Senescence-initiated Reversal of Drug Resistance: Specific Role of Cathepsin L

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ABSTRACT

The present study was undertaken to verify whether induction of senescence could be sufficient to reverse drug resistance and, if so, to determine the underlying mechanism(s). Our findings indicated that co-treatment of drug-resistant neuroblastoma cells with doxorubicin, at sub-lethal concentrations, in combination with the pan-caspase inhibitor, Q-VD-OPH, elicited a strong reduction of cell viability that occurred in a caspase-independent manner. This was accompanied by the appearance of a senescence phenotype, as evidenced by increased p21/WAF1 expression and senescence-associated β-galactosidase activity. Experiments using specific inhibitors of major cellular proteases other than caspses have shown that inhibition of cathepsin L, but not proteasome or cathepsin B, was responsible for the senescence-initiated reversal of drug resistance. This phenomenon appeared to be general because it was valid for other drugs and drug-resistant cell lines. A nonchemical approach, through cell transfection with cathepsin L small interfering RNA, also strongly reversed drug resistance. Further investigation of the underlying mechanism revealed that cathepsin L inhibition resulted in the alteration of intracellular drug distribution. In addition, in vitro experiments have demonstrated that p21/WAF1 is a substrate for cathepsin L, suggesting that inhibition of this enzyme may result in p21/WAF1 stabilization and its increased accumulation. All together, these findings suggest that cathepsin L inhibition in drug-resistant cells facilitates induction of senescence and reversal of drug resistance. This may represent the basis for a novel function of cathepsin L as a cell survival molecule responsible for initiation of resistance to chemotherapy.

INTRODUCTION

Apoptosis has been shown to be a major pathway leading to neoplastic cell death upon exposure to chemotherapeutic agents and has become the focus of many experimental therapeutic investigations (reviewed in Refs. 1 and 2). The common belief is that cytotoxic drugs induce DNA damage, leading to activation of the p53/Fas/caspase-8 system and initiation of a mitochondria-mediated apoptotic death. The occurrence of this pathway has been confirmed for a variety of drugs and types of cancer (3–6), leading to the assumption that impairment of apoptotic pathways could be sufficient to explain the development of resistance to chemotherapy. Although this was demonstrated in various hematopoietic malignancies (7, 8), it was not necessarily valid for most solid tumors (8–11). More importantly, studies designed specifically to inhibit apoptosis often did not lead to increased cell survival (12–16); unexpectedly, they resulted in a switch to a non-apoptotic cell death. The nature of this “default death” has been the focus of recent investigations, some of which attribute this type of cell death to necrosis (12, 17) or some undefined causes (15, 16). In a recent study (18), we have shown that depending on the drug concentration and duration of exposure, doxorubicin was able to induce senescence, apoptosis, and a necrotic type of cell death. In addition, inhibition of doxorubicin-induced apoptosis by the pan-caspase inhibitor (Q-VD-OPH) resulted in a switch to a cell death with features of senescence. This suggested that whether or not apoptosis is inhibited, senescence must be inhibited for cancer cells to survive chemotherapy toxicity and become drug resistant. In light of this, forcing cancer cells to undergo senescence may be sufficient to sensitize them to chemotherapy and reverse drug resistance.

The present study, using the fact that Q-VD-OPH was able to accelerate doxorubicin-induced senescence in cancer cells, was undertaken to determine the importance of senescence in initiating drug resistance reversal. Validation of this hypothesis as well as identification of the underlying mechanism(s) was also addressed.

MATERIALS AND METHODS

Reagents. Cell lines including human neuroblastoma SKN-SH, murine neuroblastoma Neuro2A, osteosarcoma OSA (SaOS), leukemia HL-60, and the p21/WAF1 plasmid were purchased from American Type Culture Collection (Rockville, MD). DMEM and fetal bovine serum were obtained from Bio-Whittaker (Walkersville, MD). Doxorubicin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT), cisplatin, vinblastine, and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside were purchased from Sigma (St. Louis, MO). Q-VD-OPH was from Enzyme Systems Products, Inc. (Livermore, CA). Lactacytin, cathepsin B inhibitor [N-(3-trans-propylcarba-moyloxoiran-2-carboxyl)-t-isoleucyl-t-proline], cathepsin L inhibitor #1 [Z-Phe-Tyr(Bu)-diazomethylketone], cathepsin K inhibitor [1,3-Bis(N-CBZ-Leu-NH)-2-propane1,3-di(N-carbobenzyloxy-t-leucylamino) acetone], and cathepsin S inhibitor (Z-Phe-Leu-COCHOH2O) were from Calbiochem-Novabiochem (San Diego, CA). Cathepsin L substrate (Z-Phe-Arg)-R110 was purchased from Molecular Probes (Eugene, OR). Purified cathepsin L was from Biomol (Plymouth Meeting, PA). Antibodies to p21/WAF1 and to β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies to cleaved caspase-3 and secondary antibodies conjugated to horseradish peroxidase were from Cell Signaling Technologies (Beverly, MA). Antibody to cleaved (active form of cathepsin L) was purchased from Novus Biologicals Inc. (Littleton, CO). Enhanced chemiluminescence reagents (ECL) were from Amersham (Arlington Heights, IL). Immobilon-P transfer membrane for Western blot was purchased from Millipore (Bedford, MA).

Cell Culture, Drug Treatment, and Cytotoxicity Assay. SKN-SH, Neuro2A, and OSA were cultured in DMEM and HL-60 in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a 95% air/5% CO2 atmosphere. Doxorubicin-resistant variants of these cells were obtained from Novus Biologicals Inc. (Littleton, CO). Enhanced chemiluminescence reagents (ECL) were from Amersham (Arlington Heights, IL). Immobilon-P transfer membrane for Western blot was purchased from Millipore (Bedford, MA).

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Western Blot Analysis. Cells were seeded in 25-cm2 flasks in DMEM containing 10% fetal bovine serum and cultivated for 24 h before the addition of doxorubicin in the presence or absence of protease inhibitors. After incubation for an additional 24 h, proteins were solubilized with lysis buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 100 mM NaF, 1 mM MgCl2, 1.5 mM EGTA, 10% glycerol, 1% Triton X-100, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride]. Equal quantities of protein were separated by electrophoresis on a 12% SDS-PAGE gel and transferred to Immobilon-P membranes. Proteins of interest were identified by reaction with specific primary and secondary antibodies linked to horseradish peroxidase. Reactive bands were detected by chemiluminescence.
Senescence-associated β-Galactosidase (SA-β-Gal) Staining. Cells were seeded into 24-well plates in DMEM culture medium. After 24 h, drugs were added, and incubated with 10 μM doxorubicin for 5 days. SA-β-Gal staining was performed as described previously (20). In brief, cells were fixed for 5 min in 3% formaldehyde, washed, and incubated at 37°C with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (1 mg/ml), dissolved in a solution containing 40 mM citric acid (pH 6.5), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl₂. After 24 h incubation, photographs were taken with a phase microscope.

Fluorescence Microscopy. Cells were seeded on coverslips and incubated in DMEM containing 10% fetal bovine serum for 24 h. Doxorubicin was added at 10⁻⁷ M in the presence or absence of cysteine protease inhibitors and incubated for 30 min at 37°C. The cells were then washed twice with cold PBS; the intracellular localization of doxorubicin was examined by fluorescence microscopy (excitation, 480 nm; emission, 560 nm), and photographs were taken.

Rhodamine-123 uptake was evaluated by incubating the cells with (10 μg/ml rhodamine) for 30 min at 37°C in the presence or absence of cathepsin L inhibitor (10 μM), after which the culture medium was removed and cells were washed twice with PBS before determining the intracellular localization of rhodamine-123. For the efflux experiment, cells were washed with PBS after rhodamine uptake and incubated in fresh medium without dye for an additional 30 min, either in the presence or absence of cathepsin L inhibitor. Cells were then visualized under fluorescence microscopy (excitation, 480 nm; emission, 560 nm) and photographed.

Measure of Purified Cathepsin L Activity. Cathepsin L activity was measured according to the manufacturer’s procedure. Briefly, the purified enzyme (200 ng) was incubated with the inhibitor (10 μM) in a 96-well plate for 15 min at room temperature in 100 μl of reaction buffer [100 mM sodium acetate (pH 5), 1 mM EDTA, and 4 mM DTT]. One hundred microliters of substrate were added and incubated for an additional 30 min at room temperature. Fluorescence was measured in a plate reader (Victor Multilabel Counter; Perkin-Elmer) at 380-nm excitation and 450-nm emission wavelengths.

siRNA Design and Transfection. The Human Cathepsin L siRNA was designed in our laboratory from the human cathepsin L cDNA sequence AAGTGGAAGGCAGTGACAA (91–111) and was synthesized by Dharmaco (Lafayette, CO). On the day before transfection, 3 × 10⁵ drug-resistant osteosarcoma cells (OSA-R) were seeded in 6-well plates and grown in 2.5 ml of DMEM supplemented with 10% fetal bovine serum. After 24 h in culture, 25 μl of 20 μM stock solution of siRNA duplexes were transfected into cells with a GeneSilencer siRNA Transfection Reagent kit according to the manufacturer’s protocol (Gentherapy System). After 48 h of incubation, the cells were treated with 10⁻⁷ M doxorubicin and maintained in culture for an additional 48 h before analysis. The cells were counted, and protein lysates were used to detect cathepsin L and p21/WAF1 expression by Western blot as described above.

Production of Recombinant p21/WAF1 and Assay of Its Cleavage by Purified Cathepsin L. Plasmid pGEX-2TKcs containing the GST-CIP1 gene coding for p21/WAF1 was expressed in BL21 Star (DE3) bacteria (Invitrogen, Carlsbad, CA). Expression of recombinant p21/WAF1 protein was induced by isopropyl-1-thio-β-D-galactopyranoside (1 mM) and purified from the bacterial culture with the MicroSpin GST Purification Module (Amersham Pharmacia Biotech, Piscataway, NJ). The quantity of expressed protein was determined by Western blot using specific antibodies either to p21/WAF1 (C-19; Santa Cruz Biotechnology) or to glutathione S-transferase (GST; Amersham Pharmacia Biotech, Piscataway, NJ).

Cleavage of recombinant p21/WAF1 by purified cathepsin L was performed by incubating various amounts of the enzyme (1–5 ng) with 100 ng of p21/WAF1 and cathepsin substrate in reaction buffer [100 mM sodium acetate (pH 5), 1 mM EDTA, and 4 mM DTT]. After 20 min at room temperature, samples were boiled and separated by SDS-PAGE. p21/WAF1 was detected by specific antibodies either to p21/WAF1 (C-19) or to GST.

RESULTS

Effect of Q-VD-OPH on Doxorubicin-induced Senescence and Reversal of Drug Resistance

Previous work from this laboratory has demonstrated that the cysteine protease inhibitor, Q-VD-OPH, switches doxorubicin-induced apoptosis to senescence (18). The human neuroblastoma cell line resistant to doxorubicin SKN-SH/R (19) was used to determine whether Q-VD-OPH could accelerate doxorubicin-induced senescence in these cells and initiate the reversal of drug resistance. As shown in Fig. 1A, when this inhibitor (at 20 μM) was added to the cell culture medium in the presence of doxorubicin, decrease in cell viability was observed after 4 days of incubation, as measured by the MTT assay. Q-VD-OPH alone did not affect cellular viability. Cell cultivation in the presence of the drug combination for an additional 4 days resulted in more extensive cell death (Fig. 1A) indicating that the protease inhibitor was able to sensitize SKN-SH/R cells to doxorubicin and to reverse drug resistance.

Microscopic analyses revealed that cells treated for 8 days with the doxorubicin (10⁻⁷ M) in combination with Q-VD-OPH (20 μM) acquired morphological features typical of senescence (Fig. 1B). This finding was supported by an increased expression of p21/WAF1 (Fig. 1C) and SA-β-gal staining (Fig. 1D), both of which are known to accompany development of a senescence phenotype (20, 21). At the concentration of doxorubicin (10⁻⁷ M) used, caspase-3 was not activated by doxorubicin, Q-VD-OPH, or the combination of both (Fig.

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**Fig. 1.** Effect of Q-VD-OPH on SKN-SH/R cell response to doxorubicin. A, cells were incubated with doxorubicin (Dox.), Q-VD-OPH (20 μM), or the combination of both for 4 or 8 days. Cell viability (percentage related to control nontreated cells) was then measured by MTT assay as described in “Materials and Methods.” Values are the means of three determinations; bars, SE. Conc., concentration. B, morphological analysis of cells treated with 10⁻⁷ M doxorubicin (Dox.) and/or Q-VD-OPH (20 μM) as described above. After 8 days of incubation, photographs were taken under light microscopy. C, Western blot analysis of p21/WAF1 and cleaved caspase-3 (C-Casp-3) expression in response to treatment with 10⁻⁷ M and/or Q-VD-OPH (Q-VD.; 20 μM) for 24 h. Ctl., a positive control for expression of cleaved caspase-3. D, cells treated for 5 days with doxorubicin alone or in combination with Q-VD-OPH (Q-VD) were stained for SA-β-Gal activity as described in “Materials and Methods.”

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Fig. 2. Effect of protease inhibitors on doxorubicin-induced senescence in SKN-SH/R cells. A, morphological analysis of cells treated with the indicated inhibitors with (upper panel) or without (lower panel) doxorubicin for 5 days. Photographs were taken under light microscopy. Ctl, control; Lac, lactacystin; Q-VD, Q-VD-OPH; Csi, cathepsin L inhibitor; Cli, cathepsin B inhibitor; Cki, cathepsin K inhibitor; Cs, cathepsin S inhibitor. B, expression of p21/WAF1 in response to treatment with doxorubicin with or without protease inhibitors for 24 h. p21/WAF1 expression was determined by Western blot using the specific antibody C19. Antibody to β-actin was used as a gel loading control. Lac, lactacystin; Qvd, Q-VD-OPH; Cli, cathepsin L inhibitor; Cli, cathepsin B inhibitor; Cki, cathepsin K inhibitor; Cs, cathepsin S inhibitor. C, comparative effects of Q-VD-OPH and cathepsin L inhibitor on activation of SA-β-Gal. Cells were treated with drugs for 5 days, followed by SA-β-Gal staining as described in “Materials and Methods.” Inhib, inhibitor; Dox., doxorubicin; Q-VD, Q-VD-OPH; Cs, cathepsin L inhibitor.

1C). This suggested that activation of caspase-3 was not responsible for the diminished cellular proliferation and reversal of drug resistance by Q-VD-OPH. Because this agent is also a general cysteine protease inhibitor, it is likely that inhibition of other cysteine proteases than caspases could be implicated in initiating senescence.

Identification of Putative Cysteine Proteases Associated with Doxorubicin-induced Senescence

The ubiquitin-proteasome complex and lysosomal cathepsins represent two major families of cellular proteases known to be responsible for digesting and eliminating undesirable proteins (22, 23). The role of protease in cell survival is well described, particularly in relation to the fact that this molecule is able to degrade certain tumor suppressors and transcription factors responsible for the inhibition of cell proliferation (24). In addition, decreased activity of proteasome has been documented to be associated with cellular aging (25), suggesting a role in senescence. Although the possible implication of cathepsins in apoptotic death has been documented (26–28), their putative role in cell survival remains to be confirmed.

We have tested a series of specific inhibitors against the proteasome and lysosomal cathepsins to determine whether they affect the ability of a drug-resistant cell to undergo senescence in the presence of doxorubicin. The results shown in Fig. 2 indicate that although the proteasome inhibitor (lactacystin) and the cathepsin B inhibitor had no effect on the action of doxorubicin in SKN-SH/R cells, cathepsin L inhibitor was effective in inducing both morphological features of senescence (Fig. 2A) as well as expression of the corresponding molecular markers p21/WAF1 (Fig. 2B) and SA-β-gal (Fig. 2C). Cell treatment with cathepsin L inhibitor or with doxorubicin alone had no effect on cell morphology (Fig. 2A) or expression of these markers (data not shown). Inhibition of cathepsins K and S, which are members of the cathepsin L family (29), also resulted in induction of senescence features, both at the morphological level and expression of p21/WAF1. Cathepsin L inhibitor appeared to be the most effective, suggesting that specific targeting of this enzyme may produce a greater effect in terms of drug resistance reversal.

Differential Effect of Cathepsin L Inhibition on Doxorubicin-induced Expression of p21/WAF1 and Activation of Caspase-3 in Drug-sensitive and Drug-resistant Cells

Expression of p21/WAF1 was used as a marker to assess the ability of wild-type and doxorubicin-resistant cells to undergo senescence, and cleaved caspase-3 was used to identify apoptosis in these cells (18). Expression of these molecules was followed in the presence of increasing drug concentrations both in wild-type and drug-resistant cells (Fig. 3A). Although doxorubicin-induced expression of p21/WAF1 was initiated at 10⁻⁷ m for wild-type cells, it was elicited only at 10⁻⁶ m in their resistant counterparts. This observation is in agreement with our previous finding on the relative toxicity of doxorubicin (~100 times difference) between these two cell lines (19). Activation of caspase-3 followed a similar profile with a maximum at 10⁻⁶ m for wild-type and 10⁻⁷ m for the resistant cells (Fig. 3A). The decrease in expression of p21/WAF1 and its relationship to increased caspase-3 activity was also documented (18) and could be explained by the fact that p21/WAF1 has been reported to be a substrate for active caspases (30). Therefore, diminished expression of p21/WAF1 may be attributable to its degradation by active caspase-3.

The profiles for expression of p21/WAF1 and activated caspase-3 were both changed when cathepsin L inhibitor was added to the cells (Fig. 3B). At the doxorubicin concentration (10⁻⁸ m), cotreatment of drug-sensitive cells with this drug and the cathepsin L inhibitor did not affect significantly expression of p21/WAF1 when compared with cells treated with doxorubicin alone. However, in doxorubicin-resistant cells (Fig. 3B), cathepsin L inhibition enhanced expression of p21/WAF1 when combined with doxorubicin at a sublethal concentration of 10⁻⁷ m. These data suggested that the enhancement of doxorubicin sensitivity attributable to cathepsin L inhibition selectively affects drug-resistant cells.

Interestingly, treatment of drug-sensitive cells with 10⁻⁷ m doxorubicin in the presence of cathepsin L inhibitor led to decreased expression of p21/WAF1, and this was not associated with caspase-3 activation (Fig. 3B). In contrast, drug-resistant cells treated with
doxorubicin at $10^{-6}$ M in the presence of cathepsin L inhibitor displayed a decreased expression of p21/WAF1 that was associated with increased caspase-3 activation, suggesting a switch of cell death from senescence to apoptosis. These data indicate that enhancement of doxorubicin toxicity in response to cathepsin L inhibition affects drug-resistant cells only and highlight the specific role of senescence induction in initiating reversal of drug resistance.

**Potency of Cathepsin L Inhibition on Reversal of Drug Resistance**

MTT assay was performed to compare the effect of cathepsin L inhibition to other protease inhibitors on reversal of doxorubicin resistance in SKN-SH/R cells. As expected, molecules that were not able to induce senescence, such as lactacystin or the cathepsin B inhibitor (Fig. 2), did not affect cell response to doxorubicin after 4 days of incubation (Fig. 4A). However, Q-VD-OPH and the cathepsin L family inhibitors reversed resistance to doxorubicin in various degrees. Cathepsin L inhibitor was the most effective, suggesting that the corresponding enzyme could be the lead candidate in this process. The limited effects of cathepsin K and S inhibitors on drug toxicity may be explained by the fact that the corresponding enzymes may play a role in drug resistance, or that these inhibitors are nonspecific and can also inhibit cathepsin L.

An in vitro experiment in which the activity of purified cathepsin L was assayed in the presence of protease inhibitors was carried out to verify whether the effect of these inhibitors on the reversal of drug resistance correlates with their effects on the activity of this enzyme. Data shown in Fig. 4B indicate that cathepsin L inhibitor was the most effective, and that Q-VD-OPH and cathepsin K were also able to reduce strongly the activity of this enzyme. Their limited effect on the reversal of drug resistance may be explained by the lack of specificity toward cathepsin L. All together, these findings indicate that cathepsin L may represent a primary target in reversal of resistance to doxorubicin. The role of cathepsins K in this process, although not excluded, seems to be less important than cathepsin L.

**Validation for Other Drugs and Cancer Cell Lines**

The effect of cathepsin L inhibitor was tested on drug-sensitive and drug-resistant cell lines corresponding to various cancer types and also in relation to unrelated drugs. As shown in Fig. 5, this molecule was able to reverse doxorubicin resistance in cell lines such as the human neuroblastoma cell line Neuro2A/R, the osteosarcoma cells OSA/R, and the leukemia cell line HL60/R. No effect of the drug combination was noticed on doxorubicin toxicity in all of the four drug-sensitive cell lines, in concurrence with the data shown in Fig. 3B demonstrating a lack of...
effect of the drug combination on induction of p21/WAF1 expression or caspase-3 activation in wild-type SKN-SH cells.

The cellular response to non-anthracyclin agents, such as cisplatin and vinblastine, was also investigated (Fig. 6). Doxorubicin-resistant SKN-SH/R cells were not resistant to cisplatin. Interestingly, the cellular response to cisplatin was not significantly affected by cathepsin L inhibition in both doxorubicin-sensitive and -resistant cells. This represents an additional argument in favor of the observation made earlier (Fig. 5), indicating that cathepsin L inhibition enhances cytotoxic drug response only in drug-resistant cells. In this case, because there was no resistance to cisplatin, no resistance reversal should be expected. However, in the case of vinblastine, doxorubicin-resistant cells were also resistant to this drug (Fig. 6). More importantly, cathepsin L inhibition reversed this resistance. Overall, the data presented in Figs. 5 and 6 suggest that alteration of cathepsin L function or its related pathway could be associated with development of drug resistance to more than one chemotherapeutic agent and in various cancer types.

**Cathepsin L Small Interfering RNA (siRNA) Reverses Drug Resistance**

To determine the specificity of cathepsin L inhibition in drug resistance reversal, we have transfected doxorubicin-resistant cells with cathepsin L siRNA as an independent approach to chemical inhibition. The data presented in Fig. 7 indicate that transfection of OSA/R cells with cathepsin L siRNA results in almost complete suppression of the enzyme expression as shown by Western blot (Fig. 7, upper panel). Interestingly, expression of p21/WAF1 in response to doxorubicin was up-regulated in samples treated with cathepsin L inhibitor or with siRNA to cathepsin L, suggesting a potential relationship between this enzyme and p21/WAF1. Although cell treatment with doxorubicin or siRNA alone inhibited proliferation to a
certain extent, the combination of both inhibited cell proliferation by >80% (Fig. 7, lower panel). Similar results were obtained when doxorubicin was combined with the chemical cathepsin inhibitor. The data are in support of the previous findings (Fig. 4) and constitute an independent method to demonstrate the specificity of cathepsin L inhibition and its role in reversing drug resistance in cancer cells.

**Putative Mechanisms Leading to Reversal of Drug Resistance Through Cathepsin L Inhibition**

**Effect on Cellular Localization of Doxorubicin.** To determine whether cathepsin L inhibition affects intracellular drug distribution, we have measured cellular uptake and localization of doxorubicin by fluorescence microscopy in the presence and in the absence of the enzyme inhibitor. As shown in Fig. 8, in the absence of cathepsin L inhibitor, a relatively small accumulation of doxorubicin was observed in the cytoplasm of drug-resistant cells. The precise localization could be of lysosomal nature because anthracyclins, including doxorubicin, are basic drugs that have been shown to accumulate preferentially in acidic organelles such as lysosomes (31). In the presence of cathepsin L inhibitor, a remarkable increase of doxorubicin content, mainly in the nucleus, was observed (Fig. 8).

Because these cells also express the drug efflux P-glycoprotein as part of their resistance mechanism (19), drug efflux experiments were conducted to determine whether cathepsin L inhibition could affect the function of this pump. Fig. 8 indicated that cathepsin L inhibition did not prevent doxorubicin efflux. Similar experiments conducted with rhodamine-123, a known substrate for P-glycoprotein that accumulates mainly in the mitochondria (Fig. 8), indicated that cathepsin L inhibitor did not alter cytoplasmic localization of this dye and has no effect on its efflux; therefore, it may have no effect on P-glycoprotein function. The fact that specific siRNA to cathepsin L also reversed drug resistance constitutes an additional argument against the
implication of P-glycoprotein in this process. All together, these data suggest that cathepsin L could play a role in drug resistance by facilitating doxorubicin incorporation into lysosomes, thereby preventing it from accumulating in the nucleus.

**Direct Effect of Cathepsin L on p21/WAF1.** To address the possibility that increased expression of p21/WAF1 in the presence of the combination doxorubicin/cathepsin L inhibitor could be attributable to its protection from degradation by this enzyme, we have performed *in vitro* experiments to determine whether p21/WAF1 could be a substrate for cathepsin L. In these experiments, we have studied the cleavage of recombinant GST-tagged p21/WAF1 by purified cathepsin L. Data shown in Fig. 9 indicated that cathepsin L readily cleaves p21/WAF1, suggesting that this enzyme may participate in drug resistance by cleaving and inactivating cell cycle inhibitors that were induced by cytotoxic stress. This possibility may represent a putative mechanism by which cathepsin L participates in the prevention of drug toxicity.

**DISCUSSION**

In the present study, we describe a new pathway that may influence the decision of cancer cells to undergo cell death or survive the initial treatment with chemotherapeutic agents and ultimately become drug resistant. This pathway appeared to be dependent on changes in intracellular drug distribution or the ability of cells to destabilize tumor suppressor gene products that are usually induced in the presence of cytotoxic drugs. The cysteine protease cathepsin L in particular and probably other members of its close family appeared to play an important role in this process.

Cathepsins were, until recently, considered as housekeeping molecules. However, with the aid of mice knock-out technology, specific functions have been attributed to each one of these lysosomal proteases (23). Relative to other cathepsins, cathepsin L is implicated in many key cellular functions. For instance, it has been shown that transgenic mice lacking cathepsin L suffered from dilated cardiomyopathy (32), suggesting a critical role of this enzyme for cardiac morphogenesis and function. Neural loss and brain atrophy accompanied by early death were also reported in double knock-out mice lacking both cathepsins B and L (33). Interestingly, cardiomyocytes and neurons from these mice contained multiple, large, and apparently fused lysosomes characterized by storage of electron-dense degraded material. Cathepsin L deficiency was also associated with hair loss in mice (34, 35). In *Caenorhabditis elegans*, the loss of this enzyme was found to be associated with embryonic lethality (36). In light of these findings, it appeared that cathepsin L could be a key determinant in cellular survival and conceivably implicated in the modulation of cancer cell response to chemotherapy.

Treatment of doxorubicin-resistant cells SKN-SH/R with the combination Q-VD-OPH/doxorubicin (each drug at sublethal concentrations) forced cells to undergo cell cycle arrest with phenotypic features of senescence (Fig. 1). This effect cannot be attributed to inhibition of caspases because the doxorubicin concentration used did not activate caspase-3 (Fig. 1C), suggesting that senescence-initiated reversal of drug resistance could occur independently of cellular apoptotic status. The key finding derived from this study (Fig. 1C) is that inhibition of a cysteine protease(s) results in induction of p21/WAF1 expression and cell growth arrest, suggesting that the putative cysteine protease might be considered as a survival molecule. The proteasome and lysosomal cathepsins were considered as potential candidates, because these two families of proteases can be active, even in the absence of any death stimulus. Specific inhibitors were tested to discriminate between major lysosomal proteases and the proteasome in terms of their ability to protect against doxorubicin toxicity. Although the proteasome inhibitor lactacystin did not alter cellular response to doxorubicin, cathepsin L inhibitor was found to be very effective in enhancing drug toxicity and forcing cells to undergo senescence (Figs. 2 and 3). Increased expression of the cell cycle inhibitor p21/WAF1 and the SA-β-Gal in cells treated with doxorubicin and cathepsin L inhibitor favor a senescence-initiated reversal of drug resistance (Figs. 1–3).

The effect of cathepsin L inhibition on drug toxicity appeared to be a general phenomenon because it was valid for other murine and human drug-resistant cell lines (Fig. 5). The potency of drug resistance reversal was, however, cell type dependent, suggesting that either the level of cathepsin L or other associated factors may be implicated. Interestingly, this effect on drug toxicity was valid only for drug-resistant cells (Fig. 6). We have found that these two cell types express the same amount of cathepsin L (data not shown), suggesting that differences in activation of this enzyme, its cellular localization, its endogenous inhibitors, or its downstream targets may explain the relative susceptibility of drug-resistant and -sensitive cells to cathepsin L inhibition.

To determine the specificity of cathepsin L as a target to reverse drug resistance, siRNAs for this enzyme were used to reduce its expression in drug-resistant cells (Fig. 7). The results indicated that siRNA to cathepsin L were potent inhibitors of the enzyme expression, and their action resulted in up-regulation of p21/WAF1 expression and reversal of drug resistance. In light of these results, we conclude that the observed effect on drug toxicity of the chemical cathepsin L inhibitor was mainly attributable to its action on this enzyme.

It has been reported that basic drugs such as anthracyclins preferentially accumulate in acidic organelles such as lysosomes (31, 37). This accumulation was found to be greater in drug-resistant cells than their drug-sensitive counterparts (31), suggesting that lysosomal drug sequestration may be responsible, at least in part, for development of drug resistance. Along these lines, our results (Fig. 8) indicated that cathepsin L could be the key element responsible for facilitating doxorubicin accumulation in lysosomes, and therefore, its inhibition might result in free drug movement from lysosomes to nucleus and increased drug toxicity. Another possibility is that inhibition by cathepsin L could result in stabilization of p21/WAF1 (Fig. 9) and induction of senescence.

In conclusion, this investigation has introduced the concept that forcing cells to undergo senescence, even without induction of apoptosis, may be sufficient to mediate the reversal of drug resistance. Inhibition of cathepsin L appears to play a key role in acceleration of drug-induced senescence, particularly in drug-resistant cells, suggesting that this protease may act as a survival molecule associated with development of drug resistance. Control of intracellular drug distribution and/or cleavage and inactivation of p21/WAF1 represent potential explanations for the protective action of cathepsin L.
n novel property could validate cathepsin L as a potential therapeutic target to enhance chemotherapy efficacy.

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