Vincristine Induces Dramatic Lysosomal Changes and Sensitizes Cancer Cells to Lysosome-Destabilizing Siramesine

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Abstract

Vincristine is a microtubule-destabilizing antimitotic drug that has been used in cancer therapy for over 40 years. However, the knowledge on vincristine-induced cell death pathways is still sparse. Here, we show that vincristine induces dramatic changes in the lysosomal compartment and sensitizes cells to lysosomal membrane permeabilization. In HeLa cervix carcinoma cells, vincristine induced mitotic arrest and massive cell death associated with an early increase in the lysosomal volume and lysosomal leakage followed by the activation of the intrinsic apoptosis program. In contrast, the majority of vincristine-treated MCF-7 breast carcinoma cells resisted apoptosis. Instead, they adapted to the spindle assembly checkpoint and escaped the mitotic arrest as micronucleated and senescent cells with an increase in the volume and the activity of their lysosomal compartment. Consistent with its substantial effects on the lysosomes, vincristine greatly sensitized cultured cancer cells as well as orthotopic breast cancer xenografts in mice to the cytotoxicity induced by siramesine, a sigma-2 receptor ligand that kills cancer cells by destabilizing their lysosomes. Importantly, the combination of nontoxic concentrations of vincristine and siramesine resulted in massive cell death even in MCF-7 cells that were capable of escaping vincristine-induced spindle assembly checkpoint and cell death. Similar synergism was observed when siramesine was combined with a semisynthetic vincristine analogue, vinorelbine, or with microtubule-stabilizing paclitaxel. These data strongly suggest that combination therapies consisting of microtubule-disturbing and lysosome-destabilizing drugs may prove useful in the treatment of otherwise therapy-resistant human cancers. [Cancer Res 2007;67(5):2217–25]

Introduction

The cytoskeleton of eukaryotic cells is composed of three major protein families, intermediary filaments, actin filaments, and microtubules that form filamentous structures throughout the cell. The dynamic microtubule cytoskeleton consists of α- and β-tubulin dimers (1). It is responsible for the transport and positioning of intracellular organelles and the separation of chromatids during the anaphase of mitosis (2, 3). If the dynamicity of the microtubule network is compromised, cell division is inhibited by the spindle assembly checkpoint (4, 5). Thus, it is not surprising that microtubule-targeting compounds that either destabilize (e.g., Vinca alkaloids) or stabilize (e.g., taxanes) the microtubule network are among the most effective anticancer drugs that are commonly used in the treatment of leukemias and lymphomas, as well as in the combination therapies of several nonhematopoetic cancers (6, 7).

Vincristine, a naturally occurring Vinca alkaloid, destabilizes microtubules by binding to the Vinca domain in the β-tubulin subunit (8). Low concentrations of vincristine deprive the dynamicity of microtubules, whereas high concentrations lead to a complete disassembly of the microtubule network (9). Consequently, no mitotic spindle can be formed, and cells are arrested in mitosis by spindle assembly checkpoint (4, 5). Peripheral neuropathy is the main dose-limiting side effect of vincristine. It arises due to the breakdown of transport lines in axons followed by their degeneration (10). As an attempt to develop chemotherapy with fewer side effects, several semisynthetic Vinca alkaloids with varying affinities to tubulin isomers have been developed. One such compound is vinorelbine that binds axonal microtubules with a weaker affinity than vincristine and therefore displays less neurotoxicity (6). Another strategy to avoid the neurotoxicity is to lower the dose of vincristine and combine it with other drugs. Due to the limited knowledge of the cell death pathways initiated by vincristine, the combination therapies used today may, however, be far from optimal.

Continuous treatment with microtubule poisons leads either to an execution of a cell death pathway directly from the mitosis or to an escape from the mitotic arrest followed by cell division, senescence, or delayed cell death (4, 5). The mechanisms coupling mitotic arrest to subsequent death remains almost completely unexplored. Because the microtubule cytoskeleton has an important role in the intracellular trafficking of organelles (e.g., endosomes, lysosomes, and autophagosomes), interference with its structure and dynamicity might also have lethal consequences apart from those induced by the mitotic arrest. This hypothesis is supported by the data showing that mitosis is not a prerequisite for the cytotoxicity of the microtubule-destabilizing drugs as shown by the above-mentioned vincristine-induced toxicity in postmitotic neurons in vivo (6). Moreover, another microtubule-destabilizing drug, colchicine, induces cell death in cultured postmitotic cerebellar granule cells. In this case, the cell death is initiated by the depolymerization of the microtubule cytoskeleton followed by both caspase-dependent and caspase-independent cell death pathways (11). Thus, it is interesting to note that diminished microtubule dynamicity induced by microtubule-stabilizing drugs induces leakage of lysosomal protease cathepsins into the cytosol and cathepsin-mediated cell death in non–small cell lung cancer cells (12). These data suggest that lysosomal integrity relies on the undisturbed microtubule network, and the question arises whether
the lysosomal destabilization could be a more common consequence of microtubule-targeting drugs.

Apoptosis is the best defined cell death program countering tumor growth. It is characterized by the activation of a family of cysteine proteases called the caspases that trigger apoptosis-associated morphologic changes such as the shrinkage of the cell, the condensation of the chromatin, and the disintegration of the cell into small fragments (13, 14). Most apoptosis pathways are initiated by the up-regulation or posttranslational modification of BH3-only proteins of the Bcl-2 family. They then activate proapoptotic Bcl-2 family proteins Bax and Bak that induce the mitochondrial outer membrane permeabilization and the release of cytochrome c into the cytosol. Cytosolic cytochrome c facilitates the assembly of the apoptosome, a multiprotein complex that consists of Apaf-1, caspase-9, and cytochrome c, and serves as a scaffold to activate caspase-9, which then activates the so-called effector caspases (caspase-3 and caspase-7). Cancer cells frequently harbor acquired mutations that allow them to escape spontaneous and therapy-induced apoptosis (15, 16). For example, the up-regulation of antiapoptotic Bcl-2 family members and mutations in the p53 tumor suppressor protein are common in human tumors. Apoptosis-resistant cancer cells are, however, not completely resistant to cell death, but can die via alternative cell death pathways often involving non-caspase proteases such as lysosomal cathepsins (17). Interestingly, transformation and tumor environment enhance the expression of lysosomal cysteine cathepsins and increase their microtubule-dependent secretion into the extracellular space (18–20). These changes lead to the cathepsin-mediated increase in angiogenesis and metastatic capacity, but also to an increased susceptibility to the lysosomal membrane permeabilization. The cathepsins released to the cytosol upon lysosomal membrane permeabilization can initiate the intrinsic apoptosis pathway possibly via a cleavage-mediated activation of proapoptotic Bcl-2 family members Bid and Bax (21, 22). Important, cytosolic cathepsins can also trigger caspase-independent and Bcl-2–insensitive apoptosis-like cell death pathways in apoptosis-resistant cells (17). For example, siraimesine, a sigma-2 receptor agonist that is presently being developed as an anticancer drug, destabilizes lysosomes and activates a caspase-independent cell death that is insensitive to the antiapoptotic effects of Bcl-2 (23). Siraimesine is a highly lipophilic weak base (pKₐ ~ 9), and it contains a protonable nitrogen suggesting that its anticancer function may be due to its chemical nature as a lysosomotropic detergent rather than binding to sigma-2 receptors (24).

Despite the long history of vincristine in cancer therapy, only minor attempts have been made to clarify the intracellular death pathway(s) it activates or the mechanisms by which some cancer cells resist its toxicity. The requirement of intact microtubule network for proper lysosomal function inspired us to study the effect of vincristine on the lysosomal compartment in vincristine-sensitive HeLa cervix carcinoma cells and relatively vincristine-resistant MCF-7 breast cancer cells. Prompted by our data showing that vincristine induced dramatic lysosomal changes in both cell lines, we next studied the ability of vincristine and other microtubule-targeting drugs to sensitize cells to drugs known to induce lysosomal membrane permeabilization. Our data showing the potent anticancer effects of the combination of microtubule-disturbing drugs with siraimesine both in vitro and in vivo open new exciting possibilities for cancer combination therapies.

Materials and Methods

Cell culture and treatment. Hela (human cervix carcinoma) cells were kindly provided by J. Bartek (Danish Cancer Society, Copenhagen, Denmark). MCF-7 cell line used in this study is a subclone (MCF-7-S1) of human ductal breast carcinoma cells originally selected for high sensitivity to tumor necrosis factor (TNF; ref. 25). MCF-neo, MCF-vector, MCF-casp3, MCF-Beclin-2, and MCF-Beclin-15 cells are single-cell clones of MCF-7 cells transfected with an empty vector or plasmids encoding for human caspase-3 (pcDNA-casp3) or FLAG-Beclin 1 (pCR3.1-FLAG-Beclin 1: kindly provided by Beth Levine, Columbia University, New York, NY), respectively (23, 26, 27). LC3-eGFP cells were created by transfecting MCF-7 cells with pEGFP-C1 plasmid encoding for a fusion protein consisting of rat LC3 and eGFP (kindly provided by G. Kroemer, CNRS-UMR8125, Villejuif, France) by electroporation followed by single-cell cloning by limiting dilution. The cells were cultured in RPMI 1640 with Glutamax (Life technologies, Ltd., Paisley, United Kingdom) supplemented with 6% heat-inactivated FCS (biological industries, Kib. Beit, Haemek, Israel), 100 units/mL penicillin, and 100 mg/mL streptomycin, and in the case of transfected cells also with 400 µg/mL geneticin sulfate (G-418; Invitrogen, Carlsbad, CA). The cells were maintained in a humidified atmosphere at 37°C, 5% CO₂ and regularly tested and found negative for Mycoplasma.

Siraimesine was kindly provided by Christian Thomsen (H. Lundbeck A/S, Valby, Denmark) and recombinant human TNF was provided by Anthony Cerami (Kenneth Warren Laboratories, Tarrytown, NY). Vincristine, cisplatin, paclitaxel, and etoposide were purchased from Sigma-Aldrich (St. Louis, MO), z-Val-Ala-Asp-fmk (zVAD-fmk) was from Bachem (Switzerland), N-acetyl-Leu-Leu-Nle-aldehyde (ALLN) was from Calbiochem (San Diego, CA) and N-tosyl-l-phenylalanine chloromethyl ketone was from Boehringer Mannheim (Mannheim, Germany).

Viability and cell death assays. The viability of subconfluent cells were analyzed by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described previously (25). Apoptotic cells were determined in an Olympus IX microscope with the UV channel by counting apoptotic cells. The cells were treated as indicated in 24- or 96-well plates and was removed, and 100 µL (96-well) or 200 µL (24-well) extraction buffer [250 mmol/L sucrose, 20 mmol/L HEPES, 10 mmol/L KCI, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L EGTA, and 1 mmol/L Pefabloc SC (pH 7.5)] containing 30 to 40 µg/mL (cytoplasmic fraction) or 300 µg/mL (total cellular fraction) digitonin was added, and plates were kept on ice with gentle shaking for 15 min. The enzyme activities of the samples were determined as described previously using zFR-aminotri fluoromethylcoumarin (AFc) and DEVD-ACF (Enzyme System Products, Livermore, CA) as substrates for cysteine cathepsins and caspase-3–like caspases, respectively, and Spectramax Gemini fluorometer (Molecular Devices, Sunnyvale, CA) for the measurement of the Vₘₐₙ of the liberation of the antibody-forming cell fluorescent (28). All protease activities were normalized to the lactate dehydrogenase activity analyzed by cytoxicity detection kit (Roche) of the same sample.

Immunocytochemistry. Cells plated on glass coverslips or centrifuged (600 × g for 5 min) on glass slides were washed and fixed in 4% formaldehyde in PBS for 20 min at 25°C or in ice-cold methanol/aceton (1:1) for 10 min. Samples were permeabilized 0.2% Triton X-100 in PBS for 2 min and blocked with 20% FCS in PBS for 30 min. Antibodies used included human anti-α-tubulin (Molecular Probes), anti-human cathepsin L (Transduction Laboratories, Lexington, KY), human anti-lamin B (Santa Cruz Biotechnol-ogy, Santa Cruz, CA), anti-Bax (active conformation; Cell Signaling, Danvers,

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1 M.S. Ostenfeld and M. Jäättelä, unpublished data.
MA), human anti–LAMP-2 (Southern Biotechnology Associates, Birmingham, AL) and the appropriate Alexa-488– and Alexa-594–coupled secondary antibodies (Molecular Probes). Filamentous actin (F-actin) was visualized by Alexa-594–phalloidin (Molecular Probes). The samples were mounted with antifade gold kit (Molecular Probes), and fluorescence images were taken with Zeiss 510 laser-scanning microscope with Axiovert 100M.

**Analysis of DNA content.** Subconfluent cells were trypsinized, washed, resuspended in 300 μL PBS + 3% FCS, and fixed by adding 800 μL ice-cold methanol. After 30 min at 4°C, cells were washed in PBS + 3% FCS, suspended in 200 μL staining solution (50 μg/mL propidium iodide, 5 mmol/L MgCl₂, and 10 μg/mL DNase-free RNase), incubated for 30 min at 37°C, and analyzed by flow cytometry (FACS Calibur; Becton Dickinson, Mountain View, CA).

**Analysis of cytochrome c release.** Cell membranes were permeabilized by extraction buffer [250 mmol/L sucrose, 20 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L EGTA, and 1 mmol/L Pefabloc SC (pH 7.5)] containing 150 μg/mL digitonin for 15 min. Then cells were permeabilized in 0.2% Triton X-100 in PBS and fixed in 4% formaldehyde in PBS for 10 min at 25°C. Samples were blocked with 20% FCS in PBS for 30 min and stained with anti–cytochrome c and Alexa-594–coupled secondary antibody (both from Molecular Probes) in suspension and analyzed by flow cytometry (FACS Calibur; Becton Dickinson).

**Total volume of the acidic compartment.** Cells plated in 24-well plates (50,000 cells per well) and treated as indicated were incubated with 50 nmol/L LysoTracker Red DND-99 (Molecular Probes) for 20 min in 37°C. Thereafter,
the cells were trypsinized, washed twice in PBS + 3% FCS, resuspended in 400 μL PBS + 3% FCS, and analyzed by flow cytometry (FACSCalibur). Tumor xenografts. MCF-7 cells (1.0 x 10⁷ in 100 μL PBS) were inoculated into the second axillary mammary fat pad of female FOX CHASE severe combined immunodeficiency mice treated with 0.670 μg/mL estrone (Sigma) in drinking water. The weekly i.v. treatment with 100 μL vehicle (0.9% NaCl solution) alone or with indicated concentration of vincristine and biweekly p.o. treatment with 200 μL vehicle (0.5% methylcellulose 15 in 0.9% NaCl solution) alone or with indicated concentrations of vincristine, etoposide (Eto), or paclitaxel (Pacl) for 36 h (top left), 72 h (top right), or for indicated times (bottom) were analyzed for the total cellular (top) and cytosolic (bottom) cysteine cathepsin (ZFRase) activities. Columns, mean from three independent triplicate experiments; bars, SD. *, P < 0.05; **, P < 0.01, as compared with untreated control cells.

Results

Vincristine induces apoptosis-like cell death in HeLa cells and senescent-like morphology in MCF-7 cells. To elucidate the mechanisms underlying vincristine-induced cytotoxicity, we compared the cellular responses of relatively vincristine-sensitive HeLa cervix carcinoma and resistant MCF-7 breast carcinoma cell lines. Vincristine reduced the density of HeLa cells at 25 nmol/L and higher concentrations (IC₅₀ = 50 nmol/L) and that of MCF-7 cells at concentrations over 100 nmol/L (IC₅₀ = 170 nmol/L; Fig. 1A). To induce approximately similar antiproliferative effects in the two cell lines, we then treated the HeLa cells with 100 nmol/L vincristine for 24 h and the MCF-7 cells with 300 nmol/L vincristine for 48 h. As expected, vincristine destabilized the tubulin network, but not the actin network, in both cell types and arrested them in the G2-M phase of the cell cycle (Fig. 1B and C). Interestingly, only HeLa cells rounded up, detached, and presented with apoptosis-like nuclear condensation, whereas most MCF-7 cells reattached after the failed mitosis and remained viable with a flat senescent-like morphology and massive micronucleation (Fig. 1B and D and data not shown).

Vincristine induces lysosomal changes in HeLa and MCF-7 cells. Because lysosomes and autophagosomes move along the tubulin network (29–31), we next studied the effect of vincristine on lysosome localization, volume, activity, and stability, as well as on
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We next studied the ability of vincristine to trigger the intrinsic apoptosis pathway by staining for the active form of the proapoptotic Bcl-2 family member Bax and cytochrome c and by measuring the effector caspase activity in the cell lysates. Vincristine-activated Bax induced cytochrome c release from the mitochondria to the cytosol and activated effector caspases in HeLa cells (Fig. 3A–D). The number of the cells with activated Bax and diffuse cytochrome c staining was, however, lower than expected from the number of dying cells, and the activation of effector caspases was only marginal as compared to etoposide-treated cells. Whereas 37% of HeLa cells treated with 100 nmol/L vincristine for 24 h had condensed chromatin indicative of apoptosis-like cell death (Fig. 1D), the same treatment failed to activate effector caspases and induced Bax activation and cytochrome c release only in 2.2% and 19.9% of the cells, respectively. Caspase activation became evident at the later time points, suggesting that it is rather a consequence than a cause of the vincristine-induced cell death. Furthermore, the costaining for cathepsin L and activated Bax revealed that lysosomal leakage occurred before the Bax activation. None of the vincristine-treated cells had activated Bax and vesicular cathepsin L staining, whereas 9.2% and 19.6% of the cells showed diffuse cathepsin L staining while devoid of active Bax at 24 and 48 h, respectively (Fig. 3B).

MCF-7 cells do not express functional caspase-3 due to a 47-base deletion within exon 3 of the CASP-3 gene (35). Thus, we tested whether the lack of caspase-3 could explain the resistance of MCF-7 cells to vincristine-induced apoptosis-like cell death. This was, however, not the case because vincristine failed to activate the intrinsic apoptosis pathway already upstream of caspase activation as judged by the lack of activated Bax and diffuse cytochrome c (Fig. 4A). Furthermore, ectopic caspase-3 failed to sensitize MCF-7 cells to vincristine, whereas it slightly increased the sensitivity of the cells to TNF (Fig. 4B), which kills MCF-7 cells in a caspasedependent and Bcl-2–sensitive manner (25). MCF-7 cells also harbor a monoallelic loss of the autophagy-associated tumor

Figure 3. Vincristine induces the intrinsic apoptosis pathway in HeLa cells. A, representative confocal images of HeLa cells left untreated or treated with 100 nmol/L vincristine or 150 μmol/L etoposide for 48 h were stained with antibodies to cathepsin L and the active conformation of Bax and visualized by confocal microscopy. Bar, 20 μm. B, HeLa cells were left untreated or treated with 100 nmol/L vincristine for 24 or 48 h and stained as in (A). One hundred randomly chosen cells were analyzed for vesicular versus diffuse cathepsin L staining and absence versus presence of active Bax. Data are representative of a minimum of three independent experiments. C and D, HeLa cells were treated as indicated with vincristine, etoposide, or paclitaxel and analyzed for cytochrome c release by flow cytometry (C) and effector caspase activity by a DEVDase enzyme assay (D). Columns, mean from three experiments analyzing 10,000 cells (C) or three replicate experiments (D); bars, SD. *, P < 0.05; **, P < 0.01, as compared with untreated control cells.
suppressor gene BECN1 that encodes for beclin 1 protein (26). Thus, we tested whether low beclin 1 levels and defective autophagy could contribute to the vincristine resistance of the MCF-7 cells. Ectopic beclin 1 had, however, no effect on the vincristine response of the MCF-7 cells. The cells that expressed ectopic beclin 1 and were sensitized to the autophagic cell death induced by EB1089, a chemotherapeutic analogue of vitamin D (27), responded to vincristine in a manner indistinguishable from that of the vector-transfected control cells (Fig. 4C).

Microtubule-disturbing agents show potent synergism with siramesine both in vitro and in vivo. Prompted by our data showing that vincristine induces dramatic lysosomal changes also in the relatively apoptosis-resistant MCF-7 cells, we next studied the ability of vincristine to sensitize cells to drugs that induce lysosomal membrane permeabilization. No increase in the cytotoxicity was observed when MCF-7 cells were treated with vincristine in combination with either cisplatin or etoposide, whereas the combination with siramesine led to a synergistic reduction in cell density (Fig. 5A). Importantly, the microscopic analysis revealed that the combination of low concentrations of vincristine and siramesine was not only cytostatic but triggered massive cell death in MCF-7 cells that were resistant to the cell death induction by vincristine alone (Fig. 5B and C). Vincristine and siramesine showed strong synergistic cytotoxicity also in HeLa cells, and similar synergism was observed both in MCF-7 and HeLa cells when vincristine was substituted either by vinorelbine, a semisynthetic vincristine analogue, or a microtubule-stabilizing agent paclitaxel (Fig. 5B and C).

We have shown earlier that p.o. administration of siramesine at daily doses ranging from 30 to 100 mg/kg has potent antitumor activity in orthotopic MCF-7 breast cancer xenograft model in mice (23). To study the therapeutic potency of the combination of vincristine and siramesine in this model, we treated mice that had tumors with a diameter of 5 to 6 mm with suboptimal doses of the two drugs (Fig. 6). Biweekly treatment with siramesine at 6 mg/kg or a single i.v. administration of vincristine (30 μg/kg) reduced the tumor growth slightly, but failed to inhibit it. Remarkably, the combination of the two treatments was significantly more potent than either treatment alone, and no significant tumor growth was observed in the mice treated with both drugs during the treatment period (Fig. 6). Importantly, the mice showed no detectable side effects during the therapy.

Discussion

Compounds that disrupt the function of the microtubule network form an important and expanding group of chemotherapeutic agents for cancer treatment. A better understanding of the cell death pathways they activate is essential to their use, particularly with regard to their optimal exploitation in combinatorial cancer chemotherapy. The data presented above introduce the lysosomes as targets of vincristine treatment. First, vincristine triggered an increase in the size of individual lysosomes and the total volume of the lysosomal compartment both in vincristine-sensitive HeLa cells and in relatively vincristine-resistant MCF-7 cells. Second, vincristine-induced apoptosis-like death of HeLa cells was preceded by lysosomal membrane permeabilization and appearance of active cysteine cathepsins in the cytosol. Most importantly, vincristine and siramesine, a sigma-2 ligand that kills cancer cells by destabilizing their lysosomes, acted synergistically. The combination of subtoxic concentrations of the two drugs resulted in dramatic cytotoxicity even in the relatively resistant MCF-7 cells.

Lysosomes are highly dynamic cytosolic organelles that receive membrane traffic input from the biosynthetic (trans-Golgi network), endocytic, and autophagic pathways (36). The increase in the size of the lysosomal compartment observed in vincristine-treated cells is not likely to be due to increased de novo synthesis of lysosomes via the biosynthetic pathway, because intact microtubule network is essential for the maintenance and function of the Golgi complex (37). Accordingly, the Golgi apparatus was completely fragmented and dispersed throughout the cytosol in vincristine-treated cancer cells used in this study (data not shown). Microtubules have also been suggested to be essential for the endocytic and autophagic pathways of membrane traffic (30, 31, 37, 38). Akin to vincristine-treated MCF-7 cells that showed a moderate accumulation of autophagosomes, vinblastine, another natural Vinca alkaloid, has been reported to increase the number of autophagosomes in other cell types. This accumulation has been viewed as a failure in the fusion of autophagosomes with the lysosomes (30), accelerated rate of autophagosome formation (38), or a combined effect of both mechanisms (31). Our data showing the lack of colocalization of the lysosomal marker (LAMP-2) with the LC3-positive autophagosomes in vincristine-treated cells (as compared with rapamycin-treated cells) supports the hypothesis that the fusion of autophagosomes and lysosomes depends on intact microtubule network. Taken together, these
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Figure 5. Microtubule-disturbing agents show potent synergism with siramesine in vitro. A, MCF-7 cells were left untreated or treated for 48 h with either vincristine alone or in combination with cisplatin (CP), etoposide, or siramesine (Sira) in concentrations as indicated. The density of the cells was determined by the MTT reduction assay and is expressed as percentage of untreated control cells. Columns, mean from three independent triplicate experiments; bars, SD. *P < 0.05 as compared with cells treated with vincristine alone. B, representative phase contrast images of HeLa and MCF-7 cells treated for 48 h with 10 nmol/L vincristine or 2 μmol/L siramesine alone or in combination. C, MCF-7 and HeLa cells were left untreated or treated with vincristine (5–25 nmol/L), vinorelbine (Vino, 5–25 mmol/L), paclitaxel (10–50 mmol/L), or siramesine (1–2 μmol/L) alone or in indicated combinations for 48 h. Cell death was determined by staining of the DNA with Hoechst 33342 and counting the percentage of cells with nuclear condensation. For each condition, three randomly chosen fields of 100 cells were counted. Columns, mean from three independent triplicate experiments; bars, SD. *P < 0.05; **P < 0.01, as compared with cells treated with vincristine, vinorelbine, or paclitaxel alone.

Data suggest that the increased volume of the lysosomal compartment does not reflect increased synthesis of the lysosomes by the classic routes. Instead, the vincristine-induced increase in the lysosomal volume could be a result of the merging of preexisting lysosomes combined with their defective turnover by exocytosis and/or autophagy. Accordingly, lysosomal exocytosis has been shown to depend on intact microtubules in hepatocytes and lymphocytes (39, 40).

Vincristine-induced destabilization of lysosomes was preceded by an accumulation of large lysosomes in MCF-7 and HeLa cells. Thus, it is interesting to note that an increase in lysosome size has been reported to correlate with their decreased stability and sensitization to nonapoptotic cell death pathways in other cell types (41). Accordingly, nontoxic concentrations of vincristine dramatically sensitized HeLa and MCF-7 cells to lysosome-destabilizing siramesine both in vitro and in vivo. Importantly, the semisynthetic Vinca alkaloid vinorelbine, which is better tolerated than vincristine, and broadly used microtubule stabilizer paclitaxel showed similar synergism when combined with siramesine. These data indicate that impairment of microtubule dynamics is enough to sensitize cancer cells and tumors to siramesine. Siramesine is a lipophilic sigma-2 receptor agonist that accumulates in the lysosomes and kills cancer cells by destabilization of their lysosomes in a Bcl-2–insensitive manner (23). Therefore, the increased volume and decreased stability of lysosomes in vincristine-treated cells might explain the synergism observed between vincristine and siramesine. Alternatively, defect autophagy in vincristine-treated cells could contribute to the sensitization. We have recently shown that siramesine-induced cell death is accompanied by an induction of massive autophagy that serves a cytoprotective function (1). γ-Irradiation and arsenic trioxide have been suggested to trigger similar cytoprotective autophagy in parallel with their cytotoxic effect (42, 43). Thus, combination therapies between these treatments and microtubule-disturbing drugs may also prove efficient.

Vincristine-treated HeLa and MCF-7 cells displayed radically different morphologies. HeLa cells died within 48 h, and the cell death was accompanied by a G2-M cell cycle arrest, lysosomal membrane permeabilization, Bax activation, cytochrome c release, effector caspase activation, and apoptosis-like chromatin condensation. Bax activation and cytochrome c release, hallmarks of the intrinsic apoptosis pathway, occurred only in a minority of dying cells, and the level of effector caspase activation was very low as compared with etoposide-treated apoptotic HeLa cells. Furthermore, these apoptosis-associated changes occurred late in the process, and an analysis at a single cell level revealed that Bax was activated only in cells that had undergone lysosomal membrane permeabilization. Thus, the activation of the intrinsic apoptosis pathway seemed to be a secondary event rather than the main cell death pathway in vincristine-treated HeLa cells. Instead, the substantial release of cathepsins into the cytosol early in the process suggests that the lysosomal membrane permeabilization is involved in this cell death pathway. Due to a high sensitivity of HeLa cells to protease inhibitors, we were unfortunately unable to define the role of individual lysosomal proteases in this process. Contrary to the HeLa cells that detached and died, vincristine-treated MCF-7 cells adapted to the mitotic arrest, reattached to the bottom of the culture wells after a failed mitosis, and stayed viable with metabolic activity and intact plasma membrane for several days. These micronucleated cells displayed a senescent flat
morbidity and enlarged lysosomal compartment, but showed no signs of apoptosis. There might be several reasons why MCF-7 cells are more resistant to vincristine-induced cell death than HeLa cells. They could, for example, express tubulin isoforms that have low affinity for vincristine (44); microtubule-stabilizing, microtubule-disturbing drugs, possess great future potential as parts of a combination therapies with lysosome-targeting drugs even in tumors with defective spindle assembly checkpoint.

Overall, our study shows that vincristine affects the lysosomal compartment in HeLa and MCF-7 cells with regard to lysosomal size and integrity, and that vincristine, as well as other microtubule-disturbing drugs, possess great future potential as parts of a combination therapies with lysosome-targeting drugs even in tumors with defective spindle assembly checkpoint.

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References

6. Figure 6. Combination treatment with vincristine and siramesine effectively inhibits the growth of orthotopic breast cancer xenografts in mice. A total of 1.0 x 10

7. 2.5–3 mm, the mice were treated with siramesine (6.5 mg/kg p.o.) at days 6, 9, and 13 (j), with vincristine (30 μg/kg i.v.) at day 7 (j), with a combination of the two drugs, or with appropriate vehicles. Points, mean tumor volumes of six mice per group; bars, SD. **, P < 0.01, level of significance between the group treated with the combination of vincristine and siramesine as compared with both single treatments.

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