**Small Molecule Therapeutics**

**Sunitinib and SU11652 Inhibit Acid Sphingomyelinase, Destabilize Lysosomes, and Inhibit Multidrug Resistance**

Anne-Marie Ellegaard1, Line Groth-Pedersen1, Viola Oorschot3, Judith Klumperman3, Thomas Kirkegaard1,2, Jesper Nylandsted1, and Marja Jäättelä1

**Abstract**

Defective apoptosis signaling and multidrug resistance are major barriers for successful cancer treatment. To identify drugs capable of targeting treatment-resistant cancer cells, we screened small-molecule kinase inhibitor libraries for compounds that decrease the viability of apoptosis-resistant human MCF7-Bcl-2 breast cancer cells. SU11652, a multitargeting receptor tyrosine kinase inhibitor, emerged as the most potent compound in the screen. In addition to MCF7-Bcl-2 cells, it effectively killed HeLa cervix carcinoma, U-2-OS osteosarcoma, Du145 prostate carcinoma, and WEHI-S fibrosarcoma cells at low micromolar concentration. SU11652 accumulated rapidly in lysosomes and disturbed their pH regulation and ultrastructure, eventually leading to the leakage of lysosomal proteases into the cytosol. Lysosomal destabilization was preceded by an early inhibition of acid sphingomyelinase, a lysosomal lipase that promotes lysosomal membrane stability. Accordingly, Hsp70, which supports cancer cell survival by increasing lysosomal acid sphingomyelinase activity, conferred partial protection against SU11652-induced cytotoxicity. Remarkably, SU11652 killed multidrug-resistant Du145 prostate cancer cells as effectively as the drug-sensitive parental cells, and subtoxic concentrations of SU11652 effectively inhibited multidrug-resistant phenotype in Du145 prostate cancer cells. Notably, sunitinib, a structurally almost identical and widely used antiangiogenic cancer drug, exhibited similar lysosome-dependent cytotoxic activity, albeit with significantly lower efficacy. The significantly stronger lysosome-targeting activity of SU11652 suggests that it may display better efficacy in cancer treatment than sunitinib, encouraging further evaluation of its anticancer activity in vivo. Furthermore, our data provide a rationale for novel approaches to target drug-resistant cancers by combining classic chemotherapy with sunitinib or SU11652.

**Introduction**

Apoptosis is an evolutionary conserved cell death program responsible for the removal of excess and damaged cells during development and in response to a variety of stresses (1, 2). It is initiated by extracellular stimuli or intracellular stresses that lead to the activation of caspase proteases and controlled demolition of the cell from within. During tumorigenesis, it is important for the transformed cells to evade apoptosis, and defective apoptosis signaling is one of the major hallmarks of cancer cells (3). Apoptosis-resistant cancer cells are, however, still able to undergo controlled cell death through other mechanisms such as necroptosis and lysosomal cell death pathways (4, 5). The latter cell death program is of special interest in the cancer field because transformation and invasiveness are associated with the enlargement of the lysosomal compartment, increased activity of lysosomal proteases cathepsins, and decreased lysosomal membrane stability (6–8). Invasive tumor cells secrete cathepsins and other lysosomal hydrolases into the extracellular space where they promote tumor growth, angiogenesis, and cancer invasion (9–11). On the other hand, the large size and high content of cathepsins render tumor lysosomes sensitive to lysosomal membrane permeabilization and subsequent leakage of cathepsins into the cytosol (6, 12). Cytosolic cathepsins can activate the intrinsic caspase-dependent apoptosis pathway or caspase-independent apoptosis-like cell death upon minor release, whereas substantial release of cathepsins leads to uncontrolled necrotic cell death (13). Importantly, the permeabilization of the lysosomal membrane and resulting caspase-independent cell death can occur in Bcl-2–expressing apoptosis-resistant cancer cells (14–17).
Apart from the apoptosis resistance acquired during tumor initiation and progression, cancers treated with chemotherapy often develop drug resistance by increasing the expression of multidrug resistance proteins (MDR), P-glycoproteins of the ATP-binding cassette transporter family that can effectively efflux unrelated drugs (18). In addition, the cancer-associated lysosomal changes contribute to the development of multidrug resistance (19). Cancer-associated increase in the number and size of lysosomal vesicles and reduction in lysosomal pH leads to increased sequestration of weakly basic chemotherapeutic drugs in this compartment (20, 21). Moreover, multidrug resistant cells show an increased rate of exocytosis, which potentially enhances the drug excretion from the cell and decreases the extracellular pH, thereby reducing the diffusion of drugs into the cell (22).

The aim of this study was to identify compounds capable of killing therapy resistant cancer cells. For this purpose, we screened two small-molecule kinase inhibitor libraries (160 compounds) for their ability to reduce the viability of apoptosis resistant Bcl-2–overexpressing MCF7 breast cancer cells. Prompted by the high number of known platelet-derived growth factor receptor (PDGFR) inhibitors among the hits (4 out of 13), we investigated these compounds in more detail and identified SU11652 as an effective inducer of nonapoptotic cell death. The further investigation of SU11652 and the chemically almost identical and clinically approved anticancer drug sunitinib indicated that their death-inducing potential was unrelated to their ability to inhibit the PDGFR. The rapid accumulation of SU11652 and sunitinib in the lysosomes lead us to investigate and show their ability to induce lysosomal cell death and to revert drug resistance. Hence, our data identify lysosomal cell death as a novel cytotoxic mechanism for this class of compounds and reveal a potential for sunitinib in the treatment of multidrug-resistant cancers.

Materials and Methods

Cell lines and reagents

MCF7 cell line used in this study is a TNF-sensitive subclone (MCF7-S1) of human ductal breast carcinoma cells (23). Its origin has been confirmed by RNA Seq. MCF7-pCEP, and MCF7-Bcl-2 cells are single-cell clones generated as described in ref. (24). U-2-OS human osteosarcoma cells, HeLa human cervix carcinoma, and Du145 prostate carcinoma cells were obtained from American Type Culture Collection and used in low (<20) passages after delivery. Multidrug-resistant variants of Du145 cells (Du145-MDR) were created by culturing the cells with increasing concentrations (5–25 nmol/L) of docetaxel for 3 months. The parental cells (Du145-P), used as the control for MDR cells, were cultured in parallel in normal growth medium. HeLa-eGFP-CD63 cells (not authenticated by the authors) were kindly provided by Dr. J.P. Luzio (University of Cambridge, Cambridge, UK; ref 25). All of the above mentioned cell lines were grown in RPMI-1640 (Life technologies Ltd.) supplemented with 6% heat-inactivated fetal calf serum (Biological industries). WEHI-vector and WEHI-Hsp70 cells are single cell clones of WEHI-S mouse fibrosarcoma cells successfully transfected with an empty vector or a vector encoding for the HSPA1A gene encoding for human Hsp70 (26). NIH3T3 fibroblasts were transduced with an empty pBabe-puro retrovirus (Babe; provided by C. Holmberg, University of Copenhagen, Denmark) or pBabe-puro encoding for c-srcY527F (Src; provided by S. Courtneidge, Van Andel Research Institute, Grand Rapids, Michigan) as described previously (8). The identities of these cell lines were confirmed by RNA Seq. Both the WEHI cells and the NIH3T3 cells were grown in Dulbecco modified Eagle medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum and nonessential amino acids (Gibco). All cells were kept at 37°C in a humidified atmosphere containing 5% CO2.

InhibitorSelect 96-Well Protein Kinase Inhibitor Library I and II (Calbiochem, cat.no. 539744 and 539745, respectively) were dissolved in dimethyl sulfoxide (DMSO) and stored at −80°C. Upon dilution in growth medium, the inhibitors were added directly to the growth medium of the cells. PDGFR inhibitor III, IV, and V (Calbiochem, cat. no. 521232, 521233, 521234, respectively), SU11652 (Calbiochem, cat. no. 572660), and sunitinib Malate (Selleck Chemicals, cat. no. S1042) were dissolved in DMSO and stored at −20°C. Just before treatment, the inhibitors were diluted in growth medium and directly added to the growth medium of the cells.

AlexaFluor594-coupled Dextran was purchased from Invitrogen; Benzoyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (zVAD-fmk) from Bachem; human TNF-α from Millipore; necrostatin-1, desipramine, docetaxel, rapamycin, and concanamycin A from Sigma.

Viability and cell death assays

Cell viability and cell death were measured with MTT (Sigma-Aldrich) reduction assay and lactate dehydrogenase (LDH) cytotoxicity assay (Roche), respectively, as previously described (27). The nuclear condensation was analyzed in cells stained with 4 μg/mL Hoechst 33342 for 10 minutes using an inverted Olympus IX-71 microscope connected to an Olympus DP72 digital camera and using Cell’P software.

To assess clonogenic survival, the cells were seeded in 6-well plates at a low density (2,000 cells/well) and treated as indicated. Four days after the treatment, the cells were washed with PBS and incubated with crystal violet/ methanol for 15 minutes, washed twice in water, and dried. Colonies were counted manually.

Immunoblotting

Primary antibodies used included murine monoclonal antibodies against MDR1/3 (sc-13131; Santa Cruz Biotechnology) and GAPDH (Biogenesis). Immunodetection of proteins separated by 10% SDS-PAGE and transferred to nitrocellulose was conducted using appropriate
peroxidase-conjugated secondary antibodies from Dako, ECL Western blotting reagents (Amersham), and Luminiscent Image Reader (LAS-4000, Fujifilm).

**Immunocytochemistry**

Cells were plated on glass coverslides, treated as indicated, fixed in ice-cold methanol for 3 minutes, and permeabilized with 0.3% Triton X-100 for 20 minutes. Notably, the fixation completely abolishes the autofluorescence of SU11652 and sunitinib. Cells were stained with antibodies recognizing lysosome-associated membrane glycoprotein 2 (LAMP-2; Southern Biotechnology Associate) and cathepsin-L (BD Transduction Laboratories) or cathepsin-B (Oncogene Research Products), and bax (Cell Signaling Technology) followed by AlexaFluor488- or AlexaFluor594–coupled secondary antibodies (1:1,000; Molecular Probes). The samples were mounted with Pro-Long Gold antifade (Molecular Probes) and confocal fluorescent images were obtained with Carl Zeiss Axiovert 100M confocal microscope using LSM 510 software.

**Immuno-electron microscopy**

Cells grown in T75 flasks and treated as indicated, were fixed by adding freshly prepared 4% w/v formaldehyde (Polysciences) in 0.1 mol/L phosphate buffer (pH 7.4) to an equal volume of culture medium for 5 minutes, followed by postfixation in 4% w/v formaldehyde at 4°C overnight. Ultrathin cryosectioning and immunogold labeling were carried out as previously described (28). In brief, fixed cells were washed with PBS containing 0.05 mol/L glycine, scraped gently from the dish in PBS containing 1% gelatin, and pelleted in 12% gelatin in PBS. The cell pellet was solidified on ice and cut into small blocks. For cryoprotection, blocks were infiltrated overnight with an equal volume of culture medium for 5 minutes, and permeabilized with 0.3% Triton X-100 for 20 minutes. Notably, the fixation completely abolishes the autofluorescence of SU11652 and sunitinib. Cells were stained with antibodies recognizing lysosome-associated membrane glycoprotein 2 (LAMP-2; Southern Biotechnology Associate) and cathepsin-L (BD Transduction Laboratories) or cathepsin-B (Oncogene Research Products), and bax (Cell Signaling Technology) followed by AlexaFluor488- or AlexaFluor594–coupled secondary antibodies (1:1,000; Molecular Probes). The samples were mounted with Pro-Long Gold antifade (Molecular Probes) and confocal fluorescent images were obtained with Carl Zeiss Axiovert 100M confocal microscope using LSM 510 software.

**Lysosomal pH and enzyme activities**

The pH of the acidic compartment (late endosomes and lysosomes) was analyzed 1 hour after treatment, followed by washing of the cells in PBS and staining with 25 nmol/L LysoTracker-Red DND-99 (Molecular Probes), and 4 μg/mL Hoechst-33342 for 15 minutes and measurement of fluorescent intensity with NucleoCounter-3000 (Chemometec; excitation 530 nm, emission 600–750 nm). Total cellular acid sphingomyelinase activity was measured with 6-hexadecanoylamino-4-methylumbelliophosphorycholine (HMU-PC) assay as described previously (30). The total cathepsine activity (zFRase) was measured using benzoyloxycarbonyl-Phe-Arg-amino-trifluoromethylcoumarin (zFR-AMC; Enzyme System Products) probe as described (27), and the N-acetyl-glucosaminidase (NAG) activity was measured using NAG substrate solution (Sigma-Aldrich) as described (6). The substrate hydrolysis was analyzed using a SpectraMax Gemini Fluorometer (Molecular Devices).

**RNAi**

siRNAs were transfected with Oligofectamine (Invitrogen) in 96-well plates to a final concentration of 20 nmol/L, using reverse transcription according to the manufacturer’s protocol. The validated siRNAs targeting ATG5, BECN1, and ULK1 were purchased from Sigma-Aldrich; the non-targeting siRNA was AllStar Negative Control siRNA from Qiagen. The efficiency of the siRNAs was confirmed by Western blotting (not shown) and inhibition of the autophagic flux (Fig. 2B).

**Assay for autophagic flux**

Autophagic flux was analyzed by measuring luciferase activities in lysates of MCF7-RLuc-LC3wt and MCF7-RLuc-LC3mut (G120A) cells essentially, as described previously (31). Briefly, cells were plated 24 hours before treatment in the even and uneven numbered columns in 96-well plates, respectively. The cells lysed in 40 μL of passive lysis buffer (Promega) at the end of the treatment were subjected to a single freeze/thaw cycle and 6 μL of each lysate was transferred to corresponding wells in a white half volume 96-well dish (Costar). The luminescence reaction was started by addition of 80 μL of freshly made assay buffer (100 nmol/L Tris/His pH 7.4, 300 nmol/L Na-Ascorbate, 25 μmol/L Coelenterazine (Synchem s053), and the luminescence was measured in an EnSpire 2300 multilabel reader (Perkin Elmer). The reduction in the ratio of luciferase activities of MCF7-RLuc-LC3wt and MCF7-RLuc-LC3mut lysates reflects specific degradation of wild-type LC3, i.e., autophagic flux.

**Results**

**A screen for nonapoptotic cytostatic compounds**

To identify small molecules that reduce cell density in an apoptosis-independent manner, we screened two libraries containing a total of 160 different kinase inhibitors for compounds that reduce the viability of MCF7 human ductal breast carcinoma cells transduced with Bcl-2 (MCF7-Bcl-2) as well or better than the corresponding vector-transfected cells (MCF7-pCEP; Supplementary Fig. S1). Compounds were selected as hits if they, in two independent screens (2.5 μmol/L; 48 hours), reduced the viability of MCF7-Bcl-2 cells by at least 40%, and the ratio between the viability of MCF7-pCEP and MCF7-Bcl-2 cells was higher than 0.9. Thirteen compounds fulfilled these criteria (Supplementary Fig. S1). The most effective compound was SU11652, an inhibitor of several receptor kinases.
tyrosine kinases, including PDGFR and VEGF receptor (VEGFR; ref. 32). It reduced the viability of MCF7-Bcl-2 cells by more than 70% and was more effective against Bcl-2–expressing cells than control cells. Similar selectivity against Bcl-2–expressing cells was observed with casein kinase 1 inhibitor IC261.

Four of our 13 hits (SU11652 and PDGFR inhibitors III, IV, and V) were PDGFR inhibitors, and therefore, we investigated these compounds in greater detail by assessing their concentration-dependent (0.1–100 μmol/L) ability to reduce cell viability (the MTT assay) and to induce cell death (the LDH release assay). All compounds induced a dose-dependent reduction in cell viability in both MCF7-pCEP and MCF7-Bcl-2 cells, but only SU11652 was able to induce significant cell death at the concentrations tested, the IC50 and LD50 values being 2.4 μmol/L and 5.4 μmol/L in MCF7-pCEP cells as measured by the MTT and LDH assays, respectively (Fig. 1A).

SU11652 and sunitinib induce nonapoptotic and nonautophagic cell death

SU11652 is almost identical with SU11248 (sunitinib), which, based on its antiangiogenic and direct antitumor properties, was in 2006 approved for the treatment of cancer. Because of its clinical relevance and striking similarity with SU11652 (Fig. 1B), we included sunitinib to our further investigation. Akin to SU11652, sunitinib induced a dose-dependent cell death in MCF7 cells in a Bcl-2–independent manner (Fig. 1A). Notably, the 48-hour IC50 and LD50 values for sunitinib in MCF7-pCEP cells were, however, considerably higher than those for SU11652 in the MTT (15.2 μmol/L vs. 2.4 μmol/L) and the LDH release (34.6 μmol/L vs. 5.2 μmol/L) assays, respectively. The effect of sunitinib on MCF7 cell growth and survival also displayed slower kinetics than SU11652, the cytotoxicity becoming evident between 24–48 hours and 12–18 hours, respectively (Supplementary Fig. S2A). SU11652 was also a more effective inducer of cell death than sunitinib in human cervix carcinoma (HeLa) and osteosarcoma (U-2-OS) cells, the 24-hour LD50 values for sunitinib and SU11652 being 23.8 μmol/L and 3 μmol/L in HeLa cells and 16.2 μmol/L and 3.6 μmol/L in U-2-OS cells, respectively (Fig. 1C). Importantly, sunitinib-induced cytotoxicity displayed selectivity towards transformed cells as analyzed by comparing the colony forming ability of immortalized fibroblasts transduced either by an empty vector or activated c-Src oncogene (Fig. 1D).

The dying cells in response to both compounds displayed morphologic characteristics of both apoptosis–like cell death (rounding and detachment of individual cells) and necrosis (formation of large blebs; Supplementary Fig. S2B). Pharmacologic inhibitors of apoptotic caspases (zVAD-fmk) and necrosis-inducing receptor–interacting serine/threonine-protein kinases (necrotomin-1) failed, however, to protect the cells from SU11652 and sunitinib (Fig. 1E).

Sunitinib has been reported to induce autophagic cell death in H9c2 cardiomyocytes (33), and SU11652 is a potent regulator of autophagy in MCF7 cells with a strong initial (0–6 hours) induction and subsequent inhibition of autophagic flux (31). To investigate whether the cell death observed here depended on autophagy, we depleted the MCF7 cells for 3 essential autophagy proteins [autophagy protein 5 (Atg5), Beclin-1, and serine/threonine protein kinase Ulk1] by validated siRNAs. The significant inhibition of rapamycin-induced autophagic flux in siRNA-treated MCF7 cells confirmed successful depletion of the targeted proteins (Fig. 1F). However, none of the three siRNAs protected MCF7 cells against SU11652 or sunitinib (Fig. 1G). Taken together, these data indicate that the cytotoxicity induced by SU11652 and sunitinib in MCF7 cells is independent of both autophagy and apoptosis.

SU11652- and sunitinib-induced cell death depends on their lysosomal accumulation

As reported earlier in other cells (34, 35), microscopic analysis of SU11652- and sunitinib-treated MCF7 cells using their autofluorescence properties revealed that the two compounds rapidly accumulated in cytosolic vacuoles (Fig. 2A). These vacuoles were identified as lysosomes based on the colocalization with fluorochrome-labeled dextran (Fig. 2B). Using time-lapse video microscopy, the lysosomal accumulation of the drugs was evident as early as 1–2 minutes after their addition (Supplementary Fig. S3), and they localized almost exclusively to this compartment 1 to 24 hours after the treatment (Fig. 2A and B). In agreement with the basic nature of SU11652 and sunitinib, their lysosomal accumulation was associated with decreased staining with the pH sensitive lysosomal probe, LysoTracker-Red (Fig. 2C), and the neutralization of the lysosomal pH gradient by an inhibitor of vacuolar H+ -ATPase (concanamycin A) resulted in their diffuse distribution (Fig. 2B and Supplementary Fig. S3). Remarkably, concanamycin A-induced relocalization of the drugs to the cytosol was associated with dramatically reduced cytotoxicity (Fig. 2D). Thus, lysosomal localization seems to be essential for the cytotoxic action of SU11652 and sunitinib.

SU11652 and sunitinib induce lysosomal cell death

Prompted by the lysosomal accumulation of SU11652 and sunitinib, we investigated their effects on lysosomal compartment in further detail. Immunostaining of MCF7 cells for LAMP-2 revealed that the drug treatment resulted in the enlargement and aggregation of LAMP-2–positive vesicles (Fig. 3A). Similar lysosomal changes were evident in SU11652- and sunitinib-treated HeLa-eGFP-CD63 cells that express CD63 antigen tagged with an enhanced GFP as a marker for the lysosomal compartment (Fig. 3B). Immunostaining of HeLa-eGFP-CD63 cells for lysosomal cathepsin-L protease revealed that both SU11652 and sunitinib induced a partial leakage of cathepsin-L to the cytosol in a time-dependent manner.
Figure 1. SU11652 and sunitinib induce nonapoptotic and nonautophagic cell death. A, MCF7-pCEP and MCF7-Bcl-2 cells were treated for 48 hours with indicated concentrations of PDGF receptor inhibitors PDGFRI III, PDGFRI IV, PDGFRI V, SU11652, or sunitinib, and cell density (top) and cell death (bottom) were measured by the MTT reduction and LDH release assays, respectively. B, schematic drawing of the chemical structures of SU11652 and sunitinib. The only difference is indicated with an “X” that refers to Cl in SU11652 and F in sunitinib. C, HeLa (left) and U-2-OS (right) cells were treated with indicated concentrations of SU11652 or sunitinib for 24 hours, and cell density (top) and cell death (bottom) were measured as in A. D, clonogenic survival of NIH3T3-vector (Vector) and c-srcY527F (Src) cells treated with indicated concentrations of sunitinib. E, MCF7 cells were pretreated with DMSO, 10 µmol/L zVAD-fmk, or 10 µmol/L necrostatin-1 for 1 hour before 24-hour treatment with 4 µmol/L SU11652, 15 µmol/L sunitinib, or 20 ng/mL TNF (positive control for apoptosis). Cell density was measured as in A. **P < 0.01 when compared with similarly treated cell in the absence of the inhibitor. F, the lysates of MCF7-RLuc-LC3wt (LC3wt) and MCF7-RLuc-LC3mut (LC3mut) cells treated with the nontargeting siRNA (NT), or siRNAs targeting ATG5, BECN1, or ULK1 for 48 hours were analyzed for luciferase activity. When indicated, 100 nmol/L rapamycin was added 6 hours before the analysis to induce an autophagic flux depicted by a reduction in the LC3wt/LC3mut luciferase activity ratio (31). G, MCF7 cells treated with oligofectamine alone (none), or with indicated siRNAs for 48 hours were treated with vehicle (DMSO), 4 µmol/L SU11652, or 15 µmol/L sunitinib for 24 hours before the analysis of cell density (left) and cell death (right) by MTT and LDH release assays, respectively. Error bars, SD for a minimum of three independent triplicate experiments. **P < 0.05; ***P < 0.001; ****P < 0.0001 when comparing vector-transduced cells with similarly treated Bcl2- (A) or c-srcY527F-transduced (E) cells, zVAD plus TNF-treated cells with TNF-treated cells (E) or cells transfected with specific siRNAs with similarly treated cells transfected with nontargeting siRNA (F).
significant leakage being evident after 8–10 hours (Fig. 3B and C). A similar lysosomal leakage visualized by staining for cathepsin-B was observed in drug-treated MCF7 cells (Fig. 3D).

Lysosomal membrane permeabilization can be either an initial cell death-inducing event or an enhancing event occurring downstream of mitochondrial outer-membrane permeabilization (5). To reveal the sequence of these events, we quantified lysosomal membrane permeabilization (cytosolic cathepsin-B) and initiation of mitochondrial outer membrane permeabilization (activated Bax foci) in MCF7 cells treated with SU11652 or sunitinib for 8 hours. The majority of affected cells displayed diffuse cathepsin-B staining in the absence of Bax foci and some cells had both cathepsin leakage and Bax foci, whereas practically no cells had Bax foci in the absence of cathepsin leakage (Fig. 3D). On the contrary, the majority of the affected TNF-treated cells (control for apoptosis) displayed Bax foci alone (Fig. 3D). These data together with the rapid accumulation of the drugs into the lysosomal compartment (Fig. 2A), and the lack of protection by Bcl-2 (Fig. 1A) strongly suggest that lysosomal leakage is an early and crucial event for the cell death induction by SU11652 and sunitinib.

To get clues to the mechanism by which the drugs induce lysosomal leakage, we analyzed the ultrastructure of SU11652-treated MCF7 cells by immuno-electron microscopy, employing antibodies against LAMP-1 and...
Almost half of the LAMP-2–positive vesicles (multivesicular bodies, late endosomes, and lysosomes) in SU11652-treated cells showed a strikingly altered, collapsed morphology with extractions of luminal material and a poorly defined outer membrane (Fig. 4). Importantly, such changes were not observed in any control cells. Although we cannot exclude that these morphologic alterations are induced during preparation of the samples, they do indicate a significant SU11652‐induced change in the membrane properties of late endosomes and lysosomes. Notably, SU11652 did not alter the organelle morphology of mitochondria, endoplasmic reticulum, and Golgi apparatus (Fig. 4 and Supplementary Fig. S4), further supporting the direct action of SU11652 on the endolysosomal compartment.  

**SU11652 and sunitinib inhibit acid sphingomyelinase activity**  
The observed effects of SU11652 on the lysosomal membrane prompted us to investigate the effect of SU11652 and sunitinib on the lysosomal sphingolipid metabolism. Acid sphingomyelinase, a lysosomal enzyme that hydrolyses sphingomyelin to ceramide and phosphocholine, promotes lysosomal membrane stability (36). To evaluate whether the destabilization of lysosomal membranes by SU11652 and sunitinib was associated with reduced acid sphingomyelinase activity, we treated MCF7 cells for 1 hour with the drugs and analyzed the acid sphingomyelinase activity in the cell lysates. Remarkably, SU11652 inhibited the acid sphingomyelinase activity as effectively as desipramine, a known inhibitor of lysosomal acid sphingomyelinase.
Sphingomyelinase (37), and both SU11652 and sunitinib reduced the activity of acid sphingomyelinase significantly at the concentrations used in this study to kill approximately 50% of the MCF7 cells (Figs. 5A and 1A). Contrary to the membrane-associated acid sphingomyelinase, neither drug reduced the activity of the luminal NAG, and the lumenal cysteine cathepsin activity was reduced clearly less than the acid sphingomyelinase activity (Fig. 5B).

In cancer cells, a small portion of Hsp70 localizes to lysosomes where it enhances the membrane association and activity of acid sphingomyelinase, thereby stabilizing the lysosomal membrane and protecting cells against lysosomal cell death (36, 38). To examine whether the overexpression of Hsp70 could attenuate the SU11652-and sunitinib-induced cell death, we treated WEHI-S murine fibrosarcoma cells expressing ectopic Hsp70 (WEHI-Hsp70) and the appropriate vector-transfected control cells (WEHI-vector) with the two compounds. Hsp70 protected the cells significantly from the toxicity of both compounds (Fig. 5C and D), supporting the idea that SU11652- and sunitinib-induced cytotoxicity is mediated by reduced acid sphingomyelinase activity and lysosomal membrane destabilization.

**SU11652 and sunitinib inhibit multidrug resistance**

The lysosomotrophic nature of SU11652 and sunitinib as well as their ability to inhibit acid sphingomyelinase, increase lysosomal pH, and induce lysosomal membrane permeabilization are properties associated with compounds capable of reverting multidrug resistance (39-41). To investigate whether SU11652 and sunitinib can kill drug-resistant cancer cells and revert multidrug resistance, we created drug resistant Du145 prostate cancer cells by long-time culture with increasing concentrations of docetaxel. The obtained cells, which expressed MDR1 and displayed resistance against docetaxel, vincristine, paclitaxel, and etoposide, were as sensitive to SU11652 and sunitinib as the parental cells (Fig. 6A-C). More importantly, low nontoxic doses of both SU11652 (1 μmol/L) and sunitinib (5 μmol/L)
effectively inhibited the multidrug-resistant phenotype in the prostate cancer cells (Fig. 6D and E).

Discussion

Defective apoptosis signaling is one of the prime obstacles for successful cancer treatment. The aim of this study was to identify small molecules that inhibit cancer cell growth in an apoptosis-independent manner. For this purpose, we screened two small-molecule kinase inhibitor libraries for compounds that effectively inhibit the growth of apoptosis resistant MCF7-Bcl-2 cells. SU11652, a cell-permeable pyrrole-indolinone compound that acts as a potent, reversible, and ATP-competitive inhibitor of PDGFR, VEGFR, and other receptor tyrosine kinases (32), scored as the most potent cytostatic agent in this screen. Prompted by its ability to induce extensive cell death in apoptosis resistant MCF7-Bcl-2 cells as well as in HeLa cervix carcinoma and U-2-OS osteosarcoma cells at low micromolar concentration, we studied its cytotoxic mechanism of action in greater detail.

SU11652 was introduced in 2003 by SUGEN as a member of a class of putative anticancer compounds that inhibit receptor tyrosine kinases essential for angiogenesis, tumor cell proliferation, and tumor cell survival (32). Sunitinib (SU11248), a highly related member of this compound family (chlorine in SU11562 substituted by fluorine in sunitinib), was, however, chosen for clinical development. Today, sunitinib is the standard of care in the first-line treatment of advanced renal cell carcinoma, and it is approved for the treatment of imatinib-resistant gastrointestinal stromal tumors and nonoperable pancreatic neuroendocrine tumors (U.S. Food and Drug Administration). Furthermore, its clinical efficacy is being evaluated in a broad range of solid tumors, including breast, lung, thyroid, and colorectal cancers. Surprisingly, sunitinib was significantly less efficient than SU11652 in inducing cell death in all cancer cells tested here. Taking into consideration the practically identical kinase inhibitory profiles of the two compounds (32), this difference suggests that their cytotoxicity is mediated by activities other than the well-documented inhibition of receptor tyrosine kinases. Supporting this hypothesis, other tested inhibitors of PDGFR (PDGFR inhibitors III, IV, and V) that effectively inhibited the growth of MCF7-Bcl-2 cells failed to induce detectable cell death, and none of the tested...
VEGFR inhibitors (VEGFR tyrosine kinase inhibitors II and III, and VEGFR2 kinase inhibitors I, II, III, and IV) scored in our initial screen (data not shown). Furthermore, the LD<sub>50</sub> values of SU11652 and sunitinib in MCF7, U-2OS, and HeLa cells ranged from 3–35 \( \mu \text{mol/L} \), being more than 100 times higher than their reported IC<sub>50</sub> values for cellular PDGFR (0.01–0.1 \( \mu \text{mol/L} \)) and VEGFR (0.005–0.05 \( \mu \text{mol/L} \)) kinase activities (32).

Because of the low (<1 \( \mu \text{mol/L} \)) plasma levels of sunitinib in treated patients, its antitumor activity has been expected to be mainly due to the inhibition of receptor tyrosine kinases in vascular endothelial cells. A recent study revealed, however, that due to a significant accumulation of sunitinib in tumor tissue, it can display direct cytotoxicity against cancer cells at clinically relevant concentrations (34). Studies investigating mechanisms of sunitinib-induced cancer cell death are, however, limited to a few reports showing the activation of caspases and appearance of apoptotic features (42–44). The data presented above show for the first time that sunitinib and SU11652 are powerful inducers of nonapoptotic lysosome-dependent cell death. This conclusion is based on the data showing that the inhibition of their pH-dependent lysosomal accumulation effectively reduced their cytotoxic potential, whereas ectopic Bcl-2 and caspase inhibition were without an effect. Moreover, sunitinib and SU11652 induced an early (8–10 hours after the addition of the drugs) leakage of lysosomal hydrolases into the cytosol, which took place clearly before the modest activation of Bax or permeabilization of the plasma membrane. Also the extensive ultrastructural changes in lysosomal membranes preceded cell death and occurred in the absence of detectable ultrastructural changes in other organelles. While we cannot exclude the ability of sunitinib and SU11652 to induce classic apoptosis in some target cells, it should be noted that...
the observed apoptotic features in other studies could be due to secondary apoptosis caused by lysosomal leakage and subsequent cathepsin-mediated activation of Bax (5, 45).

The abundance of collapsed endolysosomal vesicles with poorly defined membranes in SU11652-treated cells suggested that the drug-induced lysosomal permeabilization could be caused by the alterations in lysosomal lipid metabolism. Indeed, both sunitinib and SU11652 inhibited the activity of the lysosome-stabilizing acid sphingomyelinase 1 hour after the addition of the drugs, i.e., clearly before the lysosomal membrane permeabilization. This inhibition could not be explained by the heightening of the lysosomal pH because concanamycin A that induces a similar pH change had no significant effect on the acid sphingomyelinase activity. Furthermore, sunitinib and SU11652 reduced the pH-sensitive cysteine cathepsin activity less than that of acid sphingomyelinase and had no effect on the activity of NAG. Thus, it is interesting to note that sunitinib and SU11652 are hydrophobic basic drugs with high pKa values, characteristics associated with desipramine and many other cationic amphiphilic drugs that concentrate in acidic lysosomes up to 1,000-fold and act as functional inhibitors of acid sphingomyelinase (37). As basic substances, cationic amphiphilic drugs inhibit acid sphingomyelinase activity by interfering with the electrostatic attraction between acid sphingomyelinase and bis(monoacylglycerol)phosphate, which serves as an essential docking lipid for acid sphingomyelinase during sphingomyelin hydrolysis (46, 47). On the other hand, lysosomal Hsp70 stabilizes the association of acid sphingomyelinase and bis(monoacylglycerol)phosphate thereby enhancing lysosomal acid sphingomyelinase activity and stabilizing lysosomal membranes (36). Thus, the ability of ectopic Hsp70 to protect cells against sunitinib and SU11652 support the idea that the reduced sphingomyelin catabolism promotes the lysosomal membrane permeabilization induced by these drugs. Furthermore, sunitinib- and SU11652-induced inhibition of acid sphingomyelinase may contribute to their ability to inhibit the multidrug-resistant phenotype because many other functional inhibitors of acid sphingomyelinase (39) as well as siRNA-mediated depletion of acid sphingomyelinase (L. Groth-Pedersen and M. Jäättela, unpublished data) display a similar ability to resensitize multidrug-resistant cancer cells to chemotherapy.

The development of targeted cancer therapies such as sunitinib and other kinase inhibitors has been largely motivated by the expectation of reduced therapy-associated adverse effects. The emerging clinical data show, however, that targeted therapies can also cause severe side effects that often lead to dose reduction or treatment interruption (48). One of the severe side effects caused by sunitinib is cardiotoxicity, primarily left ventricular dysfunction (49). On the basis of the ability of RNAi-mediated depletion of Beclin 1 to inhibit sunitinib-induced cytotoxicity in human H9c2 heart cardiocytes, Zhao and colleagues have suggested that autophagic death of cardiocytes contributes to sunitinib-induced cardiotoxicity (33). In contrast, siRNA-mediated depletion of Beclin 1 and two other essential autophagy proteins (ULK1 and Atg5) had no protective effect against the cytotoxicity induced by sunitinib and SU11652 in MCF7 cancer cells. Instead, the inhibition of autophagy showed a tendency to sensitize the cells to the drugs. The protective role of sunitinib-induced autophagy in cancer cells is supported by enhanced cell death of sunitinib-treated rat pheochromocytoma cells upon genetic or pharmacologic inhibition of autophagy (50). Thus, inhibition of autophagy may be a promising therapeutic option for improving the antitumor effect of sunitinib while protecting the heart from the toxic effects.

Taken together, our data show that apart from its receptor tyrosine kinase inhibitory activity, sunitinib has direct effects on cancer cell lysosomes, which can be exploited to kill cancer cells directly or to resensitize drug-resistant cancer cells to classic chemotherapy. Furthermore, the significantly enhanced lysosome-targeting activity of SU11652 suggests that it may display better efficacy in cancer treatment than sunitinib and encourages further evaluation of its anticancer activity.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A.-M. Ellegaard, T. Kirkegaard, J. Nylandsted, M. Jäättela
Development of methodology: J. Nylandsted, M. Jäättela
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.-M. Ellegaard, L. Groth-Pedersen, V. Oorschot, J. Klumperman, M. Jäättela
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.-M. Ellegaard, L. Groth-Pedersen, V. Oorschot, J. Klumperman, J. Nylandsted, M. Jäättela
Writing, review, and/or revision of the manuscript: A.-M. Ellegaard, J. Klumperman, T. Kirkegaard, M. Jäättela
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.-M. Ellegaard, V. Oorschot, M. Jäättela
Study supervision: T. Kirkegaard, Jesper Nylandsted, M. Jäättela

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Anne-Marie Ellegaard, Line Groth-Pedersen, Viola Oorschot, et al.


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