Cathepsin L inhibition suppresses drug resistance in vitro and in vivo: a putative mechanism

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1Department of Pediatrics, Children’s Memorial Research Center, Children’s Memorial Hospital, Chicago; 2Molecular Pharmacology and Biological Chemistry, The Feinberg School of Medicine, Northwestern University, Chicago, Illinois; and 3Pharmaceutical Research Institute, Albany College of Pharmacy and Health Sciences, Rensselaer, New York

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Zheng X, Chu F, Chou PM, Gallati C, Dier U, Mirkin BL, Mousa SA, Rebbaa A. Cathepsin L inhibition suppresses drug resistance in vitro and in vivo: a putative mechanism. Am J Physiol Cell Physiol 296: C65–C74, 2009. First published October 29, 2008; doi:10.1152/ajpcell.00082.2008.—Cathepsin L is a lysosomal enzyme thought to play a key role in malignant transformation. Recent work from our laboratory has demonstrated that this enzyme may also regulate cancer cell resistance to chemotherapy. The present study was undertaken to define the relevance of targeting cathepsin L in the suppression of drug resistance in vitro and in vivo and to understand the mechanism(s) of its action. In vitro experiments indicated that cancer cell adaptation to increased amounts of doxorubicin over time was prevented in the presence of a cathepsin L inhibitor, suggesting that inhibition of this enzyme not only reverses but also prevents the development of drug resistance. The combination of the cathepsin L inhibitor with doxorubicin also strongly suppressed the proliferation of drug-resistant tumors in nude mice. An investigation of the underlying mechanism(s) led to the finding that the active form of this enzyme shuttles between the cytoplasm and nucleus. As a result, its inhibition stabilizes and enhances the availability of cytoplasmic and nuclear protein drug targets including estrogen receptor-α, Bcr-Abl, topoisozerase-II, histone deacetylase 1, and the androgen receptor. In support of this, the cellular response to doxorubicin, tamoxifen, imatinib, trichostatin A, and flutamide increased in the presence of the cathepsin L inhibitor. Together, these findings provided evidence for the potential role of cathepsin L as a target to suppress cancer resistance to chemotherapy and uncovered a novel mechanism by which protease inhibition-mediated drug target stabilization may enhance cellular visibility and, thus, susceptibility to anticancer agents.

Drug resistance; topoisozerase; histone deacetylase 1; estrogen receptor

THE DEVELOPMENT OF RESISTANCE TO CHEMOTHERAPY represents an adaptive biological response by tumor cells that leads to treatment failure and patient relapse. In recent years, it has become obvious that cancer cells can develop resistance not only to classical cytotoxic drugs but also to newly discovered targeted therapies (1, 27). Important progress has been made in identifying putative mechanisms responsible for this phenomenon (3), such as alterations in drug transport and drug- or target-modifying enzymes (9, 10, 19). Subsequently, a number of drug resistance-reversing agents affecting the above mechanisms were discovered, and, although they were very effective in vitro, most did not perform as well in vivo (31). In view of the significance of new drug discoveries in this area, research interest has been redirected toward the identification of alternative drug resistance mechanisms that can be targeted to enhance or at least to preserve the efficacy of existing therapies.

Recent findings from our laboratory have demonstrated that the cellular ability to escape from senescence (a state of irreversible growth arrest) plays an important role in the development of drug resistance (36). In the search for molecular targets to force cancer cells into senescence, we have identified lysosomal cathepsin L as a key player in this process and demonstrated that targeting this enzyme by either chemical inhibitors or short interfering (si)RNAs facilitated the reversal of resistance to doxorubicin and etoposide (36). These findings stimulated further inquiries to determine whether targeting cathepsin L could be used to prevent the onset of drug resistance, to define the validity of this approach with regard to other drugs in vitro and in vivo, and to dissect the underlying mechanism(s). Our results provided evidence that targeting cathepsin L alters the behavior of drug-resistant cancer cells in vitro and in vivo and uncovered a novel mechanism by which this enzyme facilitates the development of drug resistance, namely through proteolysis and the elimination of drug targets, rendering cancer cells “invisible” to drugs. Based on these findings, protease inhibition-mediated drug target stabilization was proposed as a mechanism to suppress resistance to chemotherapy in cancer.

MATERIALS AND METHODS

Human neuroblastoma SKN-SH and osteosarcoma SaOS2 cells were purchased from the American Type Culture Collection (Rockville, MD). Drug-resistant cells were generated by continuous incubation of parental cell lines with stepwise increases in drug concentrations over a period of 3–6 mo. Resistant cells were generated by continuous incubation of parental cell lines with stepwise increases in drug concentrations, ranging from 10−10 to 10−4M, over a period of 3–6 mo. At the end of the selection, cells were tested for resistance to drugs using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay as previously described (22). Briefly, cells were seeded at 104 cells/well in 96-well plates and incubated with the drug for 96 h. Ten microliters of MTT solution (5 mg/ml) was added to each well and incubated for 4 h at 37°C. Cells were then solubilized by the addition of 100 μl of 10% SDS + 0.01 M HCl and incubated for 15 h at 37°C. The optical density of each well was measured at 570 nm.

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determined in an ELISA plate reader using an activation wavelength of 570 nm and a reference wavelength of 650 nm. The percentage of viable cells was determined by a comparison with untreated control cells.

The following reagents were obtained from the companies cited: DMEM and FBS (BioWhittaker, Walkersville, MD); the cathepsin L inhibitor napsul-Ile-Trp-CHO (iCL; BioMol, Plymouth Meeting, PA); doxorubicin and anti-β-actin (Sigma, St. Louis, MO); reagents for siRNA transfection (Gene Therapy Systems, San Diego, CA); antibody to cathepsin L (Novus Biologicals, Littleton, CO); anti-topoisomerase (Topo)-IIα and anti-Bcr-Abl (Abcam, Cambridge, MA); anti-estrogen receptor-α (ERα; Bethesda Laboratories, Montgomery, TX); anti-histone deacetylase 1 (HDAC1) and anti-histone H3 (Cell Signaling Technologies); secondary antibodies conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA); enhanced chemiluminescence (ECL) reagents (Amersham, Arlington Heights, IL); and immobilon-P transfer membranes for Western blots (Millipore, Bedford, MA).

**Western Blot Analysis**

Cells were seeded in DMEM containing 10% FBS, and, after 24 h, doxorubicin and/or iCL were added to the culture medium and incubated for the indicated times. Cells were then lysed in 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 PMSF, and equal quantities of protein were separated by electrophoresis on a 12% SDS-PAGE gel and transferred to immobilon-P membranes. The expression of cathepsin L, Topo-IIα, β-actin, histone H1, ERα, HDAC1, and Bcr-Abl were identified by a reaction with specific primary antibodies (as described above) in PBS for 1 h, followed by a wash (3 times with PBS) and an incubation for 1 h with secondary antibodies linked to horseradish peroxidase. After an additional wash (3 times with PBS), reactive bands were detected by chemiluminescence (Bio-Rad).

**siRNA Design and Transfection**

Human cathepsin L siRNA was designed from the human cathepsin L cDNA sequence [5′-AAGTGGAAGGCGATGCACAAC-3′ (91–111)] and was synthesized by Dharmaco (Lafayette, CO). On the day before transfection, 3 × 10^5 drug-resistant osteosarcoma cells were seeded in six-well plates and grown in 2.5 ml of DMEM supplemented with 10% FBS. 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 Systems). After 48 h of incubation, cells were lysed, and protein extracts were used to detect cathepsin L, TopoIIα, and β-actin expression by Western blot analysis as described above.

**Measure of Intracellular Drug Accumulation**

Doxorubicin-sensitive and -resistant cells were seeded in 12-well plates and incubated for 24 h. Doxorubicin (1 μM) or rhodamin 123 (10 μM) were then added in the absence or presence of iCL (10 μM) and incubated for 30 min, after which cells were washed three times with PBS. Photographs were then taken under fluorescence microscopy. For doxorubicin, the excitation and emission wavelengths used were 480 and 560 nm respectively. For rhodamin 123, the excitation and emission wavelengths were 505 and 534 nm, respectively.

**PCR**

Cells cultivated in 25-cm² flasks were treated with iCL (20 μM) and incubated for the indicated times. RNA extraction was performed using the GeneAmp RNA PCR kit (Applied Biosystems) according to the manufacturer’s procedures. The primers consisted of the following sequences of the Topo-IIα gene: sense primer 5′-CACAACTGGCC-CTCCTCTTCTGCGAC-3′ and antisense primer 5′-GGGCAACCTTTACTTCTCGTTT-3′. PCR was performed for the amplification of the fragments as follows: 5 μl of 1:10 PCR mix (50 mM Tris·Cl, 440 mM KCl, and 12 mM MgCl2), 50 pmol of both sense and antisense primers, 2.5 μl of 2 mM dNTPs, 5 μl of cDNA, and 1 unit of Taq polymerase (Perkins-Elmer, Wellesley, MA) were mixed in a total volume of 50 μl. The cycling conditions were 2 min of pre-denaturation at 95°C followed by 33 cycles of 1-min denaturation at 95°C, 1 min of annealing at 53°C, and 1 min of extension at 72°C. The PCR products were separated on a 2% agarose gel.

**Animal Experiments**

The animal protocol used in this study was approved by the Animal Care and Use Committee of the Children’s Memorial Research Center (no. 2006-29). Nude mice (strain CD1, Charles River Laboratories, Wilmington, MA) of ~5–6 wk of age and weighing ~30 g received a subcutaneous implantation of drug-resistant cell lines (10^5 cells in 100 μl). When tumors were ~50 mm^3 in size, the animals were pair matched and divided into four groups of five mice as follows: 1) vehicle-treated controls, 2) mice treated with iCL (20 mg/kg), 3) mice treated with doxorubicin (1.5 mg/kg), and 4) mice treated with a combination of both drugs (iCL and doxorubicin). A total of three injections (separated by 3 days) were performed. Mice were weighed and checked for clinical signs of drug toxicity and lethality. Tumor measurements were made with a caliper every 3 days for up to 26 days and converted to tumor volumes using the formula \( W \times L^2/2 \) (where \( W \) is the width of the tumor mass and \( L \) is its length) to generate tumor growth curves.

**Statistical Analysis**

Data are expressed as means ± SE. Differences in measured variables between the experimental and control groups were assessed by Student’s t-test. Statistical calculations were performed using the Statview statistical package (Abacus Concepts, Berkeley, CA). P values of <0.05 were considered as statistically significant.

**RESULTS**

**Cathepsin L Inhibition Prevents the Development of Resistance to Doxorubicin**

To complement our previous findings on drug resistance reversal by cathepsin L inhibition, we determined whether this approach could also prevent cancer cells from becoming drug resistant. For this, human neuroblastoma (SKN-SH) and osteosarcoma (SaOS2) cell lines were chosen because the corresponding tumors are known for their aggressiveness and ability to become resistant to therapy (5, 18, 22). Cells, cultured in a 3T3 mode, were subjected to treatment with 10⁻⁹ M doxorubicin either alone or in combination with iCL (10 μM), and the surviving fraction was calculated after each passage (Fig. 1, A and B). Doxorubicin is a drug often used as a frontline therapy for neuroblastoma (4, 20) and osteosarcoma (7, 15). The choice of iCL (napsul-Ile-Trp-CHO) used in this study was based on our previous findings (36) showing that, among all the protease inhibitors tested, this molecule was the most potent in reversing resistance to doxorubicin. In addition, our previous results (36) indicated that this compound inhibited cathepsin L with high specificity. As shown in Fig. 1, in the presence of doxorubicin alone, both SKN-SH and SaOS2 cells readily adapted to increasing
drug amounts; however, when iCL was added to the culture medium, their adaptive ability was progressively lost. Non-treated cells as well as those treated with iCL alone continued to grow, suggesting that at the concentration used (10 \mu M), iCL alone had no significant effect on cellular proliferation.

**Cathepsin L Inhibition Reverses Drug Resistance at Various Stages of Its Development**

We also asked the question whether targeting cathepsin L could reestablish drug sensitivity to its initial level in cells that have reached a given stage of resistance. For this, we used cells that were generated by selection with increasing doxorubicin concentrations ranging from $10^{-9}$ to $10^{-6}$M. As shown in Fig. 2, cell lines with different levels of resistance were generated by this approach from neuroblastoma (SKN-SH; A) and osteosarcoma (SaOS2; B) cells. However, when the resulting cell lines were subjected to treatment with iCL, their resistance to doxorubicin was reversed (Fig. 2, C and D). In agreement with our previous findings (36), the combination of drug did not affect cellular responses in wild-type cells, suggesting that alterations in drug targets and/or drug availability may be responsible for the observed differences in cellular responses between drug-sensitive and -resistant cells.

Fig. 1. Prevention of drug resistance development by cathepsin L (CL) inhibitor napsul-Ile-Trp-CHO (iCL). SKN-SH (A) and SaOS2 (B) cells were divided into the following four groups: nontreated control (Ctl) cells; cells treated with doxorubicin (Dox), which exposed to stepwise increased Dox concentrations; cells continuously exposed to 10 \mu M iCL; and cells treated with both drugs (Dox + iCL), which were exposed to stepwise increased Dox concentrations in the presence of 10 \mu M iCL. After each passage, the surviving cellular fractions were compared. Data represent averages of 3 determinations ± SE.
Effect of Cathepsin L Inhibition on the Growth of Drug-Resistant Tumors In Vivo

To further define the relevance of cathepsin L inhibition on the suppression of drug resistance, we tested the toxicity versus efficacy of iCL either alone or in combination with doxorubicin in nude mice bearing xenografts of drug-resistant cancer cells. Mice were injected with the doxorubicin-resistant neuroblastoma cell line SKN-SH/R (~100 times more resistant than parental drug-sensitive cells), and, when the tumors became palpable, mice received three injections of doxorubicin alone, iCL alone, or a combination of both. The maximal tolerated dose for doxorubicin was 2.5 mg/kg; however, in the case of iCL alone, no toxicity was detected for up to 30 mg/kg (data not shown). With regard to the efficacy of these treatments, we found that doxorubicin alone (1.5 mg/kg) had no effect on tumor growth; in contrast, iCL at 20 mg/ml alone reduced tumor growth by ~40% (Fig. 3A). More importantly, the drug combination was effective in reducing tumor growth by ~90%, suggesting a synergistic effect between the two agents. This drug combination was well tolerated, and no significant weight loss was noticed in the treated animals during the experiments (data not shown).

Putative Mechanism(s) of Action

Implication of drug transporter P-glycoprotein in mediating the action of iCL. To determine whether the drug efflux transporter P-glycoprotein (P-gp) plays a role in mediating the action of iCL, we used the intrinsic fluorescence of doxorubicin and measured its cellular distribution in the absence or presence of iCL. As shown by the fluorescence microscopy results (Fig. 4A), in the absence of iCL, drug-sensitive cells accumulated more doxorubicin than their resistant counterparts. When iCL was added to the medium, total drug accumulation as well as its distribution did not change in the sensitive cells; however, a substantial increase was noted, particularly in the nucleus of drug-resistant cells (Fig. 4B). We observed that, although treatment with iCL reduced the number of live cells, it had no effect on the accumulation and/or redistribution of this substrate in drug-resistant cells. Furthermore, siRNA to cathepsin L (Fig.
4C) reversed resistance to doxorubicin (Fig. 4D) without affecting the expression of P-gp (Fig. 4C). These results led us to conclude that iCL-induced nuclear accumulation of doxorubicin in drug-resistant cells (Fig. 4A) occurred in a P-gp-independent manner.

Protease inhibition-mediated drug target stabilization as a potential mechanism. Based on the observation that iCL induces doxorubicin accumulation mainly in the nucleus of drug-resistant cells (Fig. 4A), we hypothesized that this might be due to an enhanced accumulation of Topo-IIα, a known target of doxorubicin. It has been shown that Topo-IIα amplification predicts a favorable treatment response to tailored and dose-escalated doxorubicin-based adjuvant therapy (30). Recent experimental evidence as well as numerous, large, multicenter trials have suggested that the amplification and deletion of Topo-IIα, respectively, accounts for the sensitivity and resistance to the commonly used cytotoxic drugs anthracyclines (2, 12). Based on this, approaches leading to increased Topo availability will likely facilitate drug accumulation into the nucleus and the rescue of resistance to doxorubicin. We analyzed the expression of this enzyme in drug-sensitive and -resistant cells as well as the effect of iCL treatment on its levels. The data shown in Fig. 5A indicated that the expression of Topo-IIα was indeed reduced in doxorubicin-resistant cells. Interestingly, this was accompanied by a concomitant increase of cathepsin L expression, suggesting that a negative regulatory relationship might exist between these two enzymes. In vitro experiments in which purified cathepsin L was incubated with Topo-IIα demonstrated that the former was able to cleave the latter and that this reaction was prevented by iCL (Fig. 5B). More importantly, these in vitro findings were confirmed in intact cells (Fig. 5C), as the decrease in the expression of endogenous Topo-IIα over time was inhibited by iCL. This effect was not mediated by alterations in Topo-IIα gene expression, as indicated by the PCR analysis (Fig. 5D) showing that the overall level of the corresponding mRNA was not affected over time or in response to iCL. Therefore, the decrease in Topo-IIα amounts shown in Fig. 5C might have occurred at the posttranslational level.

To verify whether the observed changes in Topo-IIα expression in response to iCL were not the result of alterations in cell numbers, we compared the expression of this enzyme in cells subjected to treatment with iCL, doxorubicin, or both. While the inhibitor alone had no detectable effect on cellular proliferation (Fig. 5E), it induced Topo-IIα accumulation (Fig. 5F). Inversely, doxorubicin, which exerted a dramatic inhibitory effect on cellular proliferation, did not significantly affect the levels of Topo-IIα (Fig. 5, E and F). Strikingly, the decrease in Topo-IIα was inversely proportional to the increased expression of cathepsin L (Fig. 5G), and siRNA to cathepsin L induced Topo-IIα accumulation (Fig. 5H). Inhibitors of cathepsin D and cathepsin B were unable to induce the accumulation of Topo-IIα (Supplemental Fig. S1). Together, these findings led us to conclude that the accumulation of Topo-IIα in response to iCL was a result of its stabilization and not due to increased expression of the corresponding gene. This effect of cathepsin L on Topo-IIα pools may help explain the observed effect of this enzyme on the control of the cellular response to doxorubicin and the development of drug resistance.

Cellular Localization of Cathepsin L and Its Regulation by the Cancer Cell Microenvironment

The findings shown in Fig. 5 raised two important hypotheses: 1) to cleave the nuclear enzyme Topo-IIα, cathepsin L must translocate to the nucleus; or 2) the observed accumulation of cathepsin L with cell density (Fig. 5G) may be caused by either an energy shortage or the accumulation of secreted factors in the medium. To address these two hypotheses, we incubated drug-resistant SaOS2 cells for 48 h in a medium deficient in either glucose or growth factors (no FBS). Western blot analysis indicated that cathepsin L expression could be detected in both the cytoplasmic and nuclear fractions (Fig. 6). Interestingly, its expression was only slightly affected by reduced glucose; however, the removal of growth factors from the media exerted

1 Supplemental material for this article is available online at the American Journal of Physiology-Cell Physiology website.
a strong inhibitory effect on the expression of this enzyme. These findings suggest that cathepsin L (or at least its active form) does translocate from the cytoplasm to the nucleus; therefore, it may affect the availability of protein drug targets in both cellular compartments. In addition to this, we showed that the expression of cathepsin L may be controlled by growth factors in the culture medium, providing further support for the notion that the cancer cell microenvironment plays an active role in the development of drug resistance.

**Cathepsin L Inhibition Reverses the Resistance to Various drugs Through the Accumulation of the Corresponding Protein Targets**

Based on the data presented above, we asked the question of whether this concept can be generalized. For this, we studied the effects of iCL on the cellular responses to tamoxifen, etoposide, imatinib, vinblastine, and trichostatin A and determined if these effects could be mediated through direct control of the corresponding protein targets. For comparison, we used cisplatin, a drug that targets DNA. The results indicated that except for cisplatin, iCL enhanced the cellular response to all of these drugs (Fig. 7A). The corresponding protein targets (Topo-IIα for doxorubicin, ERα for tamoxifen, and Bcr-Abl for imatinib) may represent natural substrates for cathepsin L, as the expression levels of some of these targets (particularly ERα and Bcr-Abl) were indeed reduced in drug-resistant cells and iCL was able to reverse this decrease (Fig. 7B). Similar experiments were conducted using prostate cancer cells (LNCap cells) in which cathepsin L inhibition was found to induce the accumulation of the androgen receptor and enhance the cellular response to the corresponding antagonist flutamide (Supplemental Fig. S2). These findings provided further evidence that protease-mediated drug target elimination may contribute to the development of drug resistance and suggest that approaches...
leading to an increased bioavailability of drug targets (i.e., through inhibition of their degradation) may represent a novel approach to enhance cancer cell visibility to drugs and improve chemotherapy efficacy.

**DISCUSSION**

The majority of, if not all, proteins are subject to hydrolysis, and proteases, by virtue of their ability to craft the cellular proteome landscape, are likely to play a decisive...
Cathepsin L is a lysosomal protease initially believed to be implicated in the maintenance of tissue homeostasis through the degradation of unwanted proteins (oxidized, misfolded, damaged, etc.). Studies during the last decade have revealed, however, that this enzyme may have specific functions linked to the occurrence of various illnesses, including cancer, neurodegenerative, metabolic, and infectious diseases (6, 8, 16, 28). Although cathepsin L knockout was not lethal in a mouse animal model, it was associated with organ dysfunctions (29, 32, 35), highlighting the relevance of this enzyme to proper functioning of the organism. With regard to its implication in cancer, early studies have identified cathepsin L as the most secreted protein from transformed fibroblasts (25, 33), and subsequent work demonstrated that its expression was upregulated in several cancers (14), supporting a putative role as a therapeutic target for this disease. Accordingly, approaches aiming to reduce cathepsin L activity using specific chemical inhibitors or in conjunction with neutralizing antibodies were found to inhibit cancer cell proliferation (13, 24). In addition, recent work from our laboratory provided evidence that cathepsin L may play a key role in the development of drug resistance (36), thus identifying a novel function for this enzyme as a potential cause for cancer recurrence. The present investigation was designed to further define the underlying mechanism of cathepsin L action and evaluate the usefulness of its targeting for therapeutic purposes. The data presented here confirmed our previous observations and demonstrated that cathepsin L inhibition not only reverses but also prevents the development of drug resistance in vitro (Fig. 1).

Interestingly, regardless of the resistance level that cancer cells might have reached, their sensitivity to drugs is restored upon inhibition of cathepsin L (Fig. 2). These in vitro findings were also valid in vivo (Fig. 3), and a unifying mechanism to explain cathepsin L inhibition-mediated suppression of drug resistance in cancer was identified (Figs. 5–7).

Historically, the drug resistance phenomenon is almost as old as the discovery of the first anticancer agents. Over the decades, different mechanisms have been postulated, but most attempts to reverse this phenomenon have failed in vivo despite their success in vitro (31). This, in addition to the fact that tumor cells can develop resistance to virtually any type of stress, including the newly discovered targeted therapies, highlighted the need for the discovery of novel drug resistance-reversing agents to enhance or at least to preserve chemotherapeutic efficacy. Taking into consideration the fact that most anticancer agents are often designed to target molecules differentially expressed in tumor versus normal tissues, one way that cancer cells can escape drug toxicity would be to reduce the amounts of drug target(s) and become “invisible” to the drug. This can be accomplished either by silencing the expression of the corresponding gene or by physically eliminating the already expressed gene product through proteolytic degradation. Examples related to the first possibility comprise mutation in the ER gene shown to accompany the development of resistance to tamoxifen (21, 23) and mutation of the Bcr-Abl gene associated with resistance to the tyrosine kinase inhibitor Gleevec (34). Other genetic alterations leading to the silencing of drug response genes (apoptotic genes) of Bax and caspases have been also reported to be implicated in the development of resistance to chemotherapy (17). Curiously, the role of target elimination by proteolysis in mediating drug resistance has not yet been investigated.

Our finding that doxorubicin accumulation was enhanced in the nucleus of drug-resistant cells in response to treatment with iCL suggested that the drug transporter P-gp may be implicated. However, since the accumulation of rhodamine 123 was not affected by iCL (Fig. 4), and since siRNA to cathepsin L reversed drug resistance without affecting the expression of P-gp, this transporter may not play a significant role in mediating the action of iCL on doxorubicin accumulation in the nucleus. An alternative hypothesis was that iCL may induce the accumulation of the doxorubicin target Topo-IIα. The data shown in Fig. 5 and Supplemental Fig. S1 indicated that this was indeed the case. More importantly, a direct regulatory relationship between cathepsin L and Topo-IIα was demonstrated in vitro and in intact cells (Fig. 5), suggesting that iCL-induced drug target stabilization may represent a logical explanation for the reversal of drug resistance by this approach. Our findings (Fig. 6) are also in support of the recent discoveries that cathepsin L function is not solely limited to lysosomes but that active isoforms of this enzyme can be also found in the cytoplasm (26), the nucleus (11), and even in the extracellular matrix (3a). Since each one of these cellular compartments contains specific protein drug targets and since the iCL used in this study can easily diffuse through the plasma membrane to interact with the enzyme inside and outside the cell, this compound may stabilize drug targets in different cellular compartment. Examples for this are provided by the findings that nuclear and cytoplasmic drug targets, including ERα, HDAC1, Bcr-Abl, and the androgen receptor, all accumulated in cells exposed to iCL and, as a result, the...
cellular response to the corresponding drugs was enhanced (Fig. 7 and Supplemental Fig. S2).

An interesting aspect of using iCL for the treatment of drug-resistant cancers is the lack of its toxicity, as mice treated with up to 60 mg/kg displayed no signs of toxic effects. The in vivo synergistic reaction shown in Fig. 3 between this inhibitor and doxorubicin suggests that this approach may hold great promise as a therapeutic strategy to suppress resistance to chemotherapy. Further validation of these findings, with respect to additional drug targets and tumor types, is, however, needed to fully establish the concept that protease inhibition-mediated drug target stabilization may be used as an alternative approach to enhance chemotherapy efficacy.
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