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The Role of C/EBP-β LIP in Multidrug Resistance

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Abstract

Background: Chemotherapy triggers endoplasmic reticulum (ER) stress, which in turn regulates levels of the active (LAP) and the natural dominant-negative (LIP) forms of the transcription factor C/EBP-β. LAP upregulates and LIP downregulates the multidrug resistance (MDR) protein P-glycoprotein (Pgp), but it is not known how critical is their role in establishing MDR.

Methods: Cell viability was quantitated by crystal violet staining and measuring absorbance at 540 nm. Expression of various proteins was determined by immunoblotting. mRNA levels were determined by quantitative reverse transcriptase polymerase chain reaction (RT-PCR). LIP and LAP were overexpressed using expression plasmids followed by selection with blasticidin. Tumor cells expressing doxycycline-inducible LIP were orthotopically implanted in mice (n = 15 mice per group), and tumor size was measured daily by caliper. Tumor sections were stained with hematoxylin and eosin and immunostained for Pgp, proliferation, and ER stress markers.

Results: MDR cells do not express basal, chemotherapy-triggered, or ER stress–triggered LIP and fail to activate the CHOP-caspase-3 death-triggering axis upon ER stress or chemotherapy challenge. Overexpression of LIP reversed the MDR phenotype in vitro and in tumors implanted in mice. LIP was undetectable in MDR cells, probably due to its ubiquitination, which was 3.56-fold higher, resulting in lysosomal and proteasomal degradation of LIP.

Conclusions: Spontaneous and drug-selected MDR cells lack LIP, which is eliminated by ubiquitin-mediated degradation. Loss of LIP drives MDR not only by increasing Pgp expression but also by a two-fold attenuation of ER stress–triggered cell death.

Rapidly growing solid tumors are characterized by lagging angiogenesis, leading to hypoxia, nutrient deprivation, and accumulation of toxic metabolites. These pathophysiological conditions trigger endoplasmic reticulum (ER) stress (1). The ability of tumor cells to resist stress is a hallmark of aggressive cancers (2,3). The cellular response to ER stress is termed the unfolded protein response (UPR). During early UPR, cells adapt to stress (1). If ER stress persists, proapoptotic proteins such as CHOP (GADD153) and TRB3 are induced (4). Despite different modes of action, many chemotherapeutic drugs—eg, anthracyclines (5), cisplatin (6), oxaliplatin (5), 5-fluorouracil (7), paclitaxel (8)—share the ability to trigger ER stress (9). Interestingly, chemotherapy efficacy is associated with increased ER stress (10,11). Hence, activation of the ER-dependent cell death machinery mediates part of the antiproliferative activity of numerous chemotherapeutic agents.

The frequently observed constitutive or drug-induced multidrug resistance (MDR) is often established by overexpression of integral membrane ATP binding cassette (ABC) drug efflux transporters, such as P-glycoprotein (Pgp/ABCB1), MDR-related
proteins 1–9 (MRP/ABCC1-9), and breast cancer resistance protein (BCRP/ABCG2) (12,13). Pathophysiologic conditions promoting ER stress (14,15) induce the expression of Pgp by unknown mechanisms (15,16).

The transcription factor C/EBP-β, a crucial activator of the proapoptotic CHOP axis (4), is produced in three isoforms, translated from the same mRNA: the liver-enriched transcriptional activator proteins LAP and LAP* and the natural dominant negative, truncated transcriptional repressor liver-enriched inhibitory protein LIP (17). We recently found that LAP promotes tumor progression by attenuating ER–stress–triggered cell death, whereas LIP exerted opposite effects (18). Of note, LAP activates the transcription of Pgp, whereas LIP inhibits its expression (19).

The aim of this study was to investigate the possible linkage between MDR and ER stress and the role of ER stress–triggered induction of C/EBP-β LIP and LAP in MDR, in chemosensitive and chemoresistant cancer cells of different histological origin and species.

Methods

Cell Lines

HT29/MDR and A549/MDR cells were generated from parental cells as described (20). Human HT29, A549, JC (21), and Caco-2 (22) cells were from ATCC (Rockville, MD). Please see the Supplementary Methods (available online) for more details.

Cell Viability and Growth

Counting of viable cells was performed in quadruplicates and repeated four times as reported (18). Please see the Supplementary Methods (available online) for more details.

In Vivo Tumor Growth

1 x 10⁶ JC TetON LIP cells in 20 µl culture medium mixed with 20 µl Cultrex BME (Trevigen, Gaithersburg, MD) were orthotopically implanted according to (23) in six-week-old female BALB/c mice (15/group). The Institutional Animal Care and Use Committee of the Weizmann Institute of Science approved all animal protocols. Please see the Supplementary Methods (available online) for more details.

Statistical Analysis

All data in the text and figures are provided as means ± SD. The results were analyzed by two-sided analysis of variance (ANOVA). A P value of less than .05 was considered statistically significant.

Results

The Role of MDR in Resistance to ER Stress

We analyzed the response to chemotherapeutic agents and ER stress inducers in the human chemosensitive HT29 and A549 cells, in the chemoresistant counterpart (HT29/MDR and A549/MDR cells), in the constitutively chemoresistant murine JC cells and in human Caco-2 cells. HT29 cells basally express Pgp, MRP1, MRP2, and MRP3; A549 cells basally express MRP1, in line with the pattern of ABC transporters physiologically present in colon and lung mucosa (13). HT29/MDR and A549/MDR cells express higher levels of these transporters and also express MRP5 and BCRP. JC cells constitutively express Pgp and low levels of BCRP; Caco-2 cells constitutively express Pgp, MRP1, and MRP2 (Supplementary Figure 1, available online).

As expected, viability of HT29/MDR cells was higher than HT29 cells after exposure to irinotecan, a substrate of Pgp (24), 5-fluorouracil, whose resistance has been associated with Pgp, MRP1 and MRP5 (25–27), and oxaliplatin, whose resistance has been associated with Pgp, MRP1, and MRP4 (survival of HT29/MDR vs HT29 cells: P = .04 for the lowest dose of each drug, P = .03 for the intermediate and highest doses) (Figure 1A, upper panels) (28,29). HT29/MDR cells were also more resistant to ER stress inducers, including thapsigargin, tunicamycin, and breflidin A (Figure 1A, lower panels). Chemotherapeutic drugs and ER stress inducers reduced cell number (Figure 1B) and growth (Figure 1C) in HT29 cells, but not in HT29/MDR cells (P = .04 at 48 hours; P = .006 at 72 hours) (Figure 1, B and C).

Comparison of basal, ER stress-induced, and chemother-apy-induced expression of the ER stress sensors ATF6, IRE1α, phospho(Ser724)IRE1α, PERK, phospho(Thr981)PERK, eIF2α, phospho(Ser51)eIF2α did not show any difference between HT29 and HT29/MDR cells (Figure 2). Triggers of ER stress and chemo-therapeutic agents induced CHOP/GADD153, TRB3, HERPUD1, cleaved caspase-3, and both C/EBP-β LIP and LAP in HT29 cells, with different kinetics (Figure 2; Supplementary Figure 2, available online). By contrast, they did not induce C/EBP-β LIP, CHOP/GADD153, TRB3, HERPUD1, and cleaved caspase-3 in HT29/MDR cells (Figure 2; Supplementary Figure 2, available online).

Because Pgp transports chemotherapeutic drugs, but also toxins and xenobiotics (13), it could potentially efflux thapsigargin, tunicamycin, and brefildin A from HT29/MDR cells. Therefore, we examined the possible role of Pgp in the ER stress resistance phenotype of MDR cells. The Pgp inhibitor verapamil restored the intracellular accumulation of rhodamine 123 and doxorubicin in HT29/MDR cells (Supplementary Figure 3A, available online), but did not restore the toxicity of the ER stress inducers (Supplementary Figure 3B, available online), indicating that the increased activity of Pgp in HT29/MDR cells does not explain the resistance to ER stress–triggered cell death.

Thapsigargin, tunicamycin, and brefildin A modulate ABC transporters activity and expression (15,30–32). Nevertheless, we found that in HT29 and HT29/MDR cells these agents did not change the efflux activity of Pgp, did not compete with rhodamine 123 for the efflux through Pgp, and did not change the expression of Pgp, MRP1, and MRP2 at the mRNA and protein levels (Figure 2; Supplementary Figure 4, available online). Hence, the increased resistance of HT29/MDR cells to ER stress was not because of increased efflux of these ER stress inducers, neither was it due to higher expression of Pgp. The same dual resistance to chemotherapeutic drugs and to ER stress inducers was observed also with A549/MDR cells, with the constitutively chemoresistant JC cells and with Caco-2 cells. For each cell type, we studied the chemotherapeutic agent that serves as first-line treatment choice of the respective tumor type. Importantly, each cell type exhibited a different C/EBP-β LIP/LAP ratio (data not shown).

C/EBP-β gene expression is regulated by many distinct transcription pathways, transcriptional inducers and repressors (33). Such multiplicity of regulatory cues may explain the differences in C/EBP-β LAP expression levels in the different cell types. In contrast with C/EBP-β LAP, ER stress and chemotherapy did not trigger considerable induction of C/EBP-β LIP in the drug-resistant cells. This behavior led us to hypothesize that the C/EBP-β LIP/LAP ratio serves as a key mediator of the resistance to chemotherapy and to ER stress.
Figure 1. Effects of chemotherapy and endoplasmic reticulum stress inducers on chemosensitive and chemoresistant cells. A) Human chemosensitive colon cancer HT29 cells and chemoresistant HT29/MDR cells were incubated for 48 hours in the absence (0) or presence of increasing concentrations of irinotecan (CPT11), 5-fluorouracil (5FU), oxaliplatin (oPt), thapsigargin (Tg), tunicamycin (Tu), brefeldin A (Bfa). Cell viability was assessed in quadruplicate by neutral red staining. Data are presented as means ± SD (n = 4). *P ≤ .03, ≤.006 and ≤.006 for cells treated with low, intermediate, or high doses of the various agents vs untreated (“0”) cells. °P ≤ .04, ≤.03 and ≤.03 for HT29/MDR vs HT29 cells treated with low, intermediate, or high doses of the various agents (two-sided analysis of variance [ANOVA]). B) Cells (10⁵/well) were grown for 48 hours in 96-well plates in media without (Ctrl) or with irinotecan (10 μM), 5-fluorouracil (5 μM), oxaliplatin (5 µM), thapsigargin (50 nM), tunicamycin (1 μM), or brefeldin A (50 nM), then stained with crystal violet and photographed (upper panels, 1x; lower panels, bright field microscopy). For each experimental point, a minimum of five microscopic fields were examined. Bars = 500 µM. C) Cells (50,000/well) were cultured in 96-well plates, in media without (Ctrl) or with the indicated agents at concentrations as in (B). Cell counts were determined in quadruplicate following fixation and crystal violet staining at the indicated times. Data are presented as mean ± SD (n = 4). *P < .001 for all drugs: treated cells vs untreated (Ctrl) cells; °P ≤ .04 (48 hours), °P ≤ .006 (72 hours) for HT29/MDR cells vs HT29 cells (two-sided ANOVA).
The Role of LIP/LAP Ratio in the Cellular Response to ER Stress and Chemotherapy

To verify the role of LAP/LIP ratio in drug resistance, we focused on HT29/MDR cells and on JC cells, two models of acquired and constitutive MDR, respectively, with clearly detectable levels of C/EBP-β LAP and Pgp.

First, we overexpressed C/EBP-β LAP in chemosensitive HT29 cells (Figure 3A, upper panel) and C/EBP-β LAP in chemoresistant HT29/MDR cells (Figure 3A, lower panel). LAP overexpression increased cell viability of control HT29 cells and prevented cell death induced by brefeldin A (cell survival: mean ± SD = 110.5 ± 8.9%), irinotecan (cell survival: mean ± SD = 91.8 ± 3.2%), 5-fluorouracil (cell survival: mean ± SD = 95.7 ± 3.3%) and oxaliplatin (cell survival: mean ± SD = 95.0 ± 2.5%), P < .04 vs untransfected cells in all these experimental conditions (Figure 3, B and C, left panels). However, LAP overexpression did not affect the viability of drug-treated HT29/MDR cells (Supplementary Figure 5, available online). In contrast, LIP overexpression sensitized HT29/MDR cells to brefeldin A (cell survival: mean ± SD = 54.7 ± 2.7%), irinotecan (cell survival: mean ± SD = 48.8 ± 2.1%), 5-fluorouracil (cell survival: mean ± SD = 56.0 ± 5.5%), and oxaliplatin (cell survival: mean ± SD = 53.4 ± 3.7%, P < .04 vs untransfected cells in all these experimental conditions) (Figure 3, B and C, right panels).

Figure 2. Expression of endoplasmic reticulum (ER) stress–associated proteins in chemosensitive and chemoresistant cells under ER stress or chemotherapy. HT29 and HT29/MDR cells were incubated 48 hours in media without (Ctrl) or with thapsigargin (50 nM, Tg), tunicamycin (1 μM, Tun), brefeldin A (50 nM, Bfa), irinotecan (10 μM, CPT11), 5-fluorouracil (5 μM, 5FU), or oxaliplatin (5 μM, oPt). Cell extracts were resolved by SDS-PAGE and immunoblotted with specific antibodies for ATF6, IRE1α, phosphorylated IRE1α (IRE1α), PERK, phosphorylated PERK (Thr1089/Thr1122), eIF2α, phosphorylated eIF2α (eIF2α), C/EBP-β (recognizing the common C-terminal peptide of LAP and LIP), CHOP, TRB3, HERPUD1, caspase-3 (recognizing both pro-caspase-3 and cleaved caspase-3), Pgp, MRP1, MRP2. An anti-actin antibody was used as a loading control.
We next generated HT29/MDR clones stably expressing a doxycycline-inducible C/EBP-β LIP (“HT29/MDR TetON LIP” cells). Induction of LIP increased the expression of CHOP, TRB3, and cleaved caspase-3 (Figure 4A) and reduced cell viability (Figure 4B). LIP induction decreased cell proliferation as measured at 72 hours (mean ± SD 197 000 ± 10 000 vs 256 000 ± 4000 cells, P = .01). LIP induction attenuated drug resistance and increased the susceptibility to ER stress triggered cell death.
as measured at 72 hours (Brefeldin A: mean ± SD = 95,000 ± 10,000 vs 235,000 ± 13,000, P = .001; irinotecan: mean ± SD = 85,000 ± 11,000 vs 247,000 ± 13,000 cells, P < .001; 5-fluorouracil: mean ± SD = 116,000 ± 10,000 vs 240,000 ± 10,000 cells, P = .002; oxaliplatin: mean ± SD = 136,000 ± 11,000 vs 257,000 ± 12,000 cells, P = .003) (Figure 4C). LIP induction did not modify the cell cycle distribution of HT29/MDR cells, with the exception of the increased percentage of cells in sub-G1 phase (13.0 ± 3.0% with doxycycline vs 5.0 ± 1.0% cells without doxycycline, P = .04) (Supplementary Figure 6, available online), suggestive of enhanced apoptosis.

Doxycycline-mediated induction of LIP in HT29/MDR TetON LIP cells upregulated mRNA expression of CHOP, TRB3, PUMA, BIM, P62, ATG5, ATG10 and downregulated the expression of ATF4, GADD34, ASNS, SNAT2, CAT1, NARS, BCL2, HMOX1, ATG3, ATG12, MAP1LC3B (P < .001 for induced vs uninduced cells for all of these genes; Supplementary Figure 7A, available online). This pattern of gene expression confirmed earlier observations (34, 35), which attributed to C/EBP-β LIP a role in inducing apoptosis, controlling the UPR, autophagy, amino acid and protein synthesis/metabolism, and redox regulation (Supplementary Figure 7B, available online). The ER stress inducer brefeldin A further increased the expression of C/EBP-β LIP in the HT29/MDR TetON LIP clone (Figure 4A), thereby augmenting LIP activities. In the absence of doxycycline, brefeldin A, which did not induce C/EBP-β LIP in HT29/MDR cells (Figure 4A), did not modify statistically significantly the expression of the above mentioned genes (Supplementary Figure 7A, available online).

Transient overexpression of C/EBP-β LIP in HT29 cells elevated Pgp mRNA level by 3.54-fold (P < .001) (Figure 5A) and protein (Figure 5B), and increased the resistance to the Pgp substrates vinblastine (cell survival: mean ± SD = 87.0 ± 5.1% vs 61.2 ± 7.1%, P = .04) and etoposide (cell survival: mean ± SD = 81.2 ± 5.1% vs 43.1 ± 7.0%, P = .008) (Figure 5D). By contrast, transient or stable overexpression of C/EBP-β LIP in HT29/MDR cells downregulated Pgp mRNA levels by 4.2- and 4.73-fold (P < .001), respectively, (Figure 5, A and B), as well as Pgp protein (Figure 5C). Similar results were obtained with vinblastine (Transient LIP overexpression: mean ± SD = 52.8 ± 7.2% vs 92.3 ± 7.2%, P = .02; inducible LIP overexpression: mean ± SD = 42.1 ± 3.4% vs 90.3 ± 5.3%, P = .004). These cells exhibited similar responses to etoposide as well (Transient LIP overexpression: mean ± SD = 41.2 ± 4.3% vs 91.3 ± 4.7%, P = .008; inducible LIP overexpression: mean ± SD = 43.0 ± 3.2% vs 89.1 ± 2.2%, P = .003) (Figure 5D).

Stable overexpression of LIP in the constitutively chemoresistant JC cells activated the CHOP/TRB3/caspase-3 axis in response to tunicamycin, doxorubicin, or paclitaxel, and also downregulated C/EBP-β LAP (Supplementary Figure 8A, available online). LIP overexpression decreased survival of tunicamycin-treated JC cells (mean ± SD = 31.2 ± 2.2% vs 88.3 ± 4.3%, P = .002), of doxorubicin-treated cells (mean ± SD = 59.1 ± 4.2% vs 89.6 ± 3.3%, P = .01), and of paclitaxel-treated cells (mean ± SD = 46.3 ± 3.4% vs 81.7 ± 2.9%, P = .009) (Supplementary Figure 8, B and C, available online). Similarly, LIP overexpression decreased the replication rate (measured at 72 hours) of tunicamycin-treated JC cells (mean ± SD = 46,000 ± 8,000 vs 334,000 ± 14,000 cells, P < .001), of doxorubicin-treated cells (mean ± SD = 107,000 ± 12,000 vs 367,000 ± 4,000 cells, P < .001), and of paclitaxel-treated cells (mean ± SD = 75,000 ± 14,000 vs 325,000 ± 10,000 cells, P < .001) (Supplementary Figure 8D, available online). In parallel, LIP overexpression reduced Pgp expression by 34.0% (P = .04), and increased doxorubicin intracellular retention (Supplementary Figure 8, E-G, available online).

To study the impact of C/EBP-β LIP on drug resistance in vivo, we orthotopically implanted JC TetON LIP cells in BALB/c mice, and treated them with vehicle or doxorubicin, with or without doxycycline in the drinking water. Doxorubicin alone had little effect on the tumor mass; induction of C/EBP-β LIP by doxycycline was sufficient to reduce tumor progression, and doxorubicin further reduced tumor size in mice receiving doxycycline (mean ± SD = 401 ± 137 mm³ vs 1570 ± 167 mm³ in untreated mice at day 15, P < .001) (Figure 6, A and B). Doxorubicin alone did not reduce statistically significantly the extent of cell proliferation and did not induce detectable signs of ER stress, as determined by immunostaining of Ki67 and HERPUD1, but upregulated Pgp in tumors, leading to drug resistance (Figure 6, C and D). Induction of C/EBP-β LIP reduced tumor cell proliferation and increased ER stress, without affecting Pgp levels. The extent of tumor cell proliferation in mice receiving both doxycycline and doxorubicin was similar to that of doxycycline alone, whereas the extent of ER stress was further increased as determined by the ER stress marker HERPUD1. Induction of Pgp by doxorubicin was induced upon doxycycline-mediated induction of LIP, thereby providing one mechanism by which LIP might augment the cytotoxic activity of doxorubicin (Figure 6, C and D).

Post-Transcriptional Regulation of C/EBP-β LIP

We then investigated the molecular mechanisms underlying the lack of C/EBP-β LIP in chemotherapy-treated MDR cells. Sequencing of genomic C/EBP-β revealed that the chemosensitive HT29 cells and the chemoresistant HT29/MDR cells had identical DNA sequence in the ORF, 5'-UTR, 3'-UTR, and promoter regions (data not shown).

To check the stability of C/EBP-β mRNA or protein, we then treated HT29 and HT29/MDR cells with the transcription inhibitor actinomycin D, which time dependently decreased C/EBP-β mRNA, both in the absence or presence of irinotecan. C/EBP-β LAP protein also showed a progressive decrease, without appreciable differences between HT29 and HT29/MDR cells. LIP, which was induced by irinotecan in chemosensitive cells, progressively decreased in response to actinomycin D but was undetectable in HT29/MDR cells at all time points. A similar trend was observed in HT29 and HT29/MDR cells treated with the translation inhibitor cycloheximide. These results excluded that chemosensitive and chemoresistant cells differed in the stability of their C/EBP-β mRNA or protein (data not shown).

We found that the expression of ER quality control (ERQC) genes, often associated with increased protein ubiquitination (36), was attenuated in both HT29/MDR and A549/MDR cells (data not shown). Therefore, we compared the extent of C/EBP-β LAP and LIP ubiquitination in chemosensitive vs MDR cells. Indeed, C/EBP-β LIP mono-ubiquitination was 3.56-fold higher in MDR cells compared with the chemosensitive cells (P < .001), whereas the same level of mono-ubiquitinated C/EBP-β LAP was observed in these cells (Figure 7A). Inhibition of lysosomal degradation by leupeptin or ammonium chloride as well as inhibition of proteasomal degradation by MG132 prevented the disappearance of C/EBP-β LIP in the MDR cells (Figure 7, B and C, right panels). Irinotecan did not change the extent of C/EBP-β LIP mono-ubiquitination; however, it increased C/EBP-β LAP poly-ubiquitination (Figure 7, B and C, left panels).
Figure 4. The impact of C/EBP-β LIP on sensitivity of chemoresistant cells to endoplasmic reticulum stress and to chemotherapy. A) HT29/MDR TetON LIP cells, stably transfected with the inducible pcDNA4/TO expression vector for C/EBP-β LIP, were cultured for 48 hours in media without (- Doxy) or with 1 μg/mL doxycycline (+ Doxy), in the absence (Ctrl) or presence of brefeldin A (50 nM, Bfa), irinotecan (10 μM, CPT11), 5-fluorouracil (5 μM, 5FU), or oxaliplatin (5 μM, oPt). Cell extracts were resolved by SDS-PAGE and immunoblotted with specific antibodies for C/EBP-β (recognizing the C-terminal peptide), CHOP, TRB3, and caspase-3 (recognizing both procaspase-3 and cleaved caspase-3). Blotting with an anti-actin antibody served as a load control. B) HT29/MDR TetON LIP cells (10⁵/well) were cultured in 96-well plates with the indicated agents as in (A). The cells were then stained with crystal violet and photographed (upper panels, 1x; lower panels, bright field microscopy). For each experimental point, a minimum of five microscopic fields were examined. Bars = 500 μM. C) HT29/MDR TetON LIP cells (50 000/well) were cultured in 96-well plates and treated as in (A). The cell number was determined following fixation and crystal violet staining in quadruplicate at the indicated times. Data are presented as mean ± SD (n = 4). *P = .04 (24 hours), *P = .01 (48 hours), *P = .01 (72 hours) for untreated (Ctrl) “+Doxy” cells vs untreated (Ctrl) “-Doxy” cells; °P = .02 (48 hours), °P = .003 (72 hours), for “+Doxy” cells treated with Bfa vs “-Doxy” cells; °P = .001 (72 hours), for “+Doxy” cells treated with CPT vs “-Doxy” cells; °P = .02 (48 hours), °P = .002 (72 hours), for “+ Doxy” cells treated with 5FU vs “+ Doxy” cells; °P < .001 (72 hours), for “+ Doxy” cells treated with oPt vs “- Doxy” cells (two-sided analysis of variance).
Figure 5. The impact of C/EBP-β LIP on Pgp levels.

A) HT29 and HT29/MDR cells were either nontransfected (-), transfected with empty pcDNA4/TO vector (em), with a pcDNA4 expression vector for C/EBP-β LAP or with a pcDNA4 expression vector for C/EBP-β LIP. Total RNA was extracted at 48 hours post-transfection, reverse-transcribed and subjected to quantitative reverse transcriptase (qRT-PCR) polymerase chain reaction for Pgp, MRP1, and MRP2 genes. Measurements were performed in triplicate and data are presented as means ± SD (n = 3) vs the respective untreated (-) cells: *P < .001, for HT29 and HT29/MDR cells overexpressing LAP or LIP vs untransfected cells (two-sided analysis of variance [ANOVA]).

B) HT29/MDR cells stably transfected with the inducible pcDNA4/TO expression vector for C/EBP-β LIP (HT29/MDR TetON LIP) were grown in media without (-) or with (+) doxycycline (Doxy; 1 μg/mL) for 48 hours. Total RNA was extracted, reverse-transcribed, and subjected to qRT-PCR for Pgp, MRP1, and MRP2 genes. Measurements were performed in triplicate and data are presented as means ± SD (n = 3) vs the respective untreated (-) cells: *P < .001 (two-sided ANOVA).

C) Extracts of cells treated as in (A) and (B) were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Anti-actin served as a load control.

D) Cells treated as in (A) and (B) were grown for 48 hours in media without (Ctrl) or with the Pgp substrates vinblastine (1 μM, Vbl) or etoposide (1 μM, Eto). Cell viability was assessed by neutral red staining. Data are presented as means ± SD (n = 4). °P = .04 and .008 for LAP-expressing cells treated with vinblastine or etoposide vs untreated (-) cells. °°P = .02, .004, and .003 for the LIP-expressing HT29/MDR cells, untreated, treated with vinblastine, or with etoposide vs untreated (-) cells. For HT29/MDR TetON LIP cells: *P = .02, .004, and .003 for Doxy vs -Doxy treated HT29/MDR TetON LIP control cells, vinblastine (Vbl)- or etoposide (Eto)-treated cells (two-sided ANOVA).
Figure 6. The impact of C/EBP-β LIP induction on tumor growth in vivo. Six-week-old female BALB/c mice bearing a 100 mm³ tumor of constitutively chemoresistant JC TetON LIP cells were treated on days 0, 6, and 12 with saline (Ctrl) or 5 mg/kg doxorubicin (Dox) ip. LIP was induced by doxycycline (doxy) in the drinking water. Mice were killed on day 15. 

A) Tumor growth was monitored daily by caliper measure. Data are presented as mean ± SD of 15 mice per group. *P < .001 for “doxy” and “Dox + doxy” group vs “Ctrl” group; °P = .006 (day 12), °P = .007 (day 15), for “Dox + doxy” group vs “Dox” group.

B) Photograph of representative tumors (two-sided analysis of variance [ANOVA]).

C) Sections of tumors from each group of mice were stained with hematoxylin and eosin (HE) or immunostained with antibodies for Ki67 as an index of proliferation, HERPUD1 as an index of ER stress, or Pgp, followed by incubation with peroxidase-conjugated secondary antibodies. Nuclei were counter-stained with hematoxylin. Bar = 10 µm. The photograph is a representative of sections from five tumors. D) Immunostaining quantification. The percent of proliferating cells was determined by the ratio of Ki67-positive nuclei and the total cell count (hematoxylin-positive nuclei), by counting sections from five mice of each group (107–92 nuclei/field); “Ctrl” group percentage was considered 100%. The percentage of HERPUD1 and Pgp-positive cells was determined by analyzing sections from five mice of each group (109–91 cells/field), using Photoshop program. “Ctrl” group intensity was considered 100%. *P = .007 (Ki67), °P = .003 (HERPUD1), °P = .005 (Pgp), for “doxy” or “Dox + doxy” group vs “Ctrl” group; °P = .006 (Ki67), °P = .002 (HERPUD1), °P = .003 (Pgp), for “Dox + doxy” group vs “Dox” group (two-sided ANOVA).
Figure 7. The impact of proteasome and lysosome inhibitors on resistance to endoplasmic reticulum stress and chemotherapy. A) Extracts from HT29 and HT29/MDR cells were immunoprecipitated (IP) with an anti-C/EBP-β antibody (recognizing the common C-terminal peptide), and then immunoblotted (IB) with anti-mono/poly-ubiquitin (UQ) antibody or with the anti-C/EBP-β antibody. No Ab: extracts of HT29 cells precipitated in the absence of the anti-C/EBP-β antibody were used as internal control. Densitometric results were expressed as arbitrary units. *P < .001 for monoubiquitinated LIP; *P = .002 for total LIP in HT29 vs HT29/MDR cells (two-sided analysis of variance [ANOVA]). B-C) Cells were grown for 48 hours in media without (-) or with irinotecan (10 μM, CP), in the absence or presence of various combinations of the proteasome inhibitor MG132 (10 μM for the last six hours, MG), the cathepsin inhibitor leupeptin (200 μM for the last 3 hours, L), or the lysosome inhibitor NH₄Cl (50 mM for the last 3 hours, N). Cell extracts were then subjected to immunoprecipitation and immunoblotting as in (A). D) HT29/MDR cells were grown 48 hours in media without (-) or with brefeldin A (50 nM, Bfa), in the absence or presence of the proteasome inhibitor MG132 (10 μM for the last 6 hours, MG), the lysosome inhibitor NH₄Cl (50 mM for the last 3 hours, N), or both inhibitors. HT29 cells were included as controls. Cell viability was assessed in quadruplicate by neutral red staining. Data are presented as means ± SD (n = 4). *P = .014 for cells treated or not with Bfa. °P = .03, .03, and .02 for cells treated with Bfa and either MG132, NH₄Cl, or MG132 and...
The impact of C/EBP-β LIP Degradation on the MDR Phenotype

To further study the role of C/EBP-β LIP in the MDR phenotype, we studied the impact of lysosomal and proteasomal inhibitors on Pgp expression and the cellular resistance to inducers of ER stress and to chemotherapy. Both NH4Cl and MG132 partially restored the cytotoxicity of the ER stress inducer brefeldin A in the ER stress-resistant HT29/MDR cells, and their combination resulted in full recovery of the cytotoxic response (survival: mean ± SD = 47.3 ± 4.7% vs 90.5 ± 4.5%, P = .02) (Figure 7D). In parallel, these inhibitors downregulated the basal level of Pgp mRNA by 3.7-fold (P < .001), as well as the Pgp protein level in the HT29/MDR cells (Figure 7, E and F). Similarly, these inhibitors restored the cytotoxic response of HT29/MDR cells to irinotecan (survival: mean ± SD = 55.4 ± 5.3% vs 93.1 ± 3.6%, P = .007) (Figure 7G).

The Role of C/EBP-β LIP in Resistance to ER Stress and Chemoresistance in Primary Tumors

To analyze the role of C/EBP-β LIP in primary resistant human tumors of different origin, we analyzed three samples of pleural malignant mesothelioma, three samples of glioblastoma multiforme, and three samples of chronic lymphocytic leukemia, chosen for their constitutively high levels of Pgp (Figure 8A). C/EBP-β LAP was expressed in all samples, whereas only low levels of C/EBP-β LIP were found (Figure 8A). These cells were resistant to the ER stress inducer brefeldin A and to doxorubicin (Figure 8B). Furthermore, triggering of ER stress with brefeldin A did not induce C/EBP-β LIP in these cells (Figure 8C). Overexpression of C/EBP-β LIP downregulated Pgp (Figure 8D) and restored the cytotoxic efficacy of brefeldin A and doxorubicin (P = .003 and .004, respectively) (Figure 8E). A similar increase in C/EBP-β LIP reduced Pgp expression (Figure 8D) and increased response to brefeldin A; and doxorubicin was obtained by inhibiting proteasomal and lysosomal degradation using NH4Cl plus MG132 (P = .004 and P = .003, respectively) (Figure 8E).

Discussion

Tumor cell adaptation to ER stress requires activation of UPR-associated prosurvival mechanisms and attenuation of UPR-associated death-promoting mechanisms, such as those triggered by C/EBP-β LIP (2,18). LIP is necessary for promoting the nuclear translocation of CHOP (35), thereby activating the proapoptotic axis (4). Earlier studies demonstrated that both chemotherapy and ER stress induce Pgp (13,15,16) and overexpression of C/EBP-β LIP lowers Pgp levels in MDR cells (19). Our finding that MDR cells lack CHOP and cleaved caspase 3 suggest that continuous ER stress because of limited vasculature or chemotherapy selects for cells lacking C/EBP-β LIP. Our LIP complementation experiment suggests that loss of LIP drives the MDR phenotype by two mechanisms: It increases Pgp expression but also reduces ER stress-triggered cell death.

Our findings show that the absence of C/EBP-β LIP rather than the C/EBP-β LIP/LAP ratio is a major trigger of the MDR phenotype. Interestingly, complementation of LIP not only restored the cytotoxic effects of Pgp substrates but also that of the MRP substrates 5-fluorouracil and oxaliplatin, which also induce ER stress (5,7,13,26,27,29,37). Hence, LIP is likely involved in additional death-promoting mechanisms triggered by these MRP substrates. Several studies have previously shown that MDR cells are also resistant to the ER stress inducer thapsigargin. We found that these cells were also resistant to tunicamycin and brefeldin A, two ER stress inducers that are not Pgp substrates and in fact were reported to reverse the Pgp-mediated MDR (38,39). C/EBP-β LIP was recently shown to promote prosurvival mechanisms (40,41), however, these studies did not address its impact on the cellular response to ER stress or to chemotherapy.

C/EBP-β LIP is ubiquitinated and degraded by the proteasome in the early phase of the UPR, whereas it escapes degradation in the late phase (34,35). Mono-ubiquitinated proteins undergo lysosomal degradation (42,45), an event commonly taking place during late ER stress (46). Hence, our finding that C/EBP-β LIP was subjected to higher baseline mono-ubiquitination in HT29/MDR cells as compared with HT29 cells provides a plausible mechanism driving its loss in MDR cells. Cathepsin L inhibitors were found to overcome resistance to tamoxifen, flutamide, doxorubicin, imatinib, and tricostatin by blocking lysosomal degradation of the respective target proteins (47). In addition, it was suggested that ER stress renders cells more resistant to camptothecin and doxorubicin by increasing the degradation of topoisomerase I and II, an effect that was prevented by proteasome inhibitors (48). Since doxorubicin, etoposide, imatinib, and the active metabolites of camptothecin and tamoxifen are effluxed by Pgp, our study provides an additional mechanism of MDR reversal based on the regulation of the Pgp level by cathepsin L inhibitors. C/EBP-β LAP and LIP were also poly-ubiquitinated to some extent, suggesting that their level is regulated both by lysosomal and proteasomal degradation.

Our findings were demonstrated using several resistant cell lines and three resistant primary tumor cells. Nevertheless, the reproducibility in several in vitro systems, which are accessible by other scientists, is not given; validation of our conclusions based on three samples per tumor entity can provide a trend at best.

In conclusion, we propose that loss of LIP by lysosomal and proteasomal degradation following its early ubiquitination drives the MDR by two independent mechanisms: upregulation of Pgp and attenuation of ER stress–triggered apoptosis. Because C/EBP-β LIP downregulates tumor progression (18) and improves the therapeutic benefits of Pgp substrates, preventing its lysosomal degradation may be an effective tool to overcome drug resistance. Our findings were demonstrated using several resistant cell lines and three resistant primary tumor cells. Nevertheless, the reproducibility in several in vitro systems,
Figure 8. The impact of LIP expression on response of primary resistant tumor cells to endoplasmic reticulum stress and chemotherapy.  

A) Immunoblot analysis with anti-Pgp and anti-C/EBP-β (recognizing the C-terminal peptide), performed with extracts from primary tumor cells isolated from three malignant mesothelioma (MM), three glioblastoma multiforme (GBM), and three chronic lymphocytic leukemia (CLL) patients. UPN = unknown patient number. Anti-actin served as a load control.

B) Cells shown in (A) were cultured for 48 hours in media without (-) or with brefeldin A (50 nM, Bfa) or doxorubicin (5 μM, Dox). Cell viability was assessed in quadruplicate by neutral red staining. Data are presented as means ± SD (n = 4).

C) Cells of UPN 1, 6, and 9 were cultured for 48 hours in media without (-) or with (+) brefeldin A (50 nM, Bfa). Immunoblot analysis with anti-C/EBP-β of the cell extracts are shown. Anti-actin served as a load control.

D) Cells of patients shown in (C), were either

E) Data are presented as means ± SD (n = 4).
which are accessible by other scientists, is not given; validation of our conclusions based on three samples per tumor entity can provide a trend at best.

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