bearing mice with nontoxic doses of narciclasine significantly increased their survival.

Narciclasine antitumor effects were of the same magnitude as those of temozolomide, the drug associated with the highest therapeutic benefits in treating glioblastoma multiforme patients. Our results show for the first time that narciclasine, a plant growth modulator, activates Rho and stress fibers in glioblastoma multiforme cells and significantly increases the survival of human glioblastoma multiforme preclinical models. This statement is made despite the recognition that to date, irrespective of treatment, no single glioblastoma multiforme patient has been cured. [Mol Cancer Ther 2009;8(7):1739–50]

Introduction

Gliomas are the most common primary brain tumors, among which glioblastoma multiforme is the most malignant form characterized by its heterogeneity and aggressive invasive behavior into normal brain tissue (1, 2). Glioblastoma multiforme patients have a median survival expectancy of only 14 months on the current standard treatment of surgical resection to the extent feasible, followed by adjuvant radiotherapy plus temozolomide, given concomitantly with and after radiotherapy (2, 3). Glioblastoma multiforme cells are associated with such dismal prognoses because glioma cells can actively migrate through the narrow extracellular spaces in the brain, often traveling relatively long distances, making them elusive targets for effective surgical management (1, 2). In addition, invasive malignant glioma cells show decreased proliferation rates and a relative resistance to apoptosis compared with the highly cellular center of the tumor, and this may contribute to their resistance to conventional proapoptotic chemotherapy and radiotherapy (1).

The cofilin pathway has emerged recently as a central player in the generation of free barbed ends and in actin filament turnover in various motile cells, including mammary carcinoma cells, fibroblasts, Dictyostelium discoideum, and glioblastoma multiforme cells during the formation of these path-finding structures (ref. 4 for review). The actin cytoskeleton controls multiple cellular functions, including cell morphology, movement, and growth of normal as well as cancer cells including those of gliomas. Actin is one of the most abundant and highly conserved proteins among eukaryotes. The assembly/disassembly and organization of the actin cytoskeleton are regulated by actin-binding proteins including actin-depolymerizing factor, also known as cofilin. Cofilin, the substrate of LIMK1, is directly responsible for severing actin filaments and regulating actin polymerization and depolymerization during cell migration (4). The LIM kinase (LIMK) family consists of two members: LIMK1 and LIMK2. With uniquely organized signaling domains, LIMKs are regulated by several upstream signaling pathways, principally acting downstream of Rho GTPases to influence the architecture of the actin cytoskeleton by
regulating the activity of the cofilin family proteins. The LIMKs have been proposed to play an important role in tumor-cell invasion and metastasis by fine-tuning the balance between phosphorylated and nonphosphorylated cofilin, which may be a significant determinant of tumor-cell metastatic potential (5).

Rho GTPases are key regulators of the actin cytoskeleton and focal adhesions. Rho A/B/C enhance actin stress fiber formation via multiple effectors (6). One such effector pathway involves the serine/threonine kinases, Rho kinase (ROCK) I/II, which increase actin polymerization by inhibiting the actin severing activity of cofilin via LIMK1/2 (7). Notably, alterations in the control of Rho GTPases have been linked to cancer (6). Recent studies indicate that the overall activity of the cofilin pathway, and not that of any single gene within the pathway, determines the invasive and metastatic phenotype of tumor cells (4).

Narciclasine is a plant growth inhibitor isolated from Narcissus bulbs (8). It has been shown that narciclasine exhibits a wide range of inhibitory effects on plant growth including seed germination, seedling growth, and plastid development of excised radish cotyledons (inhibiting the transition of glyoxysomes and peroxisomes), and chloroplast development (9). The mode of action of narciclasine is not known, although it has been shown to inhibit protein synthesis (10), isocitrate lyase, and hydroxypropyruvate reductase activities in glyoxysomes and peroxisomes (9), respectively, and to exhibit antimitotic (11) and antiviral (12) activities. McLachlan et al. (13) showed that pancratistatin, the chemical structure of which is very close to that of narciclasine, induces rapid apoptosis in SH-SY5Y neuroblastoma cells but at pharmacologic doses. We have recently shown that narciclasine at 1 μmol/L in vitro induces marked apoptosis-mediated cytotoxicity in certain human cancer cells but not in normal fibroblasts (14). However, the compound failed to induce apoptosis in glioblastoma multiforme cells even at pharmacologic doses (15). Given the postulate that the actin network of glioblastoma multiforme cells might be affected by narciclasine, the aims of the present study were to investigate the in vitro effects of the compound on actin cytoskeleton organization, actin-depolymerizing factor/cofilin expression and phosphorylation, and small GTPase activity. Additionally, we sought to establish the antitumor activity of narciclasine in orthotopic glioblastoma multiforme models in vivo.

Materials and Methods

Glioblastoma Multiforme Cell Lines and Primocultures

The Hs683 (ATCC code HTB-138) and U373 (ATCC code HTB-17) human glioblastoma multiforme cell lines were obtained from the American Type Culture Collection and maintained in our laboratory as detailed previously (16). Use was also made of four primocultures established in our facilities as detailed previously (17). These primocultures have been labeled GL5, GL16, GL17, and GL19 in the current study.

Narciclasine Antitumor Activity against Glioblastomas

Narciclasine was isolated as described previously (14, 15). In vitro Determination of Narciclasine IC50 Growth-Inhibitory Concentration

The narciclasine IC50 concentration, the narciclasine concentration that decreased by 50% the global growth rate of a given cell population, was assessed with the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloxazolium bromide (Sigma) assay as described previously (14, 15). The cells were incubated for 72 h in the presence and absence of the narciclasine (with concentrations ranging between 10−9 and 10−5 mol/L concentrate) for the determination of narciclasine IC50 values.

Annexin V Assay

Control and treated glioblastoma multiforme cells were harvested by trypsinization, resuspended in serum-containing medium to neutralize the trypsin, and washed twice with PBS. Cells were then resuspended in ice-cold 1× binding buffer (Sigma-Aldrich) at a density of 10⁶/mL and aliquots of 500 μL stained with 5 μL Annexin V-FITC and 10 μL propidium iodide (PI; Sigma-Aldrich) as described previously (14). The fluorescence was analyzed immediately on an Epics XL.MCL flow cytometer (Beckman Coulter) equipped with a 488 nm argon laser.

DNA Fragmentation Assay

Glioblastoma multiforme cells (1.5 × 10⁵) were treated with narciclasine (100 nmol/L) for different periods (24, 48, and 72 h). At the end of each incubation period, the intact chromatin was separated from the small apoptotic DNA fragments using the method and reagents of the Suicide-Track DNA Ladder Isolation kit (Calbiochem). The purified fragments were then resolved by standard agarose gel (1.5%) electrophoresis and stained with 0.5 μg/mL ethidium bromide for visualization under UV light as described previously (14).

Determination of In vitro Cell Cycle Kinetics

Glioblastoma multiforme cells were incubated for 24, 48, and 72 h in culture medium with or without narciclasine at 100 nmol/L concentration. The identification of the glioblastoma multiforme cells in the G1, S, and G2 phases of the cell cycle was done for each experimental condition by means of flow cytometry analysis of PI.

Computer-Assisted Phase-Contrast Videomicroscopy

For human glioblastoma multiforme cell lines U373, Hs683, and GL19, cell death, mitosis, overall cell population growth and morphology with and without narciclasine treatment at 100 nmol/L, and motility with and without narciclasine at 50 nmol/L were characterized in vitro by the use of computer-assisted phase-contrast videomicroscopy as described previously (18). Cells were followed for at least 72 h. To minimize the file size of the generated video clips, they have been compressed using DivX codec. The codec and a movie player are available at http://divx.com.

Global Growth Ratio

The global growth ratio in this study was defined by the ratio of cell numbers present after 72 h incubation over those determined at the beginning of the experiment.
at $T_0$. The results were obtained by manual cell counts done in triplicate by two independent investigators. The sole source of error in these count-based cell growth measurements concerned the few cells entering or leaving the observed microscopic field. Because no cell death was observed, these counts provided the number of cell divisions occurring after 72 h under the different conditions investigated.

**Specific Analysis of Cell Division Rate and Duration**

The objects of interest, the cells undergoing division, exhibit very bright patterns compared with nondividing cells. Based on this observation, we developed a custom division detector capable of identifying cells undergoing division in time-lapse sequences. This detector method is based on an automatic event detection completed by an interactive (manual) validation/correction procedure as described previously (18). Briefly, the candidate dividing cells on each frame was first identified by an adaptive threshold-based segmentation in which only sufficiently large and bright image areas are considered as cell division candidates. In a second stage, the candidates were linked from one frame to the next to establish the cell divisions in time. At the end of the sequence analysis, all events are linked into different cell divisions, making available the number of cell divisions as well as their durations. The resulting cell division numbers displayed every 8 h evidenced cell growth curves (Fig. 2C).

**Specific Analysis of Cell Migration**

The migration levels of human glioblastoma multiforme cells and normal Cdc-25-Lu fibroblasts were characterized.

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**Figure 1.** Narciclasine effects on human glioblastoma multiforme global growth, apoptotic process, and cell cycle distribution. **A,** effects of narciclasine on the overall growth of human glioblastoma multiforme cells. The antiproliferative activity of narciclasine was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in Materials and Methods. Six human glioblastoma multiforme cell lines were evaluated: U373 and Hs683 glioblastoma multiforme permanent cell lines and four human glioblastoma multiforme primocultures (GL19, GL5, GL16, and GL17). Cells were incubated with narciclasine for 72 h at concentrations ranging from 1 nmol/L to 10 μmol/L, with semi-log concentration increases. IC$_{50}$ values for each glioblastoma multiforme cell lines. The experiments were carried out in sextuplicate. Mean ± SE, with SE included within the symbol sizes because not exceeding 3% of the mean values. **B and C,** effects of narciclasine on the apoptotic process. **B,** flow cytometry analysis of double-stained [Annexin V (AV) and PI] narciclasine-treated GL19 glioblastoma multiforme cells after treatment for 18 and 36 h with 100 nmol/L and 1 μmol/L narciclasine and in MCF7 human breast cancer cells treated for 18 h with 1 μmol/L of the compound as a positive control (Ct +). Apoptotic cells are Annexin V$^+$/PI$^-$ (early apoptosis) and Annexin V$^+$/PI$^+$ (late apoptosis), whereas necrotic cells are Annexin V$^-$/PI$^-$ and normal cells are Annexin V$^-$/PI$^-$. **C,** DNA ladder formation on treatment with narciclasine of GL19 glioblastoma multiforme cells as well as in HL-60 (Ct +, positive control included in the commercial kit) cells treated with actinomycin D. GL19 glioblastoma multiforme cells were treated for 24, 48, and 72 h with 100 nmol/L narciclasine. At the end of the incubation, low molecular weight DNA fragments (<50 kb) were extracted from an equal number of cells from each treatment and subjected to electrophoresis on a 1.5% agarose gel. Lane M, molecular weight marker. Left, sizes of the different molecular weight marker DNA bands. **D,** effects of 100 nmol/L narciclasine on the cell cycle. Black areas, proportion of cells in the G1 phase of their cell cycles; white and gray areas, proportion of cells in the S and G2-M phases, respectively. For the controls (Ct), cells were incubated with solvent alone.
by means of a device that enables the trajectories of living cells maintained in culture to be quantified (18). The greatest linear distance migrated by each cell was calculated from these trajectories. This distance refers to the Maximum Relative Distance to the Origin, the MRDO quantitative variable (18). The experiments were all done over 72 h and one image was recorded every 4 min. The analyses were carried out in triplicate, meaning that each experiment was repeated three times independently. The influence of narciclasine on cell migration levels was analyzed at 50 nmol/L during the first 48 h of treatment.

Cytology

The effect of narciclasine at 100 nmol/L on the organization of the actin cytoskeleton of glioblastoma multiforme cells was investigated by means of fluorescent probes as detailed elsewhere (15, 19). Fluorescent phallacidin conjugated with Alexa Fluor 488 (green fluorochrome; Molecular Probes/Invitrogen) was used to label fibrillary actin, whereas red fluorochrome Alexa Fluor 594-conjugated DNase I (Molecular Probes) was used to label globular actin. The relative quantification of filamentous and globular actin was carried out by means of computer-assisted fluorescent microscopy.
microscopy based on the EXPLORER software (Samba Technologies) as detailed elsewhere (19). Immunofluorescent staining of the cells was undertaken after saponin permeabilization and the use of selective antibodies against coflin when phosphorylated at position Ser3 (dilution 1:25; Bioké for Cell Signaling).

**Protein Expression Measurements**

Western blotting analyses were done as detailed previously (14). Control experiments included the omission of the incubation step with the primary antibodies (negative control). The integrity and loading of the extracts were assessed by means of tubulin immunoblotting. The proteins were detected by means of the following primary antibodies: anti-phospho-cofilin Ser3 (dilution 1:1,000; Bioké for Cell signaling), anti-phospho-LIMK1/2 (dilution 1:1,000; Bioké for Cell Signaling), anti-ROCK1 (dilution 1:1,000; Bioké for Cell Signaling), anti-phospho-serine/threonine proteins (dilution 1:1,000; BD Transduction Laboratories), and anti-tubulin (dilution 1:10,000; Abcam). Secondary antibodies were purchased from Pierce (PerbioScience). Western blots were developed using the Pierce SuperSignal Chemiluminescence System.

**Rho Activation Assay and C3 Exoenzyme Inhibition Assay**

The RhoA G-LISA assay (TebuBio for Cytoskeleton) was used and the manufacturer’s exact protocol was followed. The assay uses a 96-well plate coated with the Rho binding domain of Rho family effector proteins. The active GTP-bound form of the Rho family protein but not the inactive GDP-bound form from biological samples will bind to the plate. Bound active Rho family protein is then detected by incubation with a specific primary antibody followed by a secondary antibody conjugated to horseradish peroxidase. The signal is then developed using OPD. U373 and GL19 glioblastoma multiforme cells were serum starved for 24 h and left untreated or treated with narciclasine (100 nmol/L) in serum-free medium at 37°C with and without subsequent narciclasine treatment. The product consists of highly purified C3 transferase covalently linked to a proprietary cell-penetrating moiety via a disulfide bond. The cell-penetrating moiety allows rapid and efficient transport through the plasma membrane. Once in the cytosol, the cell-penetrating moiety is released, thereby allowing C3 transferase to freely diffuse intracellularly and inactive RhoA, RhoB, and RhoC but not related GTPases such as Cdc42 or Rac1. C3 transferase inhibits Rho proteins by ADP ribosylation on Asn41 in the effector binding domain of the GTPase. Cells were then lysed and RhoA activity was measured by the RhoA G-LISA Activation Assay (TebuBio) or alternatively cells were stained for F-actin.

**In vivo Orthotopic Xenografts**

*In vivo* orthotopic xenografts of human Hs683 and GL19 glioblastoma multiforme cells in nude immunodeficient mice were obtained as described previously (20). In each experiment, all mice (6-week-old female nu/nu mice 21-23 g; Iffa Credo, Charles River) had either Hs683 (2×10^5 cells per graft) or GL19 (4×10^5 cells per graft) tumor cells stereotactically implanted into the brain on the same day. Each experimental group contained 11 mice. Comparative evaluation of the effect of administering narciclasine and temozolomide on the survival of glioblastoma multiforme-bearing mice was undertaken to regimens defined in the legend to Fig. 6. Animals were killed when they displayed neurologic signs of the disease and/or when they have lost 20% of their body weight compared with their maximal weight during the experiment. All the *in vivo* experiments described in the present study were done based on Authorization LA1230509 of the Animal Ethics Committee of the Federal Department of Health, Nutritional Safety and the Environment (Belgium).

**Statistical Analyses**

Survival analyses were carried out by means of Kaplan-Meier curves and Gehan’s generalized Wilcoxon test. Statistical comparisons of cell division durations between the control and treated groups were established by means of the Mann-Whitney test. All the statistical analyses were carried out using Statistica (Statsoft).

**Results**

**Narciclasine Impairs Human Glioblastoma Multiforme Cell Global Growth without Inducing Apoptotic Cell Death when Evaluated *in vitro* at Its IC_{50} Growth-Inhibitory Value**

The *in vitro* antitumor effects of narciclasine against a panel of 6 human glioblastoma multiforme including 4 primary cultures and 2 established cell lines were assessed by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay (Fig. 1A) and computer-assisted phase-contrast videomicroscopy (Fig. 2A: double click on the images at time 0 h to activate the videos once the downloading procedure has been completed as detailed in Materials and Methods). The mean IC_{50} of ~50 nmol/L calculated on the 6 human glioblastoma multiforme was similar to the value we previously reported for 6 carcinoma cell lines (14) and consistent with National Cancer Institute data, which also reveal a mean IC_{50} value of 47 nmol/L for the compound across a panel of 60 cancer cell lines. 8

We recently showed that the *in vitro* growth-inhibitory activity of high narciclasine concentrations (>1 μmol/L) in carcinoma cells relates to proapoptotic effects resulting from compound-mediated triggering of the activation of the initiator caspases of the death receptor pathway (14). In the present study, we failed to show any proapoptotic effects for narciclasine in U573 (data not shown), Hs683 (data not shown), and GL19 (Fig. 1B and C) glioblastoma multiforme cells by means of two distinct techniques, a double labeling with Annexin V and PI, and a DNA laddering assay. Narciclasine was assayed at 100 nmol/L and 1 μmol/L and at its IC_{50} growth-inhibitory value on each of the three glioblastoma multiforme cell lines under study.
Figure 1D reveals that narciclasine treatment increases the proportion of GL19 glioblastoma multiforme cells in the G2 and S phases of their cell cycle. These results have also been evidenced on Hs683 and U373 cell lines (data not shown).

Narciclasine Impairs Human Glioblastoma Multiforme Cell Proliferation through a Decrease in Mitosis Rate Associated with an Increased Time to Cell Division

The data illustrated in Figs. 1 and 2A (and associated video) reveal that narciclasine displayed significant cytostatic but no cytotoxic effects on human glioblastoma multiforme. Figure 2B reveals that narciclasine indeed markedly impaired the overall growth of U373 (black columns), Hs683 (gray columns), and GL19 (open columns) glioblastoma multiforme cell lines as determined by means of the global growth ratio parameter over the 72 h period of observation (P < 0.05 for the three glioblastoma multiformes). Figure 1C reveals that narciclasine-induced impairment of GL19 glioblastoma multiforme growth resulted from a marked inhibition of the mitotic rate, whereas Fig. 1D reveals that this decrease in mitosis appears to be associated with a marked increase in the duration of mitosis. Similar data have been obtained for the Hs683 and U373 cell lines (data not shown).

Narciclasine Impairs Human Glioblastoma Multiforme Cell Migration

Video corresponding to Fig. 2A illustrate the pattern of migration (and proliferation) of human GL19 glioblastoma multiforme cells left untreated or treated with narciclasine (100 nmol/L) during a 72 h period of observation (double-clicking on the image at 0 h to animate the movie). Figure 2E shows that 50 nmol/L narciclasine (gray columns) significantly modifies the levels of migration of U373, Hs683, and GL19 glioblastoma multiforme cells compared with the control condition (black columns). These effects were more marked on glioblastoma multiforme cells (P < 0.001) compared with the effects on normal fibroblasts (Cdc-25-Lu; P < 0.05). These data are in concordance with our previously published one showing that narciclasine impairs human cancer cell and, to a lesser extent, normal fibroblast cell proliferation and migration (15).

Narciclasine Induces Actin Polymerization, Which Correlates with an Increase in Cofilin Inactivation-Related Phosphorylation Status

We first evidenced that culturing human U373 glioblastoma multiforme cells for 2 h with 100 nmol/L narciclasine markedly increased the amount of fibrillar actin (green fluorescence in Fig. 3Aa, bottom) compared with cells cultured in the absence of the compound (Fig. 3Aa, CT). We confirmed here our recent data published on U373 glioblastoma multiforme cells and also PC3 prostate cancer (15). Corresponding quantitative data obtained using computer-assisted fluorescence microscopy using a kinetic of treatment with 100 nmol/L narciclasine (Fig. 3B) show a rapid and marked increase of the amount of fibrillar actin in all glioblastoma multiforme cell lines investigated after respectively 10, 30, and 45 min incubation with GL19 (green columns), Hs683 (blue columns), and U373 (red columns). Specifically, narciclasine treatment appeared to increase the number and thickness of cytoplasmic actin-containing stress fibers (Figs. 3Aa and 5B). In contrast, narciclasine treatment of glioblastoma multiforme cells had no observable effect on cytoplasmic microtubule distribution (data not shown). As the actin-severing protein cofilin is an upstream regulator of actin polymerization in the pathway (Fig. 3C), narciclasine-induced modulation of cofilin activity was investigated by monitoring phosphorylation at Ser^3, which negatively regulates cofilin activity (4). Using antibodies detecting endogenous cofilin only when phosphorylated at Ser^3 (4), immunofluorescence and Western blot analyses revealed that 100 nmol/L narciclasine treatment markedly and rapidly (already after 15 min) increased phospho-cofilin levels in U373, Hs683, and GL19 glioblastoma multiforme cells (Fig. 3D illustrates the data obtained on GL19 glioblastoma multiforme cells) without modification of overall total cofilin levels (data not shown). This increase in the phosphorylation of cofilin was sustained for at least 3 h after narciclasine treatment (Fig. 3D). Normally, directed cell migration in two-dimensional culture involves the generation of polarity in the direction of movement, a clear distinction between the front and the rear of cells, a process resulting in part from the reorganization of the actin cytoskeleton (1). This polarity is disrupted by narciclasine treatment of glioblastoma multiforme cells (Fig. 2A, video). An early event in narciclasine-induced disruption of polarization is a change in filamentous F-actin distribution from concentrations in a particular region to azimuthal symmetry around the cell rim (Figs. 3Aa and 5B).

Narciclasine Strongly Activates Several Elements of the Cofilin Signaling Pathway

As indicated previously, (a) cofilin is the main known LIMK substrate (5, 21), itself the downstream effector of ROCK1 and both are phospho-serine/threonine protein kinases (Fig. 3C), and (b) recent studies had indicated that the overall activity of the cofilin pathway, and not that of any single gene within the pathway, determines the invasive and metastatic phenotype of tumor cells (16). Western blot analysis indicated that 100 nmol/L narciclasine significantly and rapidly increased the expression of phospho-serine/threonine protein kinases, ROCK1 and phospho-LIMK1/2, in GL19 glioblastoma multiforme cells (Fig. 5). Similar results were obtained in the U373 and Hs683 glioblastoma multiforme cell lines (data not shown). These data suggest that the narciclasine-induced increase in the amount of fibrillar actin observed in vitro (Figs. 3Aa and 5B) may relate to its activation of the cofilin pathway.

Narciclasine Strongly Activates RhoA, Whereas Inhibition of RhoA Abolishes the Effects of Narciclasine on Actin Stress Fiber Induction

Several agents induce stress fiber and adhesion complex formation in cells by transiently activating RhoA, whereas various Rho isoforms have also been implicated in cell migration (22). The family of Rho GTPases also participates in cytoskeleton architecture, cell cycle control, and gene expression (23). Accordingly, the effects of narciclasine on RhoA were evaluated and found to clearly stimulate its activity in human glioblastoma multiforme cells
For the GL19 glioblastoma multiforme primary culture, RhoA activation occurred within 3 min and was sustained for 1 h.

The C3 exoenzyme from *Clostridium botulinum* inhibits the activation of all three mammalian Rho isoforms by ADP ribosylation. Use was made therefore of a highly purified C3 transferase covalently linked to a proprietary cell-permeating moiety via a disulfide bond (cell-permeable Rho inhibitor; see Materials and Methods for details) to inhibit RhoA activation. It was found to strongly block the narciclasine-induced stimulation of RhoA activity in glioblastoma multiforme cells, notably in GL19 and U373 (Fig. 5A), and stress fiber formation in GL19 cells (Fig. 5B).

These data appear therefore to confirm that the ability of narciclasine to alter the actin cytoskeleton of glioblastoma multiforme cells is mediated, at least in part, by its ability to stimulate RhoA activation. The data reported here for GL19 and U373 glioblastoma multiforme cells were reproduced in Hs683 glioblastoma multiforme cells (data not shown).

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Treatment with Narciclasine Significantly Increases the Survival of Mice Orthotopically Xenografted with Two Invasive Human Glioblastoma Multiforme Models

The Hs683 cell line and GL19 primoculture grafted into the brains of nude immunodeficient mice both produced invasive brain tumors as illustrated in Fig. 6A and B. Xenograft-bearing mice received vehicle alone, oral temozolomide at 40 mg/kg (5 administrations per week for 5 consecutive weeks), or narciclasine at 1 mg/kg either oral (once per week for 5 weeks) or i.v. (twice per week for 5 weeks). Drug administration was initiated respectively on days 5 and 7 post-tumor grafting for the Hs683 and GL19 models. The temozolomide dose and treatment schedule were selected based on previous optimized regimens (16, 20). Narciclasine dose and treatment schedule were selected based on narciclasine toxicity study in rats after oral administration and pharmacokinetic study that we have recently published (15). In toxicity study, narciclasine (25, 10, or 1 mg/kg) was administered five times a week for 3 weeks and the no adverse effect level dose was defined to be 1 mg/kg/d p.o., with minimal acanthosis reactive changes and minor variations in some biochemistry parameters observed at this dose level considered to be nonadverse (15).

Temozolomide significantly increased the survival of both Hs683 (Fig. 6C, red curve) and GL19 (Fig. 6D, red curve) glioblastoma multiforme-bearing mice. The i.v. regimen of narciclasine at 1 mg/kg significantly (P = 0.02) increased the survival of GL19 glioblastoma multiforme-bearing mice (Fig. 6D). We have recently confirmed the oral bioavailability of narciclasine in mice to be 32% (15). The compound when given orally at the same dose five times a week for 5 consecutive weeks also significantly increased animal survival in this model (P = 0.008; data not shown). Oral treatment with narciclasine at 1 mg/kg significantly increased the survival (P = 0.004) of Hs683 glioblastoma multiforme-bearing mice (Fig. 6C). Increasing the number of doses administered per week did not increase the survival of these Hs683 glioblastoma multiforme-bearing mice (data not shown). Narciclasine appears to show similar increased survival in these models to temozolomide but at appreciable lower doses and following both oral and i.v. administration.

Discussion

Our results show for the first time that narciclasine isolated from Narcissus bulbs has antitumor activity in preclinical models of glioblastoma multiforme. Further data suggest that this anti-glioblastoma multiforme activity is at least partly linked to an overall decrease in cell proliferation rate rather than to apoptosis and results from modulation to the Rho/ROCK/LIMK/cofilin signaling pathway leading to actin stress fiber stabilization and to an increase in focal adhesion.

Available literature reports that inhibition of protein biosynthesis is the primary mode of action of narciclasine in eukaryotic cells. Although the actin cytoskeleton and the translation machinery are considered to be separate cellular complexes, growing evidence supports overlapping regulation of the two systems (24). These data reconcile the protein synthesis inhibition effects evidenced by Carrasco et al. (10) and our results.

Our results show that narciclasine increased the amount of stress fibers at least that part controlled by cofilin phosphorylation (Fig. 3D). Cofilin is a small ubiquitous protein (19 kDa) that is able to bind both monomeric (G) and filamentous (F) actin (4). By severing actin filaments, cofilin increases the number of filament ends for polymerization and depolymerization (4). Cofilin can be regulated through different upstream effectors; LIMK1 and LIMK2 and by TES1 and TES2 kinases, which phosphorylate cofilin on the Ser3 residue, thus rendering it inactive (25). Cofilin is known to be an important contributor to actin organization downstream of ROCK/LIMK (Fig. 3C; ref. 26) and its activation has been linked to the formation of cell protrusions, the assembly and stability of invadopods, and the invasion and metastasis of breast cancer cells (4, 27). Inhibition of cofilin activity in carcinoma cells with either small interfering RNA (4) or the expression of constitutively active LIMK domain (28) reduces cell motility by limiting the assembly and stability of invadopodia. Siddani et al. (29) showed that the loss of cofilin caused an amoeboid tumor cell to assume a mesenchymal-type mode of movement. These results suggest that cofilin is involved in the maintenance of directionality of migration in mesenchymal-like nonmetastatic tumor cells, such as that described previously for fibroblasts (30),...
compared with the decreased directionality on increasing turning frequency in metastatic amoeboid type tumor cells. Specific small interfering RNA-mediated down-regulation of urokinase-type plasminogen activator receptor and cathepsin B retards cofilin dephosphorylation and inhibits glioma cell migration accompanied by cytoskeletal condensation (31). The overexpression of cofilin increases the velocity of cell migration in Dictyostelium and in human glioblastoma multiforme cells (4). The spontaneous overexpression of cofilin has been detected in the invasive subpopulation of cancer cells in mammary tumors (27). Cofilin is also overexpressed in the highly invasive C6 rat glioblastoma multiforme cell line (32), and the amount of phosphorylated inactive cofilin is decreased in cell lines derived from T lymphoma (Jurkat) and carcinomas from the cervix (HeLa), colon (KM12), liver (HepG2), and kidney (COS1; ref. 33). However, given that there are four regulatory mechanisms for cofilin activity, which appear not to be linked, the activity status of cofilin in a cell cannot be assessed by measuring the ratio of phosphorylated cofilin to the total cofilin present (4). We therefore showed that narciclasine increases the expression of phospho-serine/threonine kinases, ROCK1 and phosphorylated/activated LIMK1/2 (Fig. 4), and induces the activation of RhoA (Fig. 5A) in glioblastoma multiforme cells, elements upstream of cofilin in this pathway (Fig. 3C). It has been shown that the activation pattern of LIMK1, which is highly expressed in the brain, corresponds to the phosphorylation and dephosphorylation pattern of cofilin during mitosis, which is critical for proper cytokinesis (34). Amano et al. have proposed that LIMK1 plays an important role in regulating actin-depolymerizing factor/cofilin activity during mitosis and that cofilin dephosphorylation in the late stages of mitosis is critical for cytokinesis (34). Therefore, narciclasine effects on LIMK and cofilin phosphorylation could also explain its decrease of the rate of mitosis in glioblastoma multiforme cells.

Figure 5. Narciclaise-induced stress fiber formation is Rho dependent in human glioblastoma multiforme cells. A, narciclasine activates Rho in glioblastoma multiforme cells. Serum-starved U373 and GL19 glioblastoma multiforme cells were treated with narciclasine (NCS; 100 nmol/L) for the indicated times or treated with the cell-permeable Rho inhibitor (Rho Inh) alone or the combination of the inhibitor and narciclasine or left untreated (CT2), and Rho activation was assessed. CT1, blank control. Note that Rho activity remained strongly stimulated up to 1 h after treatment with narciclasine in both glioblastoma multiforme cell lines. B, narciclasine-induced stress fiber formation is Rho-dependent. Serum-starved GL19 glioblastoma multiforme cells were preloaded without or with C3 exoenzyme (Rho inhibitor) and treated without or with narciclasine (100 nmol/L) for 10 min. Cells were then stained for the actin cytoskeleton [red fluorescence, globular (nonpolymerized) actin; green fluorescence, fibrillary (polymerized) actin]. Note that preloading with C3 exoenzyme prevented narciclasine-mediated stress fiber formation.
Importantly, the ability of narciclasine to increase the actin stress fiber content of glioblastoma multiforme cells was abrogated by preloading of cells with the Rho-inhibiting C3 exoenzyme. The question that then arises is whether Rho activation is desirable from a therapeutic perspective? In several cases, Rho overexpression and/or pathway activation have been positively correlated with malignant progression (35–37), which suggests that Rho inhibitors rather than activators might show antimetastatic activity. However, the efficacy of either approach may be tumor specific. MDA231 breast (38), BE colon, and S2962 squamous carcinoma cells (39) must elongate to invade and all three of these cell lines continue to invade when Rho signaling is down-regulated (38, 39). Thus, metastatic or invading tumors, which use "elongated" invasion, may be sensitive to Rho activation by pharmacologic agents. It has been shown that oligodendrocyte lineage transcription factor 2 inhibits the motility of a certain human glial tumor cell line by activating RhoA (22). This may explain the selective antitumor activity of farnesyl transferase inhibitors, which, although originally designed to attenuate the action of Ras proteins, are now believed to act including in glioblastoma multiforme cells at least in part by activating Rho (40–42). Conversely, metastatic tumors whose cells invade using a rounded, amoeboid movement are clearly susceptible to Rho inhibition (39).

Narciclasine isolated from Narcissus bulbs has been shown to be a plant growth inhibitor (8). Actin filaments are the main players in plant cell and pollen grain growth (43). Cofilin is involved in these plant processes (44). Plant cells and polar pollen tubes use small GTPase signaling proteins termed the Rho of plants/RAC to control actin polymerization and growth processes (45). Cell polarity also plays an important role in plant development (46). Rho of plants are homologues of Rho/RAC/CDC42 Rho family GTPases, which include key regulators of cell polarity in yeast and animals (47). Plants have evolved elaborate mechanisms for surviving unfavorable growing conditions.
found in nature (48). For example, bud growth can be inhibited by signals generated inside the plant as the direct result of unfavorable conditions and provoke a dormancy state (48). Dormancy provides an important protection mechanism until the return of stable growth-conducive conditions. In certain cases, the whole plant enters the dormant state. Such is the case in Narcissus species in which, during the dormant state, the only remaining viable part is the bulb. The link between narciclasine and dormancy in Narcissus species has never been clearly established; however, it seems that the compound may induce a “dormancy state” in cancer cells, at least in glioblastoma multiforme cells, and that the pathway involved may also have a counterpart in plant growth regulation.

In conclusion, our results show for the first time that narciclasine, a plant growth modulator, activates Rho and stress fibers in glioblastoma multiforme cells and significantly increases the survival of human glioblastoma multiforme preclinical models. This statement is made despite the recognition that to date, irrespective of treatment, no single glioblastoma multiforme patient has been cured.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


35. van Golen KL, Wu ZF, Giao XT, Bao LW, Merajver SD. RhoGTPase, a novel transforming oncogene for human mammary epithelial cells that...


Correction: Narciclasine, a Plant Growth Modulator, Activates Rho and Stress Fibers in Glioblastoma Cells

In this article (Mol Cancer Ther 2009;8:1739–50), which was published in the July 2009 issue of Molecular Cancer Therapeutics (1), the authors apologize for errors in which Figs. 3D and 4 were labeled incorrectly.

Figure 3D was initially labeled as “GL19.” In fact, the top of Fig. 3D relates to the GL19 glioma cell line, whereas the bottom of Fig. 3D relates to the Hs683 glioma cell line as indicated in the corrected Fig. 3D.

Figure 3. Narciclasine induces stress fiber formation in human glioblastoma multiforme cells. A, fluorescence microscopy visualization of the actin cytoskeleton [red fluorescence, globular (nonpolymerized) actin; green fluorescence, fibrillar (polymerized) actin] in control (CT) and narciclasine-treated (100 nmol/L) U373 glioblastoma multiforme cells after 2 hours. B, quantitative determination of the intensity (absorbance) of green fluorescence (polymerized actin) in untreated glioblastoma multiforme cells (CT) versus those treated for 10 minutes (GL19 cells: green columns), 30 minutes (Hs683 cells: blue columns), or 45 minutes (U373 cells, red columns) with 100 nmol/L narciclasine (Narci). One hundred cells were analyzed per cell line and condition. Mean ± SE. P values of statistical significance refer to the comparison (Mann–Whitney U test) between untreated and narciclasine-treated groups. ***, P < 0.001. C, postulated model summarizing the hypothesis of narciclasine-mediated actin stress fiber stabilization. D, determination by means of immunofluorescence (top; GL19 cells) and Western blotting (bottom; Hs683 cells) of the expression of phospho-cofilin (phosphorylated at Ser3) in glioblastoma multiforme cells left untreated (CT) or treated with 100 nmol/L narciclasine after 15 minutes and during a kinetic of treatment from 0 to 180 minutes, respectively.
Figure 4 was initially labeled as "GL19." In fact, both the top (PPserthreo proteins) and middle (ROCK1) refer to the U373 glioma cell line, whereas the bottom (phospho-LIMK1/2) relates to the GL19 glioma cell line as indicated in the corrected Fig. 4.

Figure 4. Involvement of several elements of the RhoA/ROCK/LIMK/cofilin pathway in the antitumor effects of narciclasine. Western blot analysis of the expression of phospho-serine/threonine protein kinases (U373 cells), ROCK1 (U373 cells), and phosphorylated LIMK1/2 (GL19 cells) in glioblastoma multiforme cells left untreated or treated with 100 nmol/L narciclasine (0–180 min). Tub, tubulin.

Reference

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Narciclasine, a plant growth modulator, activates Rho and stress fibers in glioblastoma cells

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