

Effective Tumor Cell Death by σ -2 Receptor Ligand Siramesine Involves Lysosomal Leakage and Oxidative Stress

Marie Stampe Ostenfeld,¹ Nicole Fehrenbacher,¹ Maria Høyer-Hansen,¹ Christian Thomsen,² Thomas Farkas,¹ and Marja Jäättelä¹

¹Apoptosis Department, Institute for Cancer Biology, Danish Cancer Society, Copenhagen, Denmark and

²Molecular Pharmacology and Neurochemistry, H. Lundbeck A/S, Valby, Denmark

Abstract

Acquired resistance to classic caspase-mediated apoptosis is a common problem for the treatment of human cancer. Here, we show that siramesine, a novel σ -2 receptor ligand, effectively induces caspase-independent programmed cell death in immortalized and transformed cells of various origins. Siramesine-treated tumor cells displayed increased levels of reactive oxygen species, lysosomal membrane permeabilization, chromatin condensation, and shrinkage and detachment of cells. Lipid antioxidants (α -tocopherol and γ -tocopherol), but not other tested antioxidants (butylated hydroxyanisole or *N*-acetyl cysteine), effectively inhibited siramesine-induced morphologic changes and cell death. Cathepsin B inhibitors (CA-074-Me and R-2525) conferred similar, but less pronounced protection, whereas ectopic expression of antiapoptotic protein Bcl-2, lack of wild-type p53 as well as pharmacologic inhibitors of caspases (zVAD-fmk, DEVD-CHO, and LEHD-CHO), calpains (PD150606), and serine proteases (*N*-tosyl-L-phenylalanine chloromethyl ketone and pefabloc) failed to protect cells against siramesine-induced death. Importantly, transformation of murine embryonic fibroblasts with activated *c-src* or *v-Ha-ras* oncogenes greatly sensitized them to siramesine-induced cytotoxicity. Furthermore, p.o. administration of well-tolerated doses of siramesine had a significant antitumorigenic effect in orthotopic breast cancer and s.c. fibrosarcoma models in mice. These results present siramesine as a promising new drug for the treatment of tumors resistant to traditional therapies. (Cancer Res 2005; 65(19): 8975-83)

Introduction

σ Receptors are orphan receptors with unique drug-binding profiles (1). Until recently, they have been considered as central nervous system-specific binding sites that modulate neurologic processes through an interaction with psychoactive substances like haloperidol. Accumulating evidence suggest, however, that σ receptors also regulate cell proliferation and survival and that their expression is not limited to neurons (2–5). Cellular transition from quiescent to proliferative status is associated with an increase in σ receptor expression (6), and σ receptors are abundant in human and rodent tumors of various origins (4). Pharmacologic binding studies have revealed the existence of two σ receptor subtypes with different drug-binding profiles (7, 8). Whereas the

σ -1 receptor has been cloned and characterized (9), the sequence of the σ -2 receptor has not been revealed.

Whereas σ -1 receptors promote cell growth and inhibit apoptosis (5), activation of σ -2 receptors by selective (e.g., CB-184, CB-64D, and ibogaine) as well as nonselective (haloperidol) ligands induces growth arrest and cell death in various tumor cell lines (2, 3, 10). The mechanism by which σ -2 ligands induce cytotoxicity remains, however, largely unknown. This is partially due to the fact that several σ -2 signaling studies have been done with haloperidol or other nonselective σ ligands that bind both σ -1 and σ -2 receptors. For example, the induction of reactive oxygen species and protection by vitamin E has been reported in cells treated with haloperidol (11–13). Controversy also exists whether σ -1 or σ -2 receptors are responsible for the increase in intracellular calcium ($[Ca^{2+}]_i$) observed in response to σ antagonists and agonists (5, 14). Other σ -mediated effects that seem to be involved in the regulation of cell survival include the regulation of voltage-gated Ca^{2+} and K^+ channels (15, 16) and the increase in the concentration of ceramide, a sphingolipid suggested to be involved in some apoptosis signaling pathways (17).

Normal cells respond to death stimuli by undergoing caspase-dependent apoptosis, the best characterized form of programmed cell death. In contrast, cancer cells frequently escape spontaneous and therapy-induced caspase activation due to acquired mutations in the apoptotic machinery (18–20). For example, up-regulation of antiapoptotic Bcl-2 protein and mutations in p53 tumor suppressor protein are common in human tumors. Therefore, development of novel anticancer drugs that can trigger alternative death pathways that are independent of commonly mutated apoptosis-regulating genes is of great importance. The σ receptors may represent candidate targets for such drugs, because the death signaling initiated by their activation may proceed independent of caspase activation and wild-type p53 (3). Furthermore, the increased expression of σ receptors in proliferating cells may render cancer cells especially sensitive to σ -2 agonists (21, 22).

Siramesine (Lu-28-179; 1'-[4-[1-(4-fluorophenyl)-1*H*-indol-3-yl]butan-1-yl]spiro[isobenzofuran-1(3*H*),4'-piperidine]), a piperidine-analogue that binds σ -2 receptors with a subnanomolar equilibrium dissociation constant (K_d ; refs. 23, 24), was originally developed for the treatment of anxiety and depression (25). Although the clinical trials failed to show the requested efficacy in the treatment of psychiatric disorders, they presented siramesine as a nontoxic and well-tolerated compound in humans. The lack of serious side effects, together with the increasing body of evidence suggesting that σ -2 receptors trigger cell death, prompted us to investigate the potency of siramesine as a cancer drug. Indeed, siramesine induced cell death in all immortalized and tumorigenic cell lines tested. In spite of the apoptosis-like morphology of dying cells, siramesine-induced death was independent of the major modulators of classic apoptosis (i.e., p53, Bcl-2,

Requests for reprints: Marja Jäättelä, Apoptosis Department, Institute for Cancer Biology, Danish Cancer Society, Strandboulevarden 49, DK-2100 Copenhagen, Denmark. Phone: 45-35257318; Fax: 45-35257721; E-mail: mj@cancer.dk.

©2005 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-05-0269

and known caspases). The closer analysis of the death pathway revealed an involvement of reactive oxygen species and lysosomal membrane permeabilization as well as a marked sensitization by oncogenic transformation. Importantly, p.o. administration of siramesine significantly inhibited the growth of tumor xenografts in mice in the absence of detectable side effects. Taken together, these data greatly encourage the translation of siramesine into human cancer trials.

Materials and Methods

Cells and treatments. Tumorigenic cell lines originate from murine fibrosarcoma (WEHI-S and WEHI-R) and human breast (MCF-7S1 and MDA-MB-468) and cervix (HeLa and ME-180) carcinomas. Nontumorigenic lines are immortalized human breast (HBL-100) and embryonic kidney (HEK293-A) epithelial cells or murine embryonic fibroblasts (NIH3T3). WEHI-S and WEHI-R cells represent single cell clones of WEHI-164 fibrosarcoma cells selected for their sensitivity and resistance to tumor necrosis factor (TNF), respectively (26). MCF-7S1 cell line is a highly TNF-sensitive subclone of MCF-7 cells (hereafter called MCF-7; ref. 26). Cells were propagated in DMEM (Invitrogen, Paisley, United Kingdom) supplemented with 10% heat-inactivated FCS (Biological Industries, Beit Haemek, Israel), 0.1 mmol/L nonessential amino acids (Invitrogen), and antibiotics, or in RPMI 1640 (Invitrogen) supplemented with 6% heat-inactivated FCS and antibiotics at 37°C in a humidified air atmosphere with 5% CO₂.

MCF7-pCEP-1, MCF7-pCEP-2, and MCF7-pCEP-3 and MCF7-Bcl-2-1, MCF7-Bcl-2-2, and MCF7-Bcl-2-3 are single cell clones of MCF-7 cells transfected with an empty pCEP-4-hygro vector (Invitrogen) or pCEP-4-hygro encoding for human Bcl-2, respectively, essentially as described previously (27). MCF7-neo2 and MCF7-casp3.3 are single cell clones of MCF-7 cells transfected with an empty pcDNA-3-neo vector (Invitrogen) and pcDNA-3-neo encoding for human procaspase-3 (kindly provided by V. Dixit, Genentech, San Francisco, CA), respectively. NIH3T3 cells were transduced with an empty pBabe-puro retrovirus or pBabe-puro encoding for SV40 large T antigen (SV40LT), *v-Ha-ras*, or *c-src*^{Y527F} (hereafter called *c-src*) as described previously (28). All experiments were conducted at passages six to eight after the transduction.

Siramesine (1'-[4-[1-4-fluorophenyl]-1H-indol-3-yl]-1-butyl]spiro[isobenzofuran-1(3H),4'-piperidine] selective σ -2 ligand) was synthesized at Lundbeck A/S (Copenhagen, Denmark; ref. 23). The recombinant human TNF was kindly provided by A. Cerami (Kenneth Warren Laboratories, Tarrytown) and the cathepsin B inhibitor R-2525 by B. Rydzewski (Celera Applied Biosystems, Foster City, CA). Etoposide, butylated hydroxyanisole, α -tocopherol, γ -tocopherol, glutathione ethyl ester, and *N*-acetyl-cysteine, haloperidol (σ -1/2 ligand), and (+)-pentazocine (σ -1 ligand) were from Sigma-Aldrich (St. Louis, MO); zVAD-fmk and DEVD-fmk from Bachem (Bubendorf, Switzerland); DEVD-CHO from Neosystems (Strasbourg, France); LEHD-CHO from Alexis (Montreal, Quebec); CA-074-Me from Peptides International (Louisville, KY); pepstatin A, PD150606, and calpain inhibitor ALLN from Calbiochem (La Jolla, CA); and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and pefabloc (AEBSF) from Roche Diagnostics GmbH (Penzberg, Germany). Protease inhibitors and antioxidants were added to the cells 1 hour before the death-inducing stimuli.

Detection of cell viability and death. The viability and death of the cells were analyzed by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) reduction and lactate dehydrogenase (LDH; cytotoxicity detection kit from Roche) assays essentially as described (29). The cell death mode was assessed by the type of nuclear condensation and the status of the plasma membrane using an inverted Olympus IX70 fluorescent microscope (Filter U-MWU 330-385 nm) after staining the cells with a mixture of 2.5 μ g/mL Hoechst 33342 and 0.5 μ mol/L SYTOX (Molecular Probes, Eugene, OR).

Caspase activity measurement. For the analysis of caspase 3-like activity, caspase extraction buffer [0.5% Triton X-100, 25 mmol/L HEPES, 5 mmol/L Mg₂Cl₂, 1 mmol/L EGTA, 1 mmol/L pefabloc (pH 7.5)] was added to the drug-treated subconfluent cells for 20 minutes on ice. The caspase

activity was analyzed by adding one volume of 20 μ mol/L Ac-DEVD-AFC (Biomol, Plymouth Meeting, PA) in caspase reaction buffer [100 mmol/L HEPES, 20% glycerol, 0.5 mmol/L EDTA, 0.1% CHAPS, 5 mmol/L DTT, 1 mmol/L pefabloc (pH 7.5)]. The rate of substrate hydrolysis as measured by the liberation of AFC (excitation, 400 nm; emission, 489 nm) was analyzed over 20 minutes at 30°C using a Spectramax Gemini fluorometer (Molecular Devices, Sunnyvale, CA).

Immunodetection of proteins. Immunodetection of proteins separated by SDS-PAGE and transferred to nitrocellulose membranes was done with enhanced chemiluminescence Western blotting agents (Amersham Biosciences, Little Chalfont, United Kingdom). Primary antibodies raised against cathepsin L (clone S-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Hsp70 (clone 2H9, gift from Boris Margulis, Russian Academy of Sciences, St. Petersburg, Russia), Bcl-2 (clone 124, Boehringer Mannheim, Mannheim, Germany), p53 (clone DO-1, gift from Jiri Bartek, Danish Cancer Society, Copenhagen, Denmark), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Biogenesis, Poole, United Kingdom) followed by appropriate peroxidase-conjugated secondary antibodies from DAKO A/S (Glostrup, Denmark) were used.

For immunocytochemistry, cells plated on glass coverslips were washed in PBS and fixed using ice-cold methanol for 10 minutes at 25°C. The samples were blocked for 20 minutes in 5% goat serum diluted in PBS containing 0.3% Triton X-100, 1% bovine serum albumin (BSA). Primary antibodies against cathepsin L (clone 22, BD Transduction Laboratories, Lexington, KY) and cytochrome *c* (clone 6H2.B4, BD PharMingen, San Diego, CA) were diluted in PBS containing 0.25% BSA and 0.1% Triton X-100 and transferred to samples for 1 hour in darkness at 25°C. After washing, samples were incubated with Alexa 488-coupled secondary antibodies (Molecular Probes) for 30 minutes. Samples were washed in PBS containing 0.05% Tween 20 and the glass coverslips were mounted using antifade kit (Molecular Probes). Fluorescence images were subsequently taken using a Zeiss laser-scanning microscope with Axiovert 100M.

Detection of oxidative stress. Cells treated as indicated were exposed to 2 μ mol/L dihydroethidine (Molecular Probes) for 15 minutes and washed in PBS. The fluorescence intensity of ethidine, the oxidation product of dihydroethidine, was subsequently visualized in an inverted Olympus IX70 fluorescent microscope (Filter U-MWU 330-385 nm) and quantified by FACScalibur flow cytometer (Becton Dickinson, Heidelberg, Germany).

σ -2 Receptor analysis. Subconfluent cells were washed twice in PBS without Ca²⁺ and Mg²⁺, detached using a cell scraper, centrifuged for 10 minutes at 400 \times *g* at 4°C and stored at -80°C. Upon thawing, the cell pellets were resuspended in ice-cold Tris buffer [50 mmol/L Tris (pH 7.7)], homogenized using an Ultra Turrax Teflon pestle homogenizer and centrifuged at 4 \times 10⁴ *g* for 20 minutes at 4°C. The resulting pellet containing the crude membrane fraction was resuspended in ice-cold Tris buffer and stored in aliquots at -80°C until use. The protein concentrations were estimated using BCA protein assay (Pierce Biotechnology, Rockford, IL).

The binding of [³H]siramesine to crude membrane fractions was analyzed essentially as described previously (24). The membrane preparation was diluted in the Tris buffer containing 1% BSA (Sigma-Aldrich) to a final concentration of 25 μ g/mL. Nonspecific binding was defined as binding in the presence of 1,3-di-*o*-tolylguanidine (nonselective σ ligand; Sigma-Aldrich). All glass and plastic equipment used in the binding assay were coated in Tris buffer containing 1% BSA to reduce the binding of siramesine to the surfaces.

Tumor xenografts. WEHI-R4 cells (5 \times 10⁶ in 200 μ L PBS) were inoculated s.c. into the right flank of immunocompetent female BALB/c mice. MCF7-S1 cells (10 \times 10⁶ in 100 μ L PBS) were inoculated into the second axillary mammary fat pad of female FOX CHASE severe combined immunodeficient (SCID) mice treated with 0.670 μ g/mL estrone (Sigma) in drinking water. The daily p.o. treatment with 200 μ L vehicle (0.5% methylcellulose 15 in 0.9% NaCl solution) alone or with indicated concentrations of siramesine was initiated either simultaneously, 2 days before the inoculation of tumor cells, or after tumor manifestation (*r* = 2.5 mm). The tumor diameter was measured using a caliper, and the tumor volume was estimated according to the formula, $V = 4/3 \times \pi \times r^3$. All experiments were terminated at the time when the tumors in untreated

animals reached maximum tolerated size ($r = 6$ mm). All animal work was carried out in accordance with the NIH guidelines.

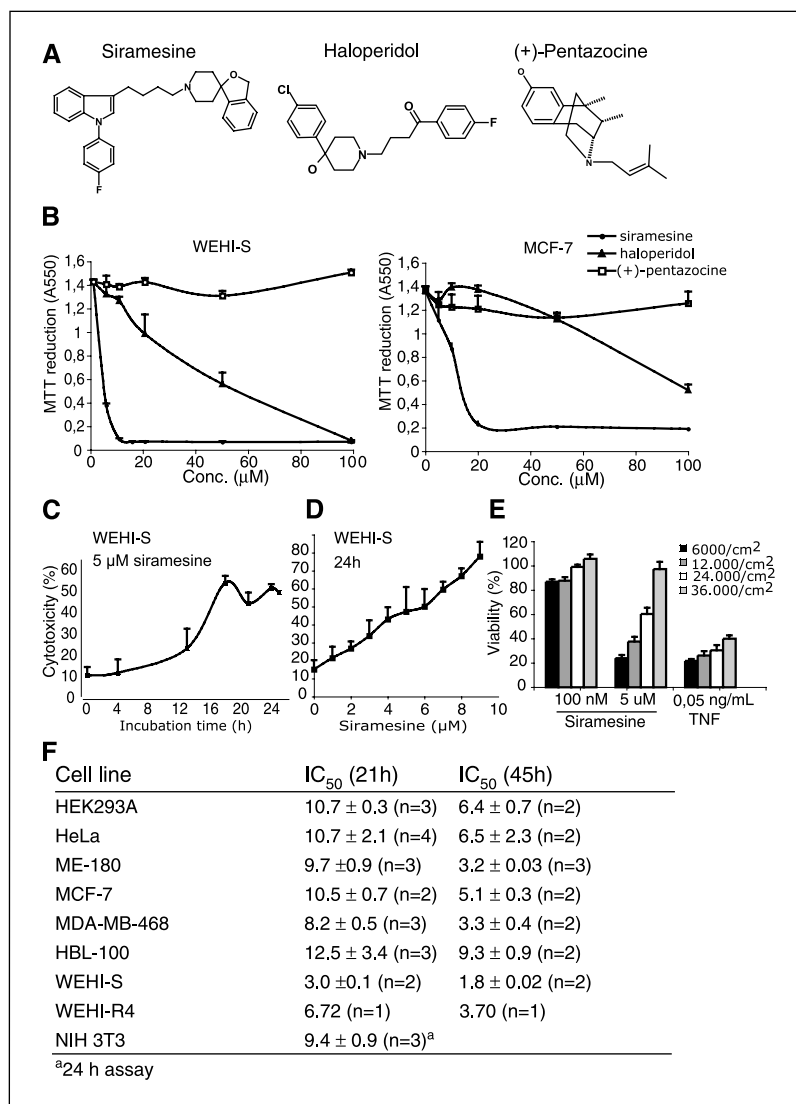
Data analysis. IC_{50} values and saturation binding kinetics [K_d , maximum number of binding sites (B_{max})] were calculated by a nonlinear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical significance between different values of IC_{50} , K_d , and B_{max} values for σ -2 site binding, and caspase activity were examined by one-way ANOVA analysis. The difference in IC_{50} values for differently transduced NIH 3T3 cells was calculated by Tukey's multiple comparison test using normalized data sets, where the IC_{50} value of vector-transduced cells served as a reference (=1). Significant protection by pharmacologic inhibitors against siramesine was tested using a one-sample t test and differences in tumor sizes between vehicle and siramesine-treated groups by Student's t test.

Results

Siramesine induces cell death in immortalized and transformed cell lines. Siramesine is a novel piperidine analogue with a high binding affinity and a 100-fold selectivity for σ -2 over σ -1 sites (Fig. 1A; refs. 23, 24). At concentrations ranging from 1 to 50 $\mu\text{mol/L}$, siramesine displayed significantly stronger antiproliferative effects on WEHI-S murine fibrosarcoma and MCF-7 human breast cancer cells than haloperidol that binds both σ -1 and σ -2 sites with high

affinity (Fig. 1B). σ -1 Ligand (+)-pentazocine did not affect cell density even when applied at 100 $\mu\text{mol/L}$. To test whether siramesine-induced reduction in cell density was associated with cell death, we analyzed its ability to induce plasma membrane permeabilization. In WEHI-S cells, siramesine induced a time- and dose-dependent plasma membrane permeabilization as measured by the release of the cytoplasmic enzyme, LDH, into the media (Fig. 1C and D). The LDH release induced by 5 $\mu\text{mol/L}$ siramesine was apparent after 13 hours treatment and reached maximum at around 18 hours. In a 24-hour assay, siramesine-induced cytotoxicity increased in a linear fashion at concentrations ranging from 1 to 9 $\mu\text{mol/L}$. In fact, siramesine caused growth inhibition in WEHI-S cells at concentrations as low as 100 nmol/L (Fig. 1E). This effect depended, however, on a lower seeding density (6,000 cells/ cm^2) compared with that used in other experiments (24,000 cells/ cm^2). Contrary to siramesine, TNF-induced cytotoxicity was not significantly affected by the cell density (Fig. 1E). In addition to WEHI-S and MCF-7 cells, all other seven cell lines tested were sensitive to siramesine with IC_{50} values in a low micromolar range (Fig. 1F). In all cell lines, siramesine inhibited cell proliferation and upon continuous exposure induced cell death.

Figure 1. Siramesine induces time- and dose-dependent cell death in immortalized and transformed cells. **A**, structural formulas of siramesine, haloperidol, and (+)-pentazocine. **B**, WEHI-S (left) and MCF-7 (right) cells were plated at a density of 24,000 cells/ cm^2 24 hours before a 24-hour treatment with indicated concentrations of siramesine, haloperidol, or (+)-pentazocine. The viability of the cells was determined by the MTT reduction assay and expressed as absorbance at 550 nm. **C** and **D**, WEHI-S cells were plated as in (B) and treated with 5 $\mu\text{mol/L}$ siramesine for indicated times (C) or with indicated concentrations of siramesine for 24 hours (D). The cell death was determined by the LDH release assay and expressed as the percentage of the released LDH of the total cellular LDH. **E**, WEHI-S cells were plated at the indicated densities 24 hours before a 24-hour treatment with 100 nmol/L or 5 $\mu\text{mol/L}$ siramesine or 0.05 ng/mL TNF. The viability of the cells was determined by the MTT reduction assay and is expressed as a percentage of that in untreated cells. **F**, the indicated cell lines were plated at 24,000 cells/ cm^2 24 hours before treatment with 0.8 to 50 $\mu\text{mol/L}$ siramesine for 21 or 45 hours. The viability was determined by the MTT reduction assay and the IC_{50} [$\mu\text{mol/L}$] of siramesine was estimated by nonlinear regression using GraphPad Prism 4.0a. The average IC_{50} value is determined from the indicated number (n) of triplicate experiments. *Points* (B-D) and *columns* (E), averages of triplicate experiments; *bars*, SD. Data are representative of a minimum of three triplicate experiments.



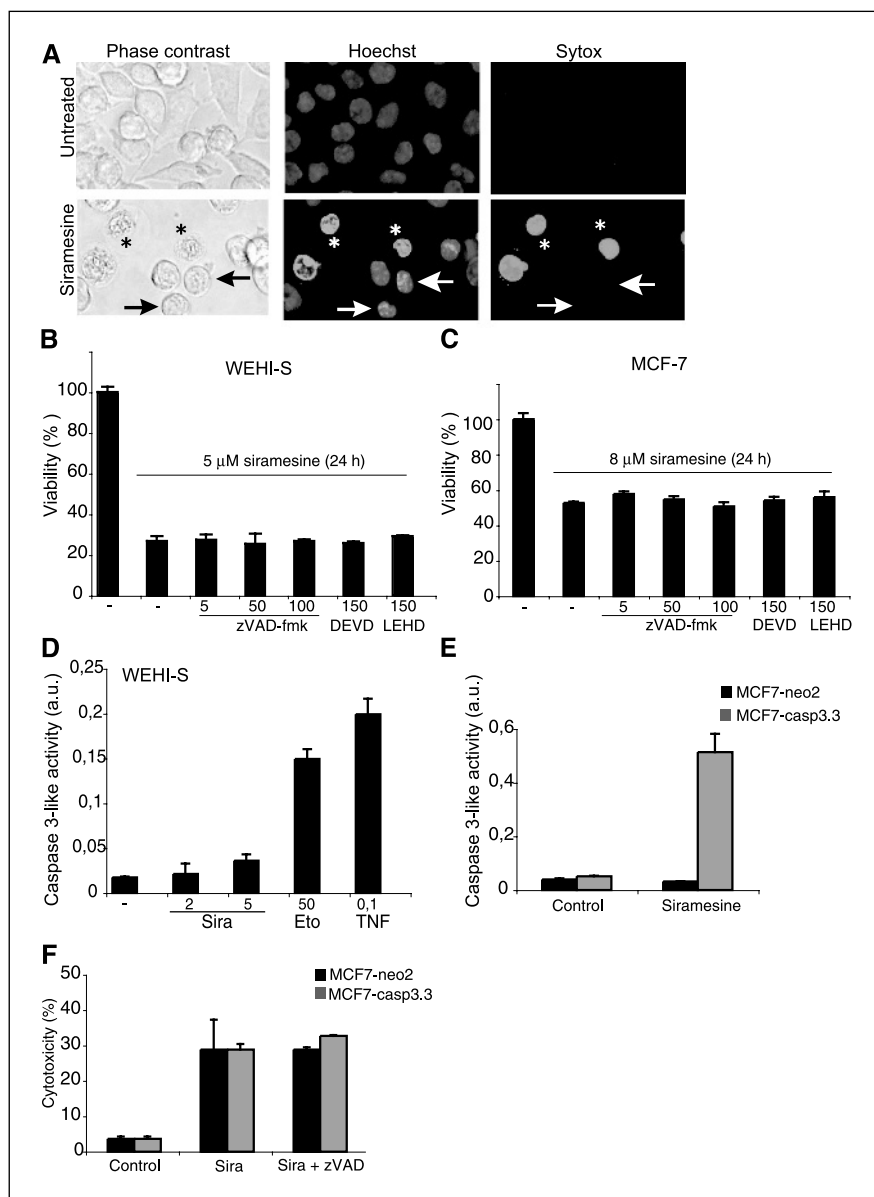


Figure 2. Siramesine induces caspase-independent apoptosis-like cell death. *A*, WEHI-S cells were left untreated or treated with 5 $\mu\text{mol/L}$ siramesine for 22 hours before staining the DNA with membrane permeable Hoechst-33342 and nonpermeable SYTOX-Green. *Arrows*, cells with condensed nuclei and intact plasma membrane; ***, cells with both nuclear condensation and loss of membrane integrity. WEHI-S (*B*) and MCF-7 (*C*) were left untreated or treated with indicated concentrations of siramesine for 24 hours. When indicated, the pan-caspase inhibitor zVAD-fmk (5, 50, or 100 $\mu\text{mol/L}$) or 150 $\mu\text{mol/L}$ specific inhibitors of caspase-3 and caspase-7 (DEVD-CHO) or caspase-9 (LEHD-CHO) were added 1 hour before siramesine. The viability of the cells was determined by the MTT reduction assay and is expressed as a percentage of untreated cells. *D*, WEHI-S cells were treated with 5 $\mu\text{mol/L}$ siramesine (*Sira*) for 22 hours and the effector caspase activity (*DEVDase*) in the cell lysates was analyzed. Measurements of WEHI-S cells treated with 50 $\mu\text{mol/L}$ etoposide (*Eto*) for 22 hours or 0.1 ng/mL TNF for 13 hours serve as positive controls. *E* and *F*, MCF-7 cells transfected with empty vector (MCF7-neo2) or caspase-3 (MCF7-casp3.3) were left untreated or treated with 8 $\mu\text{mol/L}$ siramesine for 24 hours. When indicated 10 $\mu\text{mol/L}$ zVAD-fmk was added 1 hour before siramesine. The effector caspase activity (*DEVDase*) was analyzed by fluorometric analysis of cell lysates (*E*) and the cytotoxicity by the LDH release assay (*F*). All experiments are representative of a minimum of three experiments. *B* to *F*, averages of triplicate experiments; *bars*, SD.

Siramesine triggers caspase-independent apoptosis-like cell death. Different modes of programmed cell death can be defined according to the cellular morphology (30). Necrosis-like programmed cell death is characterized by the swelling of the cell, early disruption of the plasma membrane, and lack of chromatin condensation, whereas hallmarks of apoptosis and apoptosis-like programmed cell death include blebbing of the plasma membrane, rounding and shrinkage of cells, as well as compact or loose condensation of chromatin before the plasma membrane disruption. WEHI-S cells treated with siramesine displayed plasma membrane blebbing and synchronous rounding and shrinkage of the cells followed by a loose condensation of chromatin (Fig. 2*A*). To examine whether the chromatin condensation occurred before plasma membrane disruption, DNA was stained with cell-permeable Hoechst-33342 and nonpermeable SYTOX-Green. The presence of condensed nuclei in cells that were negative for SYTOX-Green indicated that nuclear condensation occurred before the disruption of the plasma membrane (Fig. 2*A*, *arrows*). Nuclei that were both

condensed and SYTOX-Green positive are likely to represent cells in late stages of programmed cell death (Fig. 2*A*, *asterisks*). It should be noted that the condensed chromatin in siramesine-treated WEHI-S cells was clearly less compact than in TNF-treated cells (data not shown; ref. 29). Accordingly, TNF, but not siramesine, induced DNA laddering in WEHI-S cells (data not shown). Taken together, the morphologic changes induced by siramesine in WEHI-S cells were characteristic of apoptosis-like programmed cell death. Similar apoptosis-like morphologic changes were observed in siramesine-treated MCF-7 cells (data not shown).

We next investigated the role of caspases in siramesine-induced cell death. Pan-caspase inhibitor zVAD-fmk at concentrations up to 100 $\mu\text{mol/L}$ as well as inhibitors of caspase-3 (DEVD-CHO) and caspase-9 (LEHD-CHO) at 150 $\mu\text{mol/L}$ failed to confer any protection against siramesine-induced cell death in WEHI-S and MCF-7 cells as determined by the cellular morphology and the MTT reduction assay (Fig. 2*B* and *C*; data not shown). At the concentrations used, zVAD-fmk and DEVD-CHO fully attenuated

TNF-induced apoptosis in MCF-7 cells and LEHD-CHO decreased it significantly (data not shown). These data suggest that the known caspases are not involved in the cell death induced by siramesine. This finding was further supported by the absence of significant effector caspase activation in siramesine-treated WEHI-S and MCF-7 cells (Fig. 2D and E). The lack of measurable effector caspase activity in siramesine-treated MCF-7 cells could be due to the lack of functional caspase-3 in these cells (31). Therefore, we also measured the effector caspase activity in siramesine-treated MCF-7 cells expressing exogenous caspase-3 (MCF7-casp3.3). In these cells, siramesine triggered the activation of effector caspases (Fig. 2E), but this did not alter their sensitivity toward siramesine as determined by the LDH release assay (Fig. 2F). Furthermore, the abrogation of the activity of the exogenous caspase-3 by pretreatment of the cells with 10 $\mu\text{mol/L}$ zVAD-fmk failed to protect MCF7-casp3.3 cells against siramesine, indicating that the activation of caspase 3 is a secondary event, not crucial for the cell death (Fig. 2F).

Involvement of lysosomal cathepsins in siramesine-induced cell death. In several caspase-independent cell death models, cathepsins are released from lysosomes into the cytosol, where they participate in the execution of the cell (29, 32, 33). For this reason, we examined the localization of cysteine cathepsins B and L in siramesine-treated MCF-7 cells by immunocytochemistry. Contrary to untreated MCF-7 cells that displayed strong granular cathepsin B and L staining, siramesine-treated cells showed weaker and more diffuse staining pattern suggestive of lysosomal membrane permeabilization (Fig. 3A; data not shown). The weaker cathepsin L staining in siramesine-treated cells was not due to reduced levels of cathepsin L in these cells (Fig. 3B).

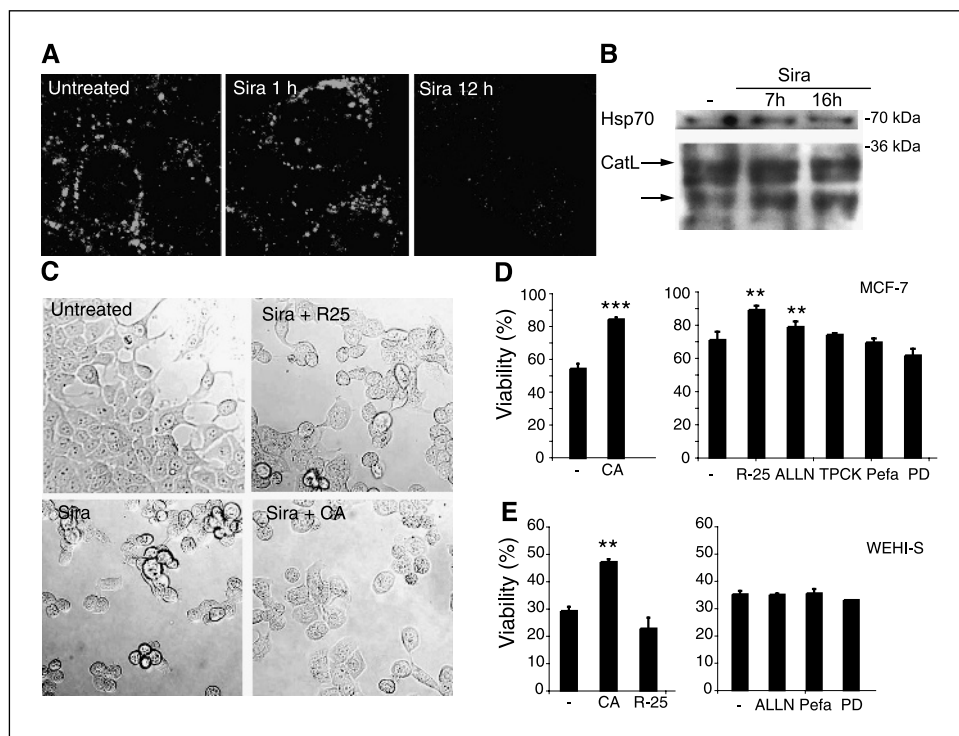
Using pharmacologic protease inhibitors, we next examined whether cysteine cathepsins participated in the execution of siramesine-treated cells. Cathepsin B inhibitors CA-074-Me and R-2525 resulted in a partial but significant protection against cell

death induced by siramesine in MCF-7 cells as analyzed by the phase-contrast microscopy and the MTT reduction assay (Fig. 3C and D). Also, ALLN, an inhibitor of calpains and cysteine cathepsins (29), increased the viability of siramesine-treated MCF-7 cells, whereas a more specific calpain inhibitor, PD150606, as well as serine protease inhibitors, TPCK and pefabloc, had no effect (Fig. 3D). Likewise, in WEHI-S cells, inhibition of cysteine cathepsins by CA-074-Me significantly attenuated the death induced by siramesine, whereas the other cysteine cathepsin inhibitors, as well as calpain and serine protease inhibitors, were without an effect (Fig. 3E).

Siramesine induces oxidative stress. To assess possible reactive oxygen species production upon siramesine treatment, we stained the cells with dihydroxyethidine that converts to highly fluorescent and DNA-binding ethidine upon oxidation. Accumulation of ethidine in WEHI-S cells treated with 5 $\mu\text{mol/L}$ siramesine was significant after 4 hours treatment (fold induction in mean fluorescence activity from channel 2H was 1.26 ± 0.07 ; $n = 4$; $P < 0.01$) and reached a maximum around 19 hours (Fig. 4A-C). To examine the role of oxidative stress in siramesine-induced cell death, WEHI-S and MCF-7 cells were pretreated with antioxidants. The lipid antioxidants α -tocopherol and γ -tocopherol (200 $\mu\text{g/mL}$), but not butylated hydroxyanisole (50-100 $\mu\text{mol/L}$), *N*-acetyl-cysteine (4-8 mmol/L), or glutathione ethyl ester (200-400 $\mu\text{mol/L}$), conferred significant protection against siramesine-induced morphologic changes and cell death in both cell lines (Fig. 4D-F; data not shown). At the concentrations used, α -tocopherol failed to confer any protection against TNF-induced apoptosis in either WEHI-S or MCF-7 cells, whereas butylated hydroxyanisole completely abrogated TNF-induced apoptosis in WEHI-S (data not shown).

To ensure that the protection observed by highly lipophilic tocopherols was not an artifact due to the formation of lipid micelles that could sequester hydrophobic siramesine and thereby inhibit its binding to the receptors, we studied the effect

Figure 3. Involvement of lysosomal cathepsins in siramesine-induced death. **A**, confocal images of MCF-7 left untreated or treated with 8 $\mu\text{mol/L}$ siramesine for 1 and 12 hours and stained with anticathepsin L. **B**, immunoblot analysis of cathepsin L (*CatL*) and heat shock protein 70 (*Hsp70*; loading control) in MCF-7 cells treated with 8 $\mu\text{mol/L}$ siramesine for 7 or 16 hours. **C** and **D**, MCF-7 cells were left untreated or treated with 8 $\mu\text{mol/L}$ siramesine for 24 hours. When indicated, 10 $\mu\text{mol/L}$ CA-074-Me (CA), 100 $\mu\text{mol/L}$ R-2525 (R25), 10 $\mu\text{mol/L}$ TPCK, 100 $\mu\text{mol/L}$ pefabloc (Pefa), 7.5 $\mu\text{mol/L}$ ALLN, or 300 $\mu\text{mol/L}$ PD150606 (PD) were added 1 hour before siramesine. The viability of the cells was determined by phase contrast microscopy (C) or MTT reduction assay (D). **E**, WEHI-S cells were left untreated or treated with 5 $\mu\text{mol/L}$ siramesine for 24 hours. When indicated, 20 $\mu\text{mol/L}$ CA-074-Me, 200 $\mu\text{mol/L}$ R-2525, 100 $\mu\text{mol/L}$ pefabloc, 7.5 $\mu\text{mol/L}$ ALLN, or 100 $\mu\text{mol/L}$ PD150606 were added 1 hour before siramesine. **D** and **E**, the viability is expressed as a percentage of cells treated with inhibitors alone. Columns, averages of triplicate experiments; bars, SD; *** $P < 0.001$, ** $P < 0.01$. All figures are representative of a minimum of three experiments.



of α -tocopherol on the binding of [3 H]siramesine to σ -2 sites in crude membrane fractions. Saturation-binding analysis revealed unaltered K_d and B_{max} values in the presence of α -tocopherol: $K_d = 0.32$ and 0.30 nmol/L and $B_{max} = 1,098$ and $1,216$ fmol/mg protein with and without α -tocopherol, respectively.

Siramesine-induced cells death is independent of p53 and cytochrome *c* release. The IC_{50} values for siramesine were comparable in breast cancer cell lines harboring wild-type (MCF-7) and mutated (MDA-MB-468) *p53* genes suggesting that the functional p53 protein is not crucial for siramesine-induced cell death (Fig. 1F). Furthermore, siramesine treatment did not lead to the stabilization of the p53 protein, a characteristic of p53-dependent death pathways (Fig. 5A), and the p53-inactivating SV40 large T antigen (SV40LT) failed to alter the sensitivity of NIH3T3 fibroblasts to siramesine (Fig. 6A and B).

During classic apoptosis, cytochrome *c* and other proapoptotic proteins are released from the mitochondrial intermembrane space into the cytosol. This step is effectively inhibited in many cancer cells by the antiapoptotic members of the Bcl-2 family. Siramesine treatment of MCF-7 cells, however, did not lead to the translocation of cytochrome *c* to the cytosol, suggesting that mitochondrial membrane permeabilization is not required for siramesine-induced cell death (Fig. 5B). This conclusion was further supported by the data showing that the expression of exogenous *Bcl-2* failed to protect cells against siramesine-induced cytotoxicity (Fig. 5C and D). It should be noted that the localization of cytochrome *c* did change upon siramesine treatment, as mitochondrial structures seemed to aggregate (Fig. 5B). Taken together, these data suggest that siramesine induces a caspase-independent apoptosis-like cell death that is not controlled by the common regulators of classic apoptosis signaling, such as p53, Bcl-2, and cytochrome *c*.

Oncogenic transformation sensitizes cells toward siramesine. To test whether oncogene-driven transformation would influence the cellular sensitivity toward siramesine, we analyzed the siramesine-induced death in NIH3T3 murine fibroblasts transformed by constitutively active *ras* (*v-Ha-ras*) or *src* (*c-src*^{Y527F})

oncogenes (28). As controls, we used NIH3T3 cells transduced with an empty pBabe-puro vector or with *SV40LT* (28). Remarkably, *v-Ha-ras* and *c-src* sensitized NIH3T3 cells toward siramesine as analyzed by the survival rate, the degree of morphologic changes, and the amount of condensed nuclei (Fig. 6A and B). The IC_{50} value for siramesine as determined by the MTT reduction assay in SV40LT-transduced cells (8.7 ± 0.5 ; $n = 3$) was not significantly different from that in vector-transduced control cells (9.4 ± 0.9 ; $n = 3$), but the IC_{50} values in *v-Ha-ras*- (7.3 ± 1 ; $n = 3$; $P < 0.01$) and *c-src*-transformed (6.4 ± 0.5 ; $n = 3$; $P < 0.001$) cells were significantly lower (Tukey's multiple comparison test, normalized data).

Cancer cells have been suggested to contain more σ -2 receptors than normal cells (4). Thus, we did saturation binding experiments using [3 H]siramesine and crude membrane fractions from vector-, *SV40LT*-, *v-Ha-ras*-, and *c-src*-transduced cells (Fig. 6C). Neither the K_d nor B_{max} values were significantly affected by *SV40LT*, *v-Ha-ras*, or *c-src* (one-way ANOVA analysis). Therefore, the transformation-dependent sensitization toward siramesine does not rely on the up-regulation of σ -2 sites but on as yet unknown oncogene-induced changes in siramesine signaling.

Antitumorigenic effect of siramesine *in vivo*. The cytotoxic potency of siramesine *in vitro* encouraged us to investigate its antitumorigenic potential *in vivo*. For this purpose, we chose WEHI-R4 cell line that is a siramesine-sensitive subclone of WEHI-164 fibrosarcoma cells capable of forming tumors in immunocompetent BALB/c mice (Fig. 1F; ref. 26), and MCF-7 breast cancer cells that form orthotopic estrogen-dependent adenocarcinomas in immunodeficient mice (34). Manifestation of s.c. WEHI-R4 tumors was observed in all untreated animals at day 3 after tumor inoculation and the initial tumor growth displayed a low variability between the animals. Remarkably, p.o. administration of siramesine at concentrations ranging from 25 to 100 mg/kg/d and initiated 2 days before the tumor inoculation resulted in a significant reduction in tumor volume (Fig. 6D). Furthermore, siramesine treatment (100 mg/kg/d) initiated simultaneously with the tumor inoculation inhibited tumor growth significantly in two additional experiments

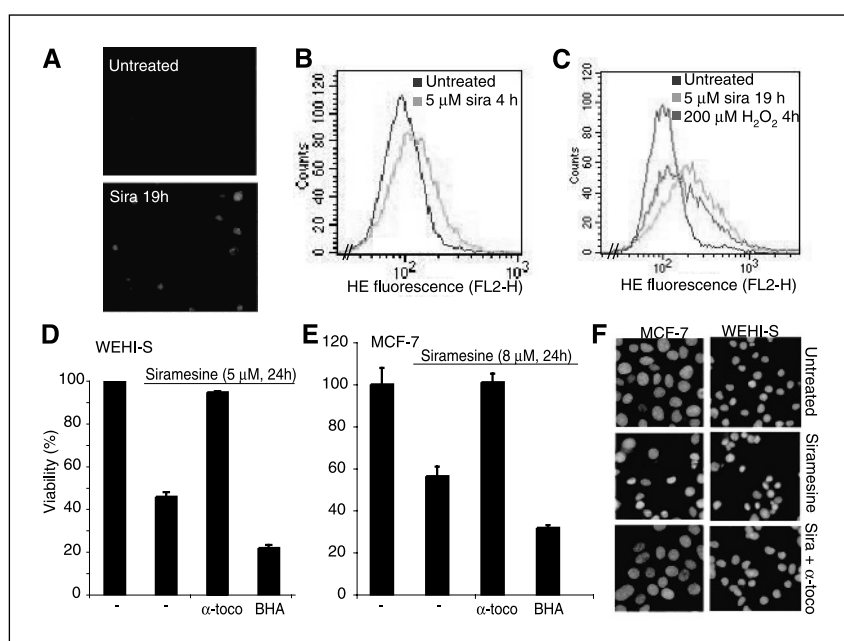


Figure 4. Siramesine induces oxidative stress. A to C, WEHI-S cells were left untreated or treated with 5 μ M/L siramesine or 200 μ M/L H_2O_2 (positive control) for the indicated times before staining with 2 μ M/L dihydroethidine and analysis by fluorescence microscopy (A) or flow cytometry (B and C). WEHI-S (D) and MCF-7 (E) were left untreated or treated with the indicated concentrations of siramesine for 24 hours. When indicated, 200 μ g/mL α -tocopherol (*α-toco*) or 100 μ M/L butylated hydroxyanisole were added 1 hour before siramesine. The viability of the cells was determined by the MTT reduction assay and is expressed as the percentage of antioxidant-treated cells. F, WEHI-S and MCF-7 cells were left untreated or treated with 5 and 8 μ M/L siramesine, respectively, before staining the DNA with Hoechst 33342. When indicated, 200 μ g/mL α -tocopherol was added to the cells 1 hour before siramesine. All experiments are representative of three experiments. Columns (D and E), averages of triplicate experiments; bars, SD.

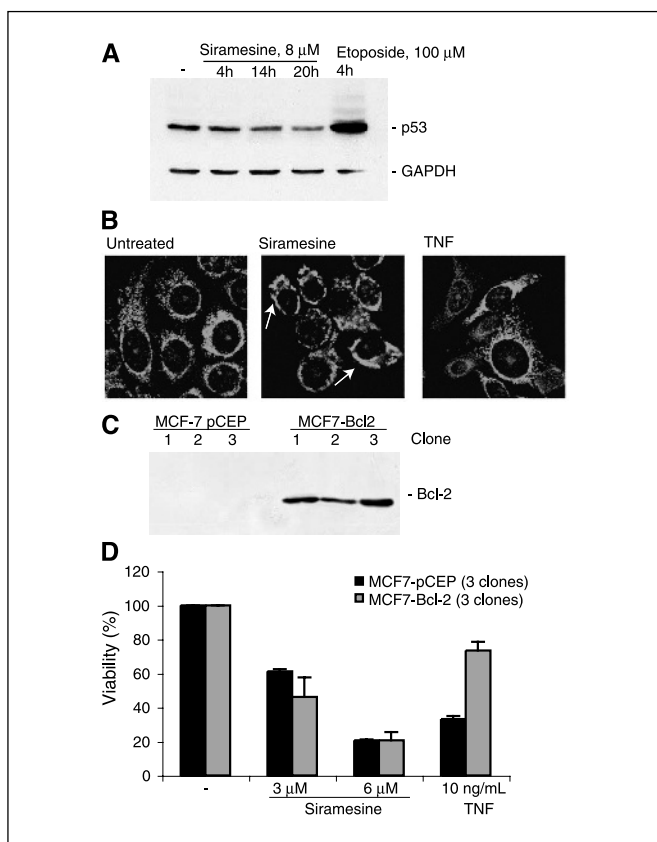


Figure 5. Siramesine does not induce p53 stabilization or cytochrome *c* release. *A*, p53 and GAPDH (loading control) protein levels in MCF-7 cells treated with 8 $\mu\text{mol/L}$ siramesine or 100 $\mu\text{mol/L}$ etoposide (positive control) for indicated times were analyzed by immunoblotting. *B*, confocal images of MCF-7 left untreated or treated with 8 $\mu\text{mol/L}$ siramesine for 18 hours and stained with anti-cytochrome *c*. Arrows, aggregation of mitochondrial structures in siramesine-treated cells. Cytochrome *c* release in similarly stained MCF-7 cells treated with 10 ng/mL TNF for 12 hours are shown as a positive control. *C* and *D*, three single cell clones of MCF-7 cells transfected with empty vector (MCF7-pCEP) or Bcl-2 (MCF7-Bcl2) were analyzed for the expression of Bcl-2 by Western blotting (*C*) and the sensitivity to indicated concentrations of siramesine and TNF (positive control) by a 45-hour MTT reduction assay (*D*). Columns, averages of three single cell clones analyzed in triplicate; bars, SD. Data are representative of four similar experiments.

(data not shown). When applied to mice with orthotopic MCF-7 breast cancer xenografts, siramesine (30 or 100 mg/kg/d) attenuated the tumor growth very potently even when administered after tumors had reached a diameter of 5 mm (Fig. 6*E* and *F*).

Discussion

The data presented above introduce siramesine, a novel selective σ -2 ligand, as an anticancer drug with great potential for the treatment of otherwise therapy-resistant cancers. This claim is supported by numerous experimental results: (*a*) all cancer cells tested, including those resistant to many other cancer drugs due to the expression of ectopic Bcl-2 or lack of functional p53 tumor suppressor protein, were susceptible to siramesine-induced caspase-independent cell death; (*b*) oncogenic transformation markedly sensitized cells to siramesine-induced cytotoxicity; (*c*) the cytotoxic potency of siramesine exceeded that of previously reported selective σ -2 ligands by inhibiting tumor cell growth at concentrations as low as 100 nmol/L; (*d*) p.o. administered siramesine significantly inhibited tumor growth in mice without

inducing any detectable side effects; and (*e*) last but not the least, siramesine has earlier been shown to be well tolerated by humans.

Siramesine induced an apoptosis-like cell death with chromatin condensation occurring before the disruption of the plasma membrane in WEHI-S and MCF-7 cells. In concordance with earlier data on other σ -2 ligands (3), siramesine-induced cell death was independent of the activation of known caspase cascades because siramesine failed to induce detectable effector caspase activation and the pharmacologic caspase inhibitors, zVAD-fmk, DEVD-CHO, and LEHD-CHO, could not block the cell death. Siramesine was, however, capable of activating caspase-3 as shown by the marked induction of DEVD-AFC cleavage in siramesine-treated MCF-7 cells expressing ectopic caspase-3. Surprisingly, the ectopic caspase-3 expression had no effect on the sensitivity of MCF-7 cells to siramesine. Thus, caspases are activated as epiphenomenon in the siramesine-induced death process, and the caspase-independent cell death pathway seems to be the prominent execution mechanism.

Oxidative stress is often involved in caspase-independent programmed cell death (35). Therefore, we examined whether siramesine treatment leads to the production of reactive oxygen species. Indeed, siramesine induced a significant increase in cellular reactive oxygen species levels several hours before morphologic signs of cell death. The lipophilic antioxidants α -tocopherol and γ -tocopherol effectively hindered both the morphologic changes and the cell death induced by siramesine, whereas hydrophilic antioxidants, such as butylated hydroxyanisole, *N*-acetyl-cysteine, and glutathione ethyl ester, failed to rescue the cells. These results are suggestive of siramesine-induced lipid peroxidation, because tocopherols, due to their lipophilic nature, are especially effective in protecting lipids from oxidation. It should, however, be noted that in addition to its function as an antioxidant, α -tocopherol has also been reported to inhibit protein kinase C and phospholipase A2, both possible mediators of cell death (36, 37).

Our data provide the first evidence for the involvement of lysosomes in σ -2 ligand-induced death. Siramesine triggered a marked release of lysosomal cathepsins into the cytosol. The release of lysosomal cathepsins into the cytosol preceded the morphologic changes and the cell death and exceeded by far that observed in TNF-treated MCF-7 and WEHI-S cells; death of these cells is known to depend on cysteine cathepsins (29). Furthermore, the siramesine-induced cytotoxicity in MCF-7 cells was partially blocked in the presence of pharmacologic cathepsin B inhibitors CA-074-Me and R-2525 and a broad-spectrum cysteine protease inhibitor ALLN. Also, in WEHI-S cells, CA-074-Me conferred partial protection, whereas other cathepsin inhibitors were without any effect. The incomplete protection observed suggests that other lysosomal hydrolases or lysosome-derived reactive oxygen species also contribute to the process. Alternatively, reactive oxygen species produced in other cellular compartments could, in parallel, induce lysosomal membrane destabilization and lysosome-independent lethal events in other cellular membranes. The induction of reactive oxygen species before the translocation of lysosomal cathepsins suggests at least a partial upstream role of oxidative stress in lysosomal membrane permeabilization. Further studies are currently undertaken to examine the role of reactive oxygen species in siramesine-induced lysosomal changes. It remains also unknown whether the induction of lysosomal leakage requires intracellular signal transduction through σ -2 receptors or whether siramesine itself can destabilize lysosomes.

Importantly, transformation of fibroblasts by oncogenic *ras* and *src* sensitized the cells dramatically to siramesine-induced death.

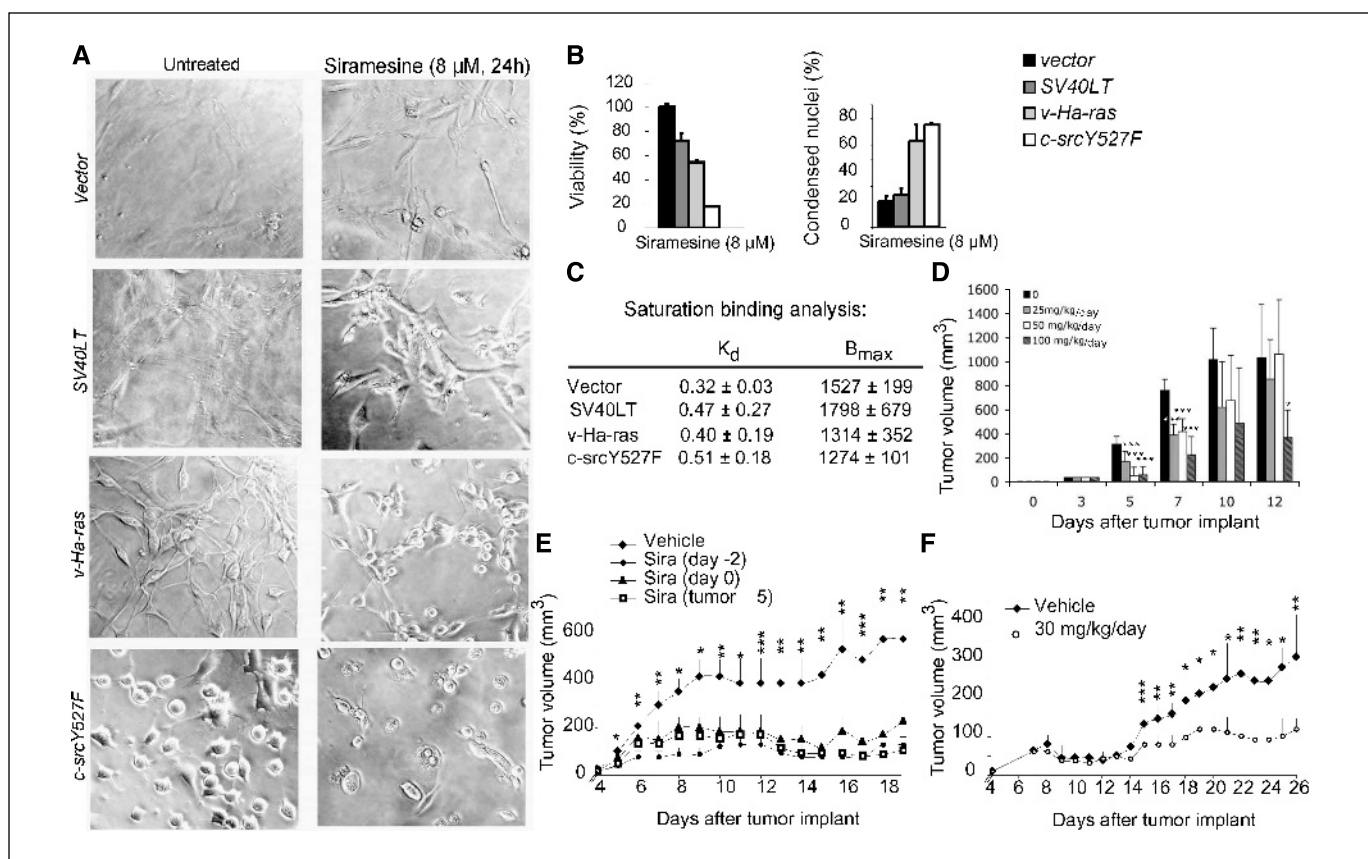


Figure 6. Siramesine displays increased efficacy against transformed murine fibroblasts *in vitro* and inhibits tumor xenograft growth *in vivo*. **A**, phase-contrast images of vector, SV40LT-, v-Ha-ras-, or c-src-transduced NIH 3T3 fibroblasts left untreated or treated with 8 μmol/L siramesine for 24 hours. **B**, the sensitivity of the same cells to 8 μmol/L siramesine as analyzed by a 24-hour MTT reduction assay (left) or counting of cells with apoptosis-like condensation of nuclei (right). Columns, averages of triplicate experiments; bars, SD. **C**, K_d (nmol/L) and the B_{max} (fmol/mg protein) of [³H]siramesine to crude membrane preparations of the same cells were determined as described in Materials and Methods. Data represent averages of three duplicate experiments. **D**, 5×10^6 WEHI-R4 fibrosarcoma cells were inoculated s.c. into the flank of immunocompetent BALB/c mice and the tumor size was measured at indicated days. Indicated daily p.o. doses of siramesine were initiated 2 days before tumor inoculation. **E** and **F**, 10×10^6 MCF7-S1 breast carcinoma cells were inoculated into the mammary fat pad of immunodeficient mice that were treated p.o. with siramesine at 100 mg/kg/d (**E**) or 30 mg/kg/d (**F**) starting either 2 days before (day 2) or simultaneously with (day 0) tumor inoculation or after tumor manifestation (diameter \geq 5 mm; **E**, tumor \geq 5 and **F**). Points, tumor volumes representing averages of five (**D** and **F**) or four (**E**) mice; bars, SD; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ indicate significant differences between vehicle- and siramesine-treated groups (**D-F**). **E**, P values are indicated only for the group treated after tumor manifestation.

Saturation binding analysis done on crude membrane fractions from transformed cells revealed unaltered σ -2 receptor density on cellular membranes. It is, however, possible that transformation-induced changes in σ receptor modality or localization are responsible for the increased susceptibility (5). Alternatively, the sensitization may rely on downstream signaling effects induced by oncogenic *ras* and *src*. *Ras* and *src* promote cell growth and motility and protect cells against classic caspase-mediated apoptosis, but sensitize cells to the TNF-induced lysosomal death pathway (28). Therefore, it is tempting to speculate that the observed sensitization toward siramesine is due to the oncogene-induced increase in the expression levels of cysteine cathepsins and/or extensive changes in lysosome trafficking (38). Accumulating evidence suggest that tumor cell lysosomes, indeed, are good targets for anticancer drugs. For example, a recent drug screening study revealed numerous lysosome-destabilizing drugs among compounds that induce p53-independent cell death (39). Moreover, a lysosomotropic agent isolated from the Hawaiian mollusc *Elysia rufescens* that is currently in phase II clinical trials has shown clinical benefits in patients with advanced and therapy resistant cancers of various origins (40).

Overexpression of antiapoptotic proteins of Bcl-2 family as well as mutations in p53 are among the most common mechanisms by which cancer cells escape spontaneous as well as therapy-induced cells death (20, 41, 42). Thus, development of anticancer agents that activate Bcl-2- and p53-independent death pathways is of great importance. Remarkably, the death pathway induced by siramesine could not be inhibited by the ectopic expression of Bcl-2 and did not depend on p53 protein. Whereas the protective role of Bcl-2 in σ receptor-induced death pathways has not been studied earlier, the independence of p53 seems specific for selective σ -2 ligands because haloperidol, a nonselective σ ligand, has been found to increase p53 expression (11).

It is a general and puzzling observation that the apoptosis-inducing potency of other σ -2 ligands is 100-1000 fold lower than predicted from the binding affinity determined on isolated cell membrane preparations. Instead, the potency seems largely to depend upon the log P value (hydrophobicity; ref. 43). Importantly, siramesine induced growth inhibition even at nanomolar concentrations. This could be due to the very hydrophobic nature of siramesine explaining the relative higher potency of this ligand compared with others. σ -2 Receptors are associated with lipid rafts,

possibly facing the cytosol (43). More efficient passage through the plasma membrane by highly hydrophobic ligands, such as siramesine, may thus explain the high potency of siramesine.

In conclusion, our data shows that siramesine is an efficient inducer of alternative apoptosis through the induction of oxidative stress and massive lysosomal permeabilization. The recent progresses in the research on tumor cell lysosomes seriously invite more effort to be invested into the detailed elucidation of lysosomal death pathways and putative anticancer drugs, like siramesine, that induce lysosomal leakage.

References

1. Guitart X, Codony X, Monroy X. Sigma receptors: biology and therapeutic potential. *Psychopharmacology (Berl)* 2004;174:301–19.
2. Brent PJ, Pang GT. Sigma binding site ligands inhibit cell proliferation in mammary and colon carcinoma cell lines and melanoma cells in culture. *Eur J Pharmacol* 1995; 278:151–60.
3. Crawford KW, Bowen WD. Sigma-2 receptor agonists activate a novel apoptotic pathway and potentiate antineoplastic drugs in breast tumor cell lines. *Cancer Res* 2002;62:313–22.
4. Vilner BJ, John CS, Bowen WD. σ -1 and σ -2 receptors are expressed in a wide variety of human and rodent tumor cell lines. *Cancer Res* 1995;55:408–13.
5. Spruce BA, Campbell LA, McTavish N, et al. Small molecule antagonists of the σ -1 receptor cause selective release of the death program in tumor and self-reliant cells and inhibit tumor growth *in vitro* and *in vivo*. *Cancer Res* 2004;64:4875–86.
6. Al-Nabulsi I, Mach RH, Wang LM, et al. Effect of ploidy, recruitment, environmental factors, and tamoxifen treatment on the expression of σ -2 receptors in proliferating and quiescent tumour cells. *Br J Cancer* 1999; 81:925–33.
7. Quirion R, Bowen WD, Itzhak Y, et al. A proposal for the classification of σ binding sites. *Trends Pharmacol Sci* 1992;13:85–6.
8. Hellewell SB, Bruce A, Feinstein G, Orringer J, Williams W, Bowen WD. Rat liver and kidney contain high densities of σ 1 and σ 2 receptors: characterization by ligand binding and photoaffinity labeling. *Eur J Pharmacol* 1994;268:9–18.
9. Kekuda R, Prasad PD, Fei YJ, Leibach FH, Ganapathy V. Cloning and functional expression of the human type 1 σ receptor (h σ R1). *Biochem Biophys Res Commun* 1996;229:553–8.
10. Vilner BJ, Bowen WD. Sigma receptor-active neuroleptics are cytotoxic to C6 glioma cells in culture. *Eur J Pharmacol* 1993;244:199–201.
11. Post A, Rucker M, Ohl F, et al. Mechanisms underlying the protective potential of α -tocopherol (vitamin E) against haloperidol-associated neurotoxicity. *Neuropsychopharmacology* 2002;26:397–407.
12. Sagara Y. Induction of reactive oxygen species in neurons by haloperidol. *J Neurochem* 1998;71:1002–12.
13. Behl C, Rupprecht R, Skutella T, Holsboer F. Haloperidol-induced cell death-mechanism and protection with vitamin E *in vitro*. *Neuroreport* 1995;7:360–4.
14. Vilner BJ, Bowen WD. Modulation of cellular calcium by σ -2 receptors: release from intracellular stores in

- human SK-N-SH neuroblastoma cells. *J Pharmacol Exp Ther* 2000;292:900–11.
15. Aydar E, Palmer CP, Djamgoz MB. Sigma receptors and cancer: possible involvement of ion channels. *Cancer Res* 2004;64:5029–35.
16. Renaudo A, Watry V, Chassot AA, Ponzio G, Ehrenfeld J, Soriani O. Inhibition of tumor cell proliferation by σ ligands is associated with K⁺ channel inhibition and p27kip1 accumulation. *J Pharmacol Exp Ther* 2004;311: 1105–14.
17. Crawford KW, Coop A, Bowen WD. σ (2) Receptors regulate changes in sphingolipid levels in breast tumor cells. *Eur J Pharmacol* 2002;443:207–9.
18. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70.
19. Pommier Y, Sordet O, Antony S, Hayward RL, Kohn KW. Apoptosis defects and chemotherapy resistance: molecular interaction maps and networks. *Oncogene* 2004;23:2934–49.
20. Jäättelä M. Multiple cell death pathways as regulators of tumour initiation and progression. *Oncogene* 2004;23: 2746–56.
21. Banerjee R, Tyagi P, Li S, Huang L. Anisamide-targeted stealth liposomes: a potent carrier for targeting doxorubicin to human prostate cancer cells. *Int J Cancer* 2004;112:693–700.
22. Everaert H, Flamen P, Franken PR, Verhaeghe W, Bossuyt A. σ -receptor imaging by means of I123-IDAB scintigraphy: clinical application in melanoma and non-small cell lung cancer. *Anticancer Res* 1997;17: 1577–82.
23. Perregaard J, Moltzen EK, Meier E, Sanchez C. σ ligands with subnanomolar affinity and preference for the σ 2 binding site. I. 3-(ω -aminoalkyl)-1H-indoles. *J Med Chem* 1995;38:1998–2008.
24. Söby KK, Mikkelsen JD, Meier E, Thomsen C. Lu 28-179 labels a σ (2)-site in rat and human brain. *Neuropharmacology* 2002;43:95–100.
25. Heading C. Siramesine H Lundbeck. *Curr Opin Investig Drugs* 2001;2:266–70.
26. Jäättelä M. Over-expression of hsp70 confers tumorigenicity to mouse fibrosarcoma cells. *Int J Cancer* 1995; 60:689–93.
27. Jäättelä M, Benedict M, Tewari M, Shayman JA, Dixit VM. Bcl-x and Bcl-2 inhibit TNF and Fas-induced apoptosis and activation of phospholipase A2 in breast carcinoma cells. *Oncogene* 1995;10:2297–305.
28. Fehrenbacher N, Gyrd-Hansen M, Poulsen B, et al. Sensitization to the lysosomal cell death pathway upon immortalization and transformation. *Cancer Res* 2004; 64:5301–10.

Acknowledgments

Received 1/26/2005; revised 6/8/2005; accepted 7/26/2005.

Grant support: Danish Cancer Society (M. Jäättelä), Danish Medical Research Council (M. Jäättelä), Association for International Cancer Research (M. Jäättelä), Meyer Foundation (M. Jäättelä), and the Novo Foundation (T. Farkas).

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.

We thank Ingrid Fossar Larsen and Charlotte Hundahl for the excellent technical assistance; Jiri Bartek, Anthony Cerami, Vishva Dixit, Mads Gyrd-Hansen, Boris Margulis, and Bob Ryzewski for valuable research tools; and Marcel Leist for general advice and stimulating discussions.

29. Foghsgaard L, Wissing D, Mauch D, et al. Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. *J Cell Biol* 2001;153:999–1010.
30. Leist M, Jäättelä M. Four deaths and a funeral: from caspases to alternative mechanisms. *Nat Rev Mol Cell Biol* 2001;2:89–98.
31. Jänicke RU, Sprengart ML, Wati MR, Porter AG. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J Biol Chem* 1998;273:9357–60.
32. Kågedal K, Zhao M, Svensson I, Brunk UT. Sphingosine-induced apoptosis is dependent on lysosomal proteases. *Biochem J* 2001;359:335–43.
33. Nylandsted J, Gyrd-Hansen M, Danielewicz A, et al. Heat shock protein 70 promotes cell survival by inhibiting lysosomal membrane permeabilization. *J Exp Med* 2004;200:425–35.
34. Karpanen T, Egeblad M, Karkkainen MJ, et al. Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res* 2001;61:1786–90.
35. Fiers W, Beyaert R, Declercq W, Vandenabeele P. More than one way to die: apoptosis, necrosis and reactive oxygen damage. *Oncogene* 1999;18:7719–30.
36. Azzi A, Ricciarelli R, Zingg JM. Non-antioxidant molecular functions of α -tocopherol (vitamin E). *FEBS Lett* 2002;519:8–10.
37. Mukherjee AK, Ghosal SK, Maity CR. Lysosomal membrane stabilization by α -tocopherol against the damaging action of *Vipera russelli* venom phospholipase A2. *Cell Mol Life Sci* 1997;53:152–5.
38. Roshly S, Sloane BF, Moin K. Pericellular cathepsin B and malignant progression. *Cancer Metastasis Rev* 2003; 22:271–86.
39. Erdal H, Berndtsson M, Castro J, Brunk U, Shoshan MC, Linder S. Induction of lysosomal membrane permeabilization by compounds that activate p53-independent apoptosis. *Proc Natl Acad Sci U S A* 2005; 102:192–7.
40. Jimeno JM. A clinical armamentarium of marine-derived anti-cancer compounds. *Anticancer Drugs* 2002; 13 Suppl 1:S15–9.
41. Vousden KH, Lu X. Live or let die: the cell's response to p53. *Nat Rev Cancer* 2002;2:594–604.
42. Cory S, Huang DC, Adams JM. The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene* 2003;22: 8590–607.
43. Gebreselassie D, Bowen WD. σ -2 receptors are specifically localized to lipid rafts in rat liver membranes. *Eur J Pharmacol* 2004;493:19–28.