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Cisplatin and a potent platinum(IV) complex-mediated enhancement of TRAIL-induced cancer cells killing is associated with modulation of upstream events in the extrinsic apoptotic pathway

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Abstract

TRAIL (TNF-related apoptosis-inducing ligand) can selectively trigger apoptosis in various cancer cell types. However, many cancer cells are resistant to death receptor-mediated apoptosis. Combination therapy with platinum complexes may affect TRAIL-induced signaling via modulation of various steps in apoptotic pathways. Here we show that cisplatin or a more potent platinum(IV) complex LA-12 used in twenty-fold lower concentration enhanced killing effects of TRAIL in human colon and prostate cancer cell lines via stimulation of caspase activity and overall apoptosis. Both platinum complexes increased DR5 surface expression in colon cancer cells. siRNA-mediated DR5 silencing rescued cells from sensitizing effects of platinum drugs on TRAIL-induced caspase-8 activation and apoptosis, showing the functional importance of DR5 in the effects observed. In addition, both cisplatin and LA-12 triggered the relocalization of DR4 and DR5 receptors to lipid rafts, and accelerated internalization of TRAIL, which may also affect TRAIL signaling. Collectively, modulations of the initial steps of the extrinsic apoptotic pathway at the level of DR5 and plasma membrane are important for sensitization of colon and prostate cancer cells to TRAIL-induced apoptosis mediated by LA-12 and cisplatin.

Introduction

The main goal of anticancer therapy is to selectively induce apoptosis in cancer cells while sparing untransformed cells and healthy tissues. One promising approach is triggering of the extrinsic apoptotic pathway by TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), a member of TNF superfamily, which has been shown to selectively induce apoptosis in various cancer cells *in vitro* and *in vivo* [1,2]. The different sensitivity/resistance to TRAIL in normal and cancer cells is still a matter of debates among the experts. Several potential ways of resistance of non-tumorigenic cells to TRAIL have been proposed so far, at the level of both surface and intracellular molecules. Furthermore, many tumor cells have been shown to be resistant to the effects of TRAIL due to deficiencies in apoptotic pathways or over-activated pro-survival signaling [3-6]. Elucidation of the molecular mechanisms of the resistance, and designing safe combination therapy using agents capable of sensitizing the cancer but not normal cells to TRAIL-induced apoptosis are important prerequisites of the successful clinical application of this cytokine.

There are five known types of TRAIL receptors. Death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) containing death domain (DD) are responsible for transmission of the apoptotic signal, while decoy receptors DcR1 (TRAIL-R3, lacking DD) or DcR2 (TRAIL-R4, with truncated DD), and a soluble receptor osteoprotegerin (OPG, TRAIL-R5) are unable to signal apoptosis. However, the cell surface expression of particular TRAIL receptor may not fully corelate with its functional importance in induction/inhibition of TRAIL apoptotic signaling. It has been published in several cancer cell types that despite the presence of both DRs at their surface, particular tumors preferentially signal through either DR4 (e.g. lymphocytic leukemia) [7] or DR5 (e.g. colon cancer) [8]. The specific DR involvement as well as differences in apoptotic signaling mediated by DR4 or DR5 remain to be answered.

Some authors also showed a higher expression of decoy receptors in non-tumorigenic compared to tumor cells. However, this phenomenon may be tissue specific, and the sole presence of surface decoy receptors has often been found not sufficient to protect the cells from apoptotic effect of TRAIL [9,10].

DR4 and DR5 mediate TRAIL-induced apoptosis by recruiting FADD (Fas-Associated DD) protein and pro-caspase-8 to form a death-inducing signaling complex (DISC). The amount of caspase-8 activated at the DISC is important factor affecting further progression of apoptotic signaling. In so-called type I cells, abundantly activated caspase-8 directly cleaves and activates effector caspases, which leads to execution of cell death. In type II cells, the amount of caspase-8 activated at the DISC is not sufficient to trigger an adequate effector caspase stimulation; therefore, amplification of the apoptotic signal via mitochondria is required [11,12]. In this case, a BH-3-only protein Bid is cleaved by caspase-8 to tBid, and translocated to mitochondria to trigger processes leading to release of proapoptotic proteins such as cytochrome c into the cytosol [12]. Cytochrome c, Apaf-1, and procaspase-9 then form an apoptosome complex where caspase-9 is activated, and the apoptotic signal is augmented.

The initial steps of TRAIL signaling have been studied intensively, especially the regulation of TRAIL receptor expression, their translocation to the cell surface, plasma membrane distribution, lipid raft (co)localization, and receptor internalization (endocytosis). Lipid rafts are dynamic plasma membrane microdomains enriched with cholesterol and sphingolipids. They play fundamental roles in diverse cellular processes, particularly in signal transduction, by promoting compartmentalization of membrane proteins and lipids [13,14]. Recent studies have suggested the role of lipid rafts as platforms for DR-mediated apoptosis signaling [15]. Relocalization of TRAIL DRs into the lipid rafts has been shown to facilitate DISC formation and caspase-8 activation-initiated apoptosis, while TRAIL-DISC assembly in

the non-raft phase of the plasma membrane resulted in inhibition of caspase-8 cleavage, and a promotion of antiapoptotic signaling [15]. Changes of the DRs distribution within the plasma membrane may therefore have a crucial impact on modulation of cell sensitivity/resistance to apoptotic signals triggered by TRAIL. Death ligand/receptor interactions may induce receptor clustering and internalization, which targets the active receptor to endocytic compartments. An essential requirement for receptor internalization in transmitting the CD95L-induced apoptotic signal has been reported [16,17]. In contrast, although being rapidly internalized, TRAIL and its DRs have been reported not to essentially require internalization for DISC formation, caspase-8 activation, and subsequent apoptosis induction in BJAB type I cells [18].

Combined treatment with chemotherapeutic drugs has been shown to overcome TRAIL resistance in many cancer cell types. Diverse molecular mechanisms have been reported to be responsible for synergistic effects of these agents to induce apoptosis of target cells. Chemotherapy may have a great impact on the crucial steps of the TRAIL signaling pathway, e.g. through increase of TRAIL DR expression [19,20], lipid raft relocalization [21,22], decrease of cFLIP protein level [23], facilitating DISC formation and caspase-8 activation [24], upregulation/downregulation of proapoptotic/antiapoptotic molecules [25], or stimulation of mitochondria [26]. The involvement of the particular events depends on the type and/or stage of cancer, type I/II cells, and the type of the selected chemotherapeutic drug(s).

Platinum complexes, e.g. cisplatin, carboplatin and oxaliplatin, belong to the most widely used chemotherapeutic agents in the treatment of solid cancers. By creating covalent bonds with DNA, they induce DNA damage signaling, which leads either to cell cycle arrest providing time to repair the damage, or immediate activation of apoptotic signaling via the intrinsic mitochondrial pathway, and killing of cancer cells. However, application of therapy using platinum drugs such as cisplatin is limited due to serious side effects and/or

development of intrinsic or acquired resistance of the cancer cells. In the past two decades, plenty of newly synthesized analogues of platinum complexes were examined, and some of them entered the clinical trials, e.g. Pt(IV) complexes. LA-12 is a novel Pt(IV) adamantylamine ligand-containing complex, currently in phase I of clinical trials. It has been shown to be more cytotoxic than satraplatin and to efficiently induce cell death in a panel of 14 cancer cell lines with various sensitivity to cisplatin [27] and in ovarian carcinoma cells with acquired (A2780cis) or intrinsic resistance to cisplatin (SK-OV-3) [28,29]. LA-12 has been shown to induce cell cycle arrest and apoptosis in various cancer cells [30,31,32,33].

In vivo studies in murine xenografts revealed a higher antitumor activity of LA-12 compared to cisplatin and Pt(IV) complex satraplatin, and enhanced tissue penetration, and lower acute systemic toxicity. Due to its improved lipophilicity, LA-12 was shown to effectively penetrate tissues and tumors and can be administered perorally [34-36]. Taken together, these data imply that LA-12 is a promising candidate for cancer therapy, with high effectivity in killing cancer cells *in vitro* and *in vivo* and low toxic side effects *in vivo*.

In the present study, we investigated the role of a novel platinum drug LA-12 in modulating colon and prostate cancer cell sensitivity to apoptotic effects of TRAIL, and compared the LA-12-mediated effects with those exerted by conventionally used cisplatin in combination with TRAIL. The molecular mechanisms responsible for the enhancement of apoptosis following combined treatments with these agents were investigated, with special focus on the most upstream events of the TRAIL signaling pathway, namely TRAIL DRs and initiator caspase-8. Both platinum complexes increased DR5 surface expression and lipid raft localization, and consequentially TRAIL-induced caspase-8 activation and apoptosis, which could be counteracted by siRNA-mediated DR5 silencing. Our results demonstrate that modulating the initial steps of the extrinsic apoptotic pathway by LA-12 and cisplatin at the

level of DR5 and plasma membrane are important events in sensitization of colon and prostate cancer cells to TRAIL-induced apoptosis.

Materials and methods

Materials and reagents

The stock solutions of cisplatin (cis-diamminedichloroplatinum(II); FW 300.1) (Sigma-Aldrich Corp.; St. Louis, MO, USA); and LA-12 ([(OC-6-43)-bis(acetato)(1-adamantylamine)ammine dichloroplatinum(IV)]; FW 552.4) (Pliva-Lachema, a.s.; Brno, Czech Republic) were freshly prepared before use. Human N-terminally His-tagged recombinant Apo2L/TRAIL (amino acids 95–281) was affinity-purified from cell lysates of the producer bacteria (*E. coli*, strain BL-21) and contaminating bacterial endotoxins were removed by Endotrap chromatography (Profos AG) [37].

Cell culture

The human colon adenocarcinoma cell line HCT-116 (obtained from Prof. B. Vogelstein) and prostate cancer PC-3 (from ATCC) epithelial cell lines were maintained in McCoy's 5A modified medium with 1.5 mM L-glutamine (Sigma-Aldrich; St. Louis, MO, USA) or F12, respectively, supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml) (both Duchefa Biochemie; The Netherlands), sodium bicarbonate (1.5 g/l, Serva; Heidelberg, Germany), and 10% heat-inactivated fetal bovine serum (HCT-116) or 10% fetal bovine serum (PC-3) (PAA Pasching, Austria). The cells were incubated in a humidified incubator at 37 °C in a 5% CO_2 atmosphere, and passaged twice a week by EDTA/PBS washing and trypsinization.

WST cytotoxicity assay

HCT-116 and PC-3 cells were seeded in 96-well plates in a density of 20,000 cells per cm^2. After 24 h (HCT-116) or 48 h (PC-3), the cells were treated with cisplatin (10 μM) or LA-12 (0.5 μM). Twenty-four hours later, various amounts of TRAIL (6.25-100 ng/ml) were added. After 24 h of incubation, tetrazolium salt WST-1 with 1-methoxy-5-methylphenazium methylsulphate (Serva; Heidelberg, Germany) was added for 4 h, and absorbance of a soluble formazan compound formed by metabolically active cells was analyzed using a microplate reader FLUOSTAR Galaxy (BMG Labtechnologies GmbH, Offenburg, Germany).

Real-time cell impedance analysis

Acea E-plates® 96 and an xCELLigence RTCA SP system including RTCA Software v1.2 (both Roche; Applied Science, Prague, Czech Republic) monitors cellular events including cell number, adhesion, viability, and morphology, and provides information about the biological status of the cells in real time by measuring electrical impedance across micro-electrodes integrated on the bottom of its special tissue culture plates [38]. First, a standard background measurement was performed using 100 µl of complete culture media. PC-3 cells were trypsinized, counted, and seeded in additional 100 µl of culture media in a final concentration of 30,000 cells per cm^2. The cells were monitored continually every hour after the seeding for a period of 48 h. Next, the cells were pretreated with cisplatin or LA-12 for 24 h followed by treatment with TRAIL (HCT-116 cells: 100 ng/ml, PC-3 cells: 12.5 ng/ml) for another 20 h. During TRAIL treatment, the cells were monitored continually every 2 min in the first 3 h, and then every 30 min. Acea E-plates® 16 and xCELLigence RTCA DP Analyzer were used for monitoring the effects of tested compounds after transfection with nontargeting siRNA (sc-37007) or siRNA targeting DR5 (sc-40237; both Santa Cruz; CA, USA). Briefly, HCT-116 cells were seeded in 200 µl of cultivation media without antibiotics

in a final concentration of 50,000 cells per cm^2 and after overnight incubation the cells were transfected with control or DR5 siRNA using Lipofectamine™ 2000 (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions. Twenty-four hours later, the cultivation medium was replaced by a fresh one with penicillin/streptomycin and the cells were treated with LA-12 (0.5 μM) for 24 hours. Finally, TRAIL (50 ng/ml) was added. During TRAIL treatment, the cells were monitored continually every 2 min for 4 hours.

Flow cytometric analysis of TRAIL receptors on the cellular surface

After 24 h of incubation with cisplatin (10 μM) or LA-12 (0.5 μM), the attached cells were harvested by gentle trypsinization, washed twice in cold PBS with 0.2% BSA, and then incubated with anti-DR4 (#HS101, 1:100), anti-DR-5 antibody (#HS201, 1:100; both FITC-conjugated; Alexis Biochemicals Corporation; Lausen, Switzerland) or anti-DR4 (anti-CD261, #1P-403-C025) and anti-DR5 (anti-CD262, #1P-461-C025; both PE-conjugated, Exbio; Vestec, Czech Republic) on ice in the dark for 45 min. The cells were washed twice, and 7-AAD or LIVE/DEAD® Fixable Dead Cell Stain Far Red (Invitrogen; Carlsbad, CA, USA) were added. After 20 min, the expression of DR4 and DR5 was assessed by flow cytometry (FACSCalibur, Becton Dickinson; San Jose, CA). CellQuestPro software was used for data acquisition and analysis. Dead cells (7-AAD or Dead Cell Stain positive) were excluded from analysis. Receptor expression on the cellular surface was expressed as a ratio of the median fluorescence index (MFI) of the specific antibody and MFI of the isotype control antibody. To minimize the possibility of antibody-receptor complex internalization during the incubation period, we performed a staining protocol based on only 10 min of incubation with both antibody and viability probe together. Both analyses gave similar results.

Internalization of TRAIL

TRAIL was labeled using Alexa Fluor 647 Microscale Protein Labeling Kit (A3000, Molecular probes; Eugene, Oregon, US) based on the manufacturer's instructions. Cells were seeded in a 12-well plate, incubated with cisplatin (10 μM) or LA-12 (0.5 μM) for 24 h, and then with Alexa Fluor 647-conjugated TRAIL for 5, 10, 20 or 30 min. Sucrose (0.25 M) was added at the same time to inhibit endocytosis of TRAIL. The plates were rapidly chilled on ice to stop internalization. The cells were washed in HEPES twice and incubated in a mixture of 0.2 M acetic acid and 0.2 M NaCl for 5 min on ice. After three washing steps in HEPES, the cells were incubated in EDTA/PBS and briefly trypsinized. Trypsin was neutralized by 2% BSA in PBS. The cells were centrifuged, resuspended in 2% BSA, stained with propidium iodide (5 μg/ml), and immediately analyzed by flow cytometer [18]. The data were analyzed using CellQuest software. Doublets, debris and dead cells (propidium iodide positive) were excluded from analysis.

Western blot analysis

The cells were harvested, washed twice in cold PBS, and lysed in 1% SDS buffer and Western blot analysis was performed as previously described [30]. Immunodetection was carried out with the following antibodies: anti-DR5 (1:1000, 210-743, Alexis, Biochemicals Corporation; Lausen, Switzerland), caspase-8 (1:500, 9746, Cell Signaling; Danvers, MA, USA), caspase-3 (1:500, sc-7272), PARP (1:500, sc-7150), lamin B (1:500, sc-6217) (all of them Santa Cruz, CA, USA), anti-DR4 (D3813, 1:500) and β-actin (A5441, 1:5000) (both Sigma-Aldrich Corp.; St. Louis, MO, USA). Densitometric quantification of the visualized bands was performed by ImageJ software (NIH, Bethesda, MD) and normalized to the expression of β-actin.

RNA isolation and real-time RT-PCR

Total RNA was isolated using a High Pure RNA Isolation Kit (Roche Applied Science, Prague, Czech Republic) according to the manufacturer's instructions. The sequences of a gene-specific primer for combination with Universal ProbeLibrary probes: DR5 (GenBank: AF012628.1), F: 5'-AGA GCC AAC AGG TGT CAA CAT; R: 5'-GCC TCC TCC TCT GAG ACC TT (probe #29, 04687612001); POLR2A (polymerase (RNA) II (DNA directed)) polypeptide F: 5′-ATCTCTCCTGCCATGACACC-3′, R: 5′-AGACCAGGCAGGGGAGTAAC-3′ (probe #1, 04684974001, Roche Diagnostics GmbH, Mannheim, Germany). The amplification reactions were carried out in a final volume of 20 μl in a reaction mixture containing 10 μl of QuantiTect Probe RT-PCR Master Mix, 0.2 μl of QuantiTect RT Mix (Qiagen; Valencia, CA, USA), 2 μl of solution of primers and probe, 5.8 μl of water, and 2 μl of RNA sample. The final concentration of each primer was 0.4 μM and the probe was 0.1 μM. The amplifications were run on the RotorGene3000 with RotorGene Real-Time Analysis Software (Corbett Research; Sydney, Australia), using the following program: 50 °C for 30 min for reverse transcription and 95 °C for 15 min for denaturation of cDNA, followed by cycling (40 repeats) 94 °C for 15 s and 60 °C for 60 s acquiring fluorescence. All PCR reactions were performed in triplicates and changes in gene expression were calculated using the comparative threshold cycle method [39] with POLR2A as a normalizing gene.

Immunofluorescent labeling TRAIL receptors and lipid rafts

PC-3 and HCT-116 cells were seeded in IBIDI 8-well coverslip chambers. The cells were washed three times in ice-cold HEPES buffer and incubated with 15 μg/ml anti-DR4 (MA1-19025) or anti-DR5 (MA1-19416, Affinity Bioreagents; Golden, CO, USA) monoclonal antibodies for 10 min on ice. After three washes, Alexa Fluor 488-conjugated secondary antibody (A-11017, Molecular Probes, OR, USA) was added at 10 μg/ml together with 4 μg/

ml Alexa Fluor 647-conjugated cholera toxin B-subunit (C-34778, Molecular Probes, OR, USA) for 10 min on ice. After three washes, the cells were fixed with 4% formaldehyde and mounted with Mowiol 4-88 (Calbiochem). The Cholera toxin B subunit binds to GM1 glycosphingolipid rich domains and serves as one of the most widely used markers for lipid rafts.

Confocal laser scanning microscopy (CLSM)

CLSM (Zeiss LSM 510) was used for colocalization measurements. Alexa Fluor 488 was excited at 488 nm, and Alexa Fluor 647 was excited at 633 nm. Their fluorescence emission was detected through 505- to 550-nm band-pass and 650-nm long-pass filters, respectively. The images were taken in multitrack mode to completely exclude channel cross-talk (although the great spectral separation minimized this already). 512 × 512-pixel, 1.5 μm thick optical sections were obtained with a 40× C-Apochromat water immersion objective (NA=1.2).

Determining colocalization from image cross-correlation

Colocalization of molecules at the few-hundred-nm scale was determined from CLSM images of double-labeled cells. The optical section was taken from the top horizontal slice of the membrane of adherent cells. The images were gated on the presence (above-background intensity) of at least one of the fluorophores. For a pair of images, x and y, the cross-correlation coefficient between the intensity distributions of cell-surface labeling was calculated as (1),

$$C = \frac{\sum_{i,j}(x_{ij} - \langle x \rangle)(y_{ij} - \langle y \rangle)}{\sqrt{\sum_{i,j}(x_{ij} - \langle x \rangle)^2 \sum_{i,j}(y_{ij} - \langle y \rangle)^2}}, \quad \textbf{[1]}$$

where x_{ij} and y_{ij} are fluorescence intensities at pixel coordinates i, j in images x and y, and $\langle x \rangle$, $\langle y \rangle$ are the mean intensities in each channel. The theoretical maximum is $C = 1$ for identical

images and a value of 0 implies independent random localization of the labeled molecules. A custom program was written in LabView to analyze the images. The average intensity of labels in the membrane was also evaluated by the program [13,14].

RNA interference

HCT-116 cells were seeded at a density of 20,000 cells per cm^2 and cultured for 12 h. Transfections were carried out in McCoy's medium without antibiotics using a Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The small interfering RNA (siRNA) targeting DR5 (40 nM; sc-40237) or nontargeting control siRNA (40nM; sc-37007; Santa Cruz Biotechnology; CA, USA) was added directly to the transfection reagent solution and incubated for 24 h. After the transfection, the medium was replaced by McCoy's medium.

Caspase activity assay

The cells were collected and caspase activity assay was performed as described previously [40] using fluorogenic caspase-8 substrate Ac-IETD-AMC (ALX-260-042-M005, Alexis, Biochemicals Corporation, Lausen, Switzerland).

Statistical analysis

The data were expressed as means ± S.D., and analyzed by ANOVA followed by a Tukey test or by a nonparametric Mann-Whitney U-test. A *P* value of less than 0.05 was considered significant. All statistical analyses were performed by the Statistica for Windows software, V. 6.1 (StatSoft, Inc., Tulsa, OK, USA).

Results

LA-12 or cisplatin enhanced cytotoxic effects of TRAIL in HCT-116 and PC-3 cell lines

After pretreatment with cisplatin (10 μM) or LA-12 (0.5 μM), the cytotoxic effects of TRAIL (6.25 – 100 ng/ml) were significantly enhanced as demonstrated by a decrease of HCT-116 and PC-3 cell metabolic activity (WST test; Figure 1a, b and e, f). Platinum drug-mediated sensitizing effects were clearly evident already when combined with the lowest concentration of TRAIL (6.25 ng/ml). Using a real time impedance analysis (system xCELLigence; Figure 1 c, d and g, h), we also demonstrated a significant decrease of cell surface adhesion following the combined treatment with cisplatin/LA-12 and TRAIL in both cell lines. The pretreatment with platinum drugs prevented recovery of the cell index value observed in cells treated with TRAIL alone.

LA-12- or cisplatin-mediated potentiation of TRAIL-induced cytotoxicity is associated with activation of the apoptotic caspase cascade

In order to examine whether the cytotoxicity induced by combination of platinum drugs and TRAIL occurred via activation of the caspase cascade and apoptotic signaling, analysis of the cleavage of caspase-8, -3, and effector caspase substrate PARP was performed (Western blotting). Pretreatment with cisplatin or LA-12, followed by TRAIL, resulted in a substantial potentiation of specific processing of pro-caspase-8, -3, and PARP in HCT-116 cells (Figure 2a) compared to TRAIL alone-treated cells. A similar increase in cleavage of pro-caspase-8 and -3 was also observed in PC-3 cells following combined treatments (Figure 2b). A corresponding significant increase in the number of cells with characteristic apoptotic changes of nuclear morphology (condensation and fragmentation of chromatin, fluorescence microscopy) was also detected in cisplatin/LA-12 and TRAIL-treated cells compared to the agents used alone (data not shown).

LA-12 and cisplatin were responsible for significant increase of surface DR5 expression in HCT-116 but not in PC-3 cells

LA-12 or cisplatin induced a significant concentration-dependent upregulation of the surface DR5 but not DR4 level in HCT-116 cells (Figure 3a). Increased surface expression of DR5 following treatment with platinum drugs in HCT-116 was also confirmed by confocal microscopy (data not shown). No significant effects of any platinum drug in concentrations used on DR4 and DR5 surface levels were detected in PC-3 cells using flow cytometric analysis (Figure 3b). LA-12-mediated changes of DR5 surface level in HCT116 cells were accompanied by an increase in DR5 mRNA (Figure 3c) and total protein level (Figure 3d). Similarly, cisplatin enhanced amounts of DR5 mRNA (Figure 3c) and total protein (Figure 3d) in HCT-116 cells. None of the two platinum complexes modulated total amount of DR4 protein in HCT-116 cells (Figure 3d).

Lipid raft localization of DR4 and especially of DR5 was increased upon treatment with cisplatin derivatives

To determine the membrane domain localization of DR4 and DR5 with respect to lipid rafts, the receptors on the cell surface were labeled with indirect immunofluorescence (specific monoclonal primary antibodies followed by Alexa Fluor 488 secondary Abs) and CTX B was used as a lipid raft marker specific to GM1 rich domains. The extent of colocalization was quantitated by calculating the Pearson's correlation coefficient from confocal microscopic optical sections of upper horizontal membrane layers from numerous single cells. Both TRAIL DR4 and DR5 receptors colocalized with GM1 rich domains in the plasma membrane of PC3 as well as HCT-116 cells. An example is shown for DR5 in HCT-116 cells (Figure 4a, b, c). After 1h of cisplatin or LA-12 treatment, the colocalization between lipid rafts and

TRAIL receptors increased in both HCT-116 (Figure 4d) and PC-3 cells (Figure 4e). This effect was more pronounced for DR5.

Cisplatin induced an increase of TRAIL internalization in both PC-3 and HCT-116 cell lines, while LA-12 was only effective in the latter one

Following treatment with TRAIL, an acute time-dependent increase of dye-conjugated TRAIL internalization was detected in both HCT-116 and PC-3 cell lines by flow cytometry. Suppression of TRAIL internalization by sucrose was used as a negative control of clathrin-dependent endocytosis [18]. Pretreatment with cisplatin resulted in a significant enhancement of TRAIL internalization, starting from 10 or 20 min of subsequent TRAIL treatment in PC-3 or HCT-116 cells, respectively (Figure 5a, b). LA-12-mediated stimulation of TRAIL internalization was detected only in HCT-116 cells following 30 min incubation with TRAIL (Figure 5b).

LA-12 or cisplatin-mediated increase of DR5 is essential for potentiation of TRAIL-induced caspase-8 activation and apoptosis in HCT-116 cells

To determine the functional role of DR5 in the LA-12/cisplatin and TRAIL-induced apoptosis, the expression of DR5 was down-regulated using specific siRNA. Transfection of HCT-116 cells with DR5 but not control siRNA resulted in a significant decrease of basal (72 %) as well as LA-12/cisplatin-induced (78/80 %) surface level of DR5 (Figure 6a). Following DR5 siRNA transfection, LA-12/cisplatin-mediated stimulation of TRAIL-induced caspase-8 processing/activation and apoptosis (demonstrated by PARP and lamin B cleavage) was significantly reduced (Figure 6b, c) compared to control siRNA-transfected cells. To further elucidate the role of DR5 in LA-12/cisplatin enhanced TRAIL-mediated cell death, the cell index was assessed using the xCELLigence system (Figure 6d). Silencing of DR5

significantly rescued the reduction of the cell index observed in TRAIL- and LA-12/TRAIL-treated cells. The results imply that cisplatin- or LA-12-mediated enhancement of TRAIL-induced cell death depends on DR5 function.

Discussion

The ability to induce apoptosis in tumor cells while sparing nontransformed cells designates TRAIL for the therapy of cancer diseases. Unfortunately, an increasing number of studies demonstrate that many primary tumors are resistant to TRAIL monotherapy [41]. A promising strategy to overcome the resistance of cancer cells and improve the clinical outcome is represented by combination therapy. By this time, combinations of TRAIL with many other antitumor agents with various mechanisms of action including DNA damage have been tested [42,43]. However, detailed description of the molecular mechanisms of combined effects of these compounds has to precede their possible clinical application. Chemotherapy-mediated enhancement of TRAIL toxicity in tumor cells has been shown to be regulated at many levels, e.g. TRAIL receptors expression and localization, DISC components expression and modulation of their assembly, integrity of mitochondria, prosurvival, and apoptotic signaling. Interactions of extrinsic and intrinsic pathways of apoptosis may also be profitable for the killing of type II cells as they rely on the mitochondrial loop to activate enough effector caspases and commit TRAIL-mediated programmed cell death. In our study, we showed that pretreatment with subtoxic concentrations of cisplatin and novel Pt(IV) complex LA-12 significantly enhanced TRAIL-induced apoptosis in human colon and prostate carcinoma cells, and we studied molecular mechanisms responsible for the observed effects.

Cisplatin has been shown to enhance the killing capacity of TRAIL in several cancer cell lines in *in vitro* [19,20,44,45], *in vivo* [46,47], and in *ex vivo* models [48]. Contradictory

studies demonstrating a cisplatin-mediated inhibition of TRAIL-induced cell death through direct inactivation of caspases have also been reported. The authors explain the discrepancy in their results compared to the majority of literature by the dependance on the duration of the treatment [49,50]. In our study, cisplatin was responsible for significant potentiation of TRAIL-induced apoptosis in human prostate and colon cancer cell lines. Importantly, similar effects on TRAIL-induced apoptosis were also induced by novel Pt(IV) complex LA-12, although a twenty-fold lower dose of LA-12 was used compared to cisplatin. The ability of LA-12 to be effective in significantly lower doses when compared to other conventional therapeutic drugs, together with its favorable pharmacokinetic profile, make LA-12 a promising candidate for combination cancer therapy. Enhancement of cell death induction following cisplatin/LA-12 and TRAIL was confirmed by cytotoxic tests, the state-of-the-art noninvasive real time cell impedance analysis method, and biochemical and morphological apoptosis assays.

The death domain-containing receptors DR4 and DR5 are important mediators of TRAIL proapoptotic signaling. Modulation of their surface expression may affect the apoptotic signal transduction and sensitivity/resistance of tumor cells to TRAIL-induced apoptosis. Some antitumor agents have been shown to increase expression of death receptors at the level of transcription, translation or posttranslational modifications. This increase may result in enhanced sensitivity to TRAIL, while evidence exists that in some models cisplatin enhanced TRAIL-induced apoptosis without modulation of DRs expression [51,52]. Cisplatin has been shown to increase expression of TRAIL receptors DR4 or DR5 in esophageal and osteosarcoma tumor cells at the mRNA level [19,20]. Various mechanisms of regulation of DRs by chemotherapeutic drugs have been suggested. In addition to p53, other transcription factors such as NF-?B or Sp-1 have been demonstrated to be involved in the regulation of DR5 expression [53,54]. In our study, cisplatin as well as Pt(IV) complex LA-12 increased

the expression of DR5 mRNA and protein, and enhanced its surface level in colon but not in prostate carcinoma cells. At the same time, these agents were not effective in modulating DR4. The precise mechanisms responsible for cisplatin/LA-12-mediate increase of the DR5 level in HCT-116 cells remain yet to be fully elucidated. We confirmed a functional role of DR5 in LA-12/cisplatin-mediated enhancement of TRAIL-induced apoptosis in colon cancer cells by showing that siRNA-mediated silencing of the DR5 surface level rescued cells from activation of caspase-8 and apoptosis induced by combination treatment. This implies that DR5 plays an important role in cisplatin/LA-12-mediated enhancement of TRAIL-induced apoptosis, being more important than DR4.

Localization of TRAIL receptor complexes in the plasma membrane and their distribution in membrane microdomains can have a significant functional impact on TRAIL signaling. Cisplatin has been shown to relocalize Fas receptors to lipid rafts and to enhance receptor clustering and apoptosis [55]. The shift of DR receptors to lipid rafts facilitated the apoptotic outcome of TRAIL by depsipeptide, quercetin, and oxaliplatin [21,22,56]. LA-12/cisplatin-mediated relocalization of DR4 and DR5 to lipid microdomains was also observed in our models of colon and prostate carcinoma cells. We suggest that in addition to the increase in surface DR levels, changes in lipid rafts localization of DR4 and DR5 may be important in LA-12/cisplatin-mediated reinforcement of TRAIL-induced apoptosis.

After the binding of death ligands to its cognate death receptors, complexes of ligand and membrane receptors can be internalized via endocytosis. Internalization of Fas receptor (CD95) has been shown to be crucial for Fas ligand-mediated formation of the DISC complex and induction of apoptosis. Inhibition of Fas internalization activated the prosurvival ERK and NF-?B signaling pathways [17]. The role of internalization of the TRAIL ligand and receptor complex for TRAIL-induced apoptosis has not been fully resolved yet. Internalization was described not to influence formation of DISC and activation of caspase-8

in a type I BJAB Burkitt lymphoma B cell line [18]. Nevertheless, transit of TRAIL to lysosomes was shown to contribute to TRAIL-induced apoptosis in the type II hepatocellular carcinoma cell line Huh-7 [57], and to participate in the activation of the lysosomal pathway of apoptosis. Internalization of TRAIL led to release of cathepsin B from lysosomes, and liberalization of cytochrome c from mitochondria [58]. Here we demonstrated that cisplatin and LA-12-mediated enhancement of TRAIL-induced apoptosis was accompanied by accelerated internalization of TRAIL, suggesting its possible role in the effects observed.

We demonstrated that cisplatin- and the novel platinum(IV) complex LA-12-mediated enhancement of TRAIL-induced apoptosis in human colon and prostate carcinoma cells were associated with modulation of upstream events of TRAIL signaling. Cisplatin and LA-12 increased the expression of DR5, stimulated the relocalization of DR4 and DR5 to lipid rafts, and accelerated the internalization of TRAIL. Furthermore, these drugs enhanced TRAIL-induced caspase-8 activation, cleavage of caspase-3 and its substrate PARP and, overall, the fraction of apoptotic cells. We demonstrated a functional role of DR5 in the reinforcement of TRAIL-activated apoptosis by cisplatin and LA-12. Our results show the complexity of interactions of signaling pathways triggered by TRAIL and cisplatin or LA-12, and highlight the striking ability of LA-12 to sensitize the cancer cells to TRAIL-induced apoptosis even when applied in significantly lower doses compared to cisplatin. Our observation will help to improve therapeutical approaches to cancer diseases in terms of more efficient killing of cancer cells, while minimizing the side effects of the therapy.

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Abbreviations: BSA, bovine serum albumin; CLSM, confocal laser scanning microscopy; DcR, decoy receptor; DD, death domain; DISC, death-inducing signaling complex; DR, death receptor; ERK, extracellular signal-regulated kinase; FADD, Fas-associated death domain protein; FITC, fluorescein isothiocyanate; FLIP, FLICE-like inhibitory protein; GM1, monosialotetrahexosylganglioside; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MFI, median fluorescence index; NF-?B, nuclear factor kappa B; OPG, osteoprotegerin; PBS, phosphate-buffered saline; siRNA, small interfering RNA; RT-PCR, reverse transcription polymerase chain reaction; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; WST-1, (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate).

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Legends to Figures

Figure 1 Pretreatment of HCT-116 and PC-3 cells with cisplatin or LA-12 intensified cytotoxic effects of TRAIL. **(a, b, e, f)** WST test was performed in HCT-116 **(a, b)** and PC-3 **(e, f)** cell lines pretreated with LA-12 (0.5 μM) **(a, e)** or cisplatin (10 μM) **(b, f)** for 24 h, and then treated with TRAIL (6.25-100 ng/ml) for further 24 h. The ability of the cells to

transform WST reagent to soluble formazan salt was measured as relative absorbance using plate reader Fluostar. **(c, d, g, h)** Real-time measurement of TRAIL toxic effects in HCT-116 **(c, d)** and PC-3 **(g, h)** cells pretreated (24 h) with vehicle, LA-12 (0.5 μM) **(c, g)** or cisplatin (10 μM) **(d, h)** and then incubated for 20 h with TRAIL, was performed employing the xCELLigence RTCA SP system as described in Materials and Methods. Time of TRAIL application is indicated by an arrow-head.

Figure 2 Subtoxic concentrations of cisplatin and LA-12 enhanced TRAIL-induced cleavage of caspase-8, -3, and PARP. HCT-116 **(a)** and PC-3 **(b)** cells were pretreated with LA-12 (0.5 μM) or cisplatin (10 μM) for 24 h, and then treated (4 h) with TRAIL (5 ng/ml). Proteins were detected by Western blotting. An equal loading was verified using anti-β-actin antibody. The results are representative of three independent experiments.

Figure 3 Total and surface level of DR5 was augmented by LA-12 and cisplatin. HCT-116 **(a)** and PC-3 cells **(b)** were untreated or treated (24 h) with LA-12 (0.1-1 μM) or cisplatin (1.5 μM), incubated with specific FITC-conjugated antibodies (Alexis), and analysis of surface DR4 and DR5 was performed using flow cytometer. DR5 mRNA **(c)** in HCT-116 cells treated (4 h) with LA-12 (0.5 μM) or cisplatin (1.5 μM), was detected by qRT-PCR, and total protein level **(d)** of DR4 and DR5 in HCT-116 cells treated (24 h) with LA-12 (0.5 μM) or cisplatin (1.5 and 10 μM) was detected by Western blotting.

Figure 4 Cisplatin and LA-12 treatment increased colocalization of TRAIL DR4 and DR5 receptors with GM1-rich domains of plasma membrane. HCT-116 **(a, b, c, d)** and PC-3 **(e)** cells were incubated (1 h) with cisplatin (10 μM) or LA-12 (0.5 μM), cell surface receptors (green) and GM1-rich lipid rafts (blue) were labeled, detected by CLSM, and differences in

the cross-correlation coefficient were noted ($p<0.005$). An example is shown for DR5 in HCT-116 cells (**a, b, c**, fields of view: 5x5 μm). Confocal optical sections of 1.5 μm in size were used to calculate cross-correlation of cell surface receptors and GM1-rich lipid microdomains for HCT-116 **(d)** and PC-3 **(e)** cells. Data are averages of the cross-correlation coefficient ± SEM of 40~60 independent measurements, normalized to control values.

Figure 5 Internalization of TRAIL is accelerated by platinum complexes cisplatin and LA-12. Cells were pretreated (24 h) with cisplatin (10 μM) and LA-12 (0.5 μM), and incubated with Alexa Fluor 647-conjugated TRAIL for 5 to 30 min. Simultaneously with TRAIL, 0.25 M sucrose was added to suppress endocytosis in appropriate samples. Flow cytometric analysis was accomplished. The amount of internalized TRAIL was expressed as percent of maximum fluorescence measured after 30 min of TRAIL incubation in HCT-116 **(a)** and PC-3 cells **(b)**.

Figure 6 siRNA-mediated silencing of DR5 prevented enhancement of TRAIL-induced apoptosis by cisplatin and LA-12 in HCT-116 cells. **(a)** Surface DR5 level in HCT-116 cells with Lipofectamine alone or transfected (24 h) with control or DR5 siRNA (40 nM), followed by 24 h of incubation with cisplatin (10 μM) or LA-12 (0.5 μM), was evaluated by flow cytometry (specific anti-DR5 PE-conjugated antibody, Exbio)**. (b, c)** Activity of caspase-8 was measured by fluorogenic assay **(b),** and cleavage of caspase-8, PARP, and lamin B **(c)** in HCT-116 cells transfected (24 h) with control or DR5 siRNA (40 nM), then treated (24 h) with cisplatin (10 μM) or LA-12 (0.5 μM), and subsequently incubated (1 h) with TRAIL (5 ng/ml), was detected by Western blotting. An equal loading was verified using anti-β-actin antibody. The results are representative of three independent experiments. **(d)** Real-time measurement of TRAIL toxic effects in HCT-116 cells transfected with control or DR5 siRNA, pretreated (24 h) with vehicle or LA-12 (0.5 μM), and then incubated (4 h) with

TRAIL (50 ng/ml) was performed employing the xCELLigence RTCA SP system. Time of TRAIL application is indicated by an arrow-head. Data were normalized to the time interval when TRAIL treatment was performed (normalized cell index).

HCT-116

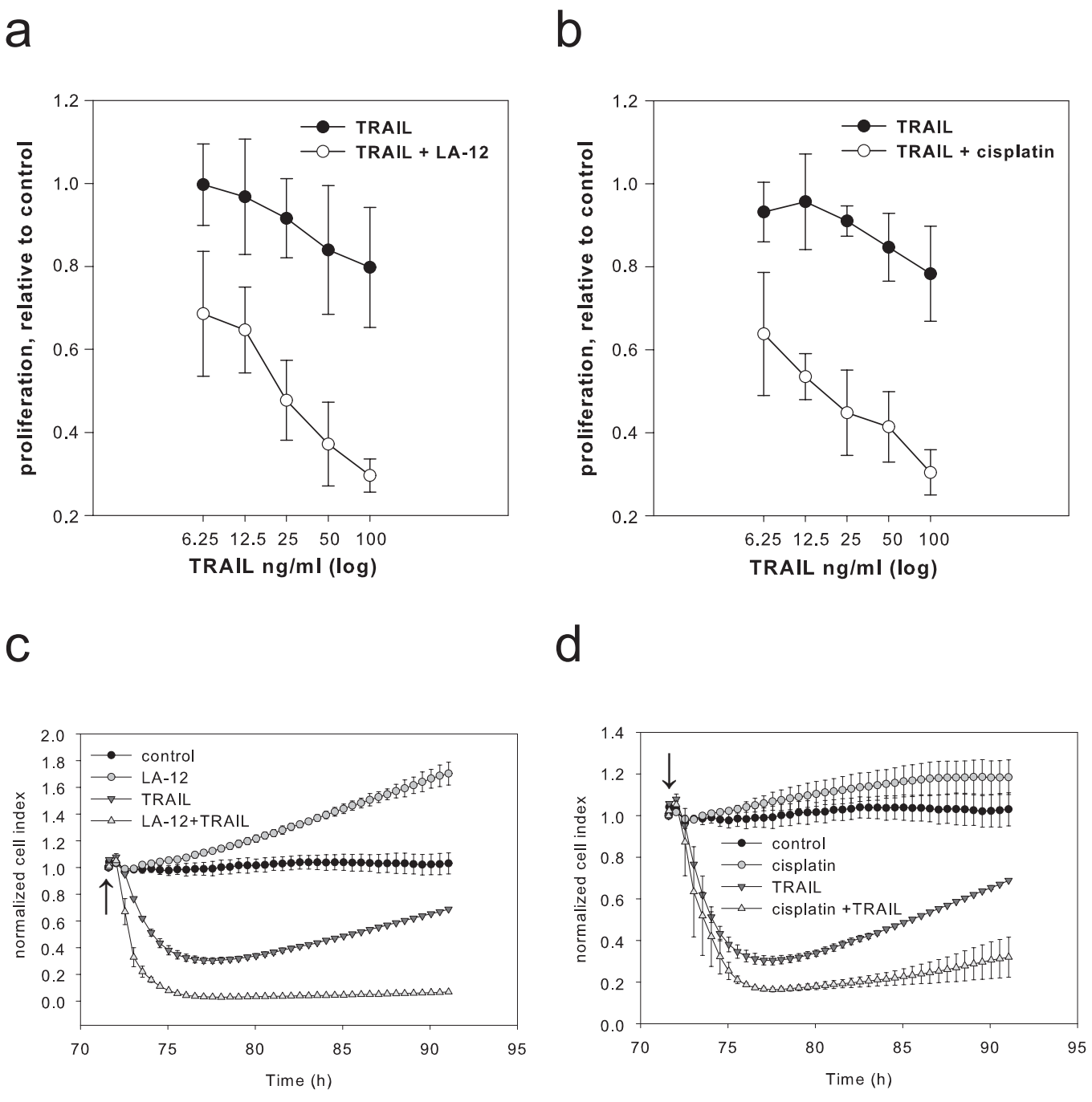
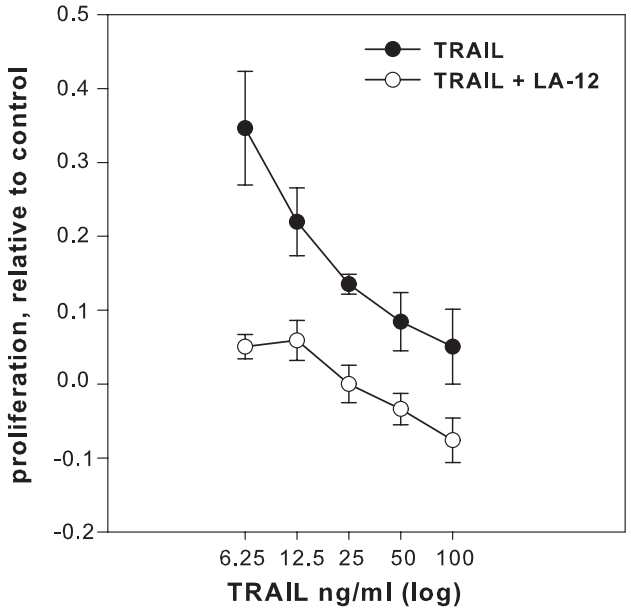


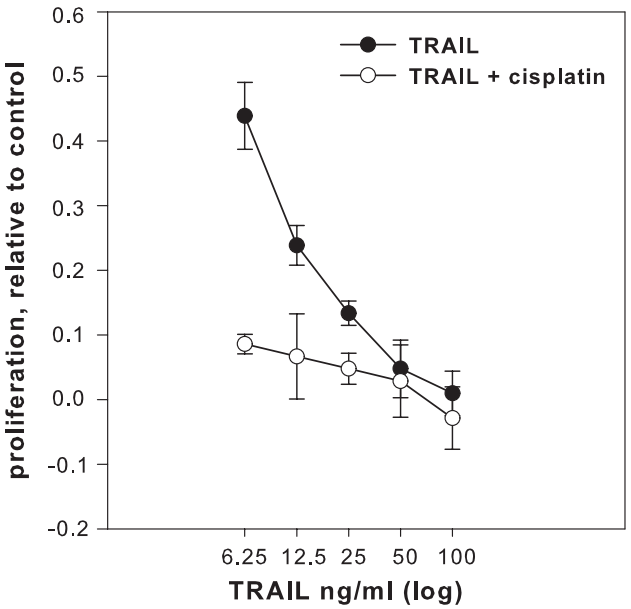
Figure 1

PC-3

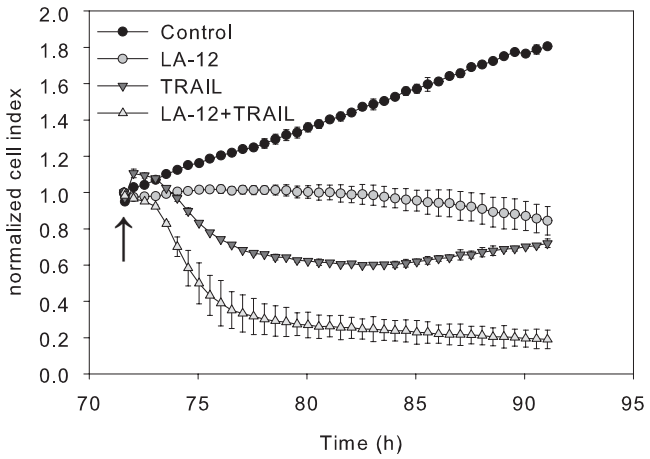
e



f



g



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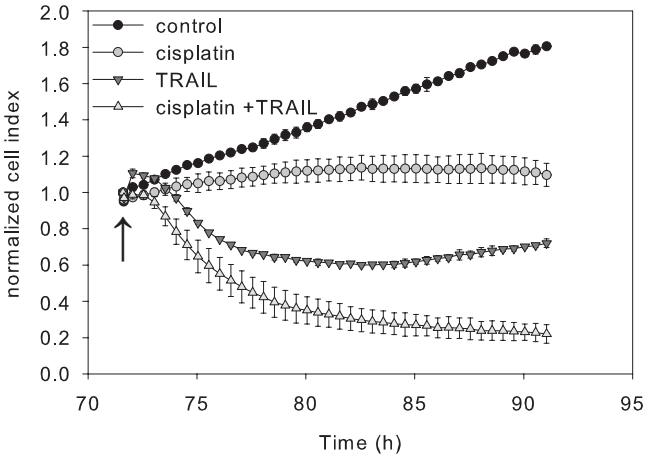


Figure 1 continued

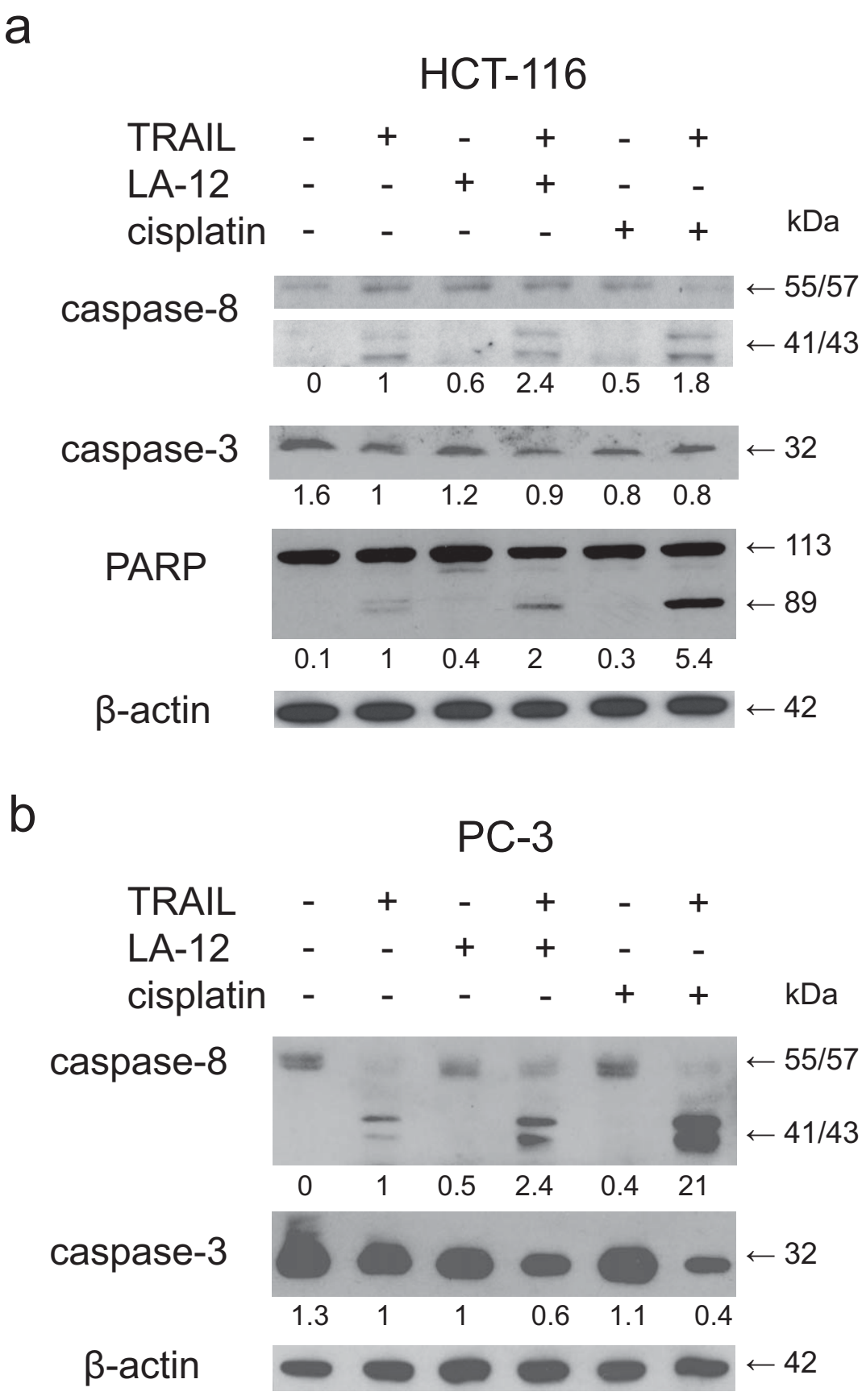


Figure 2

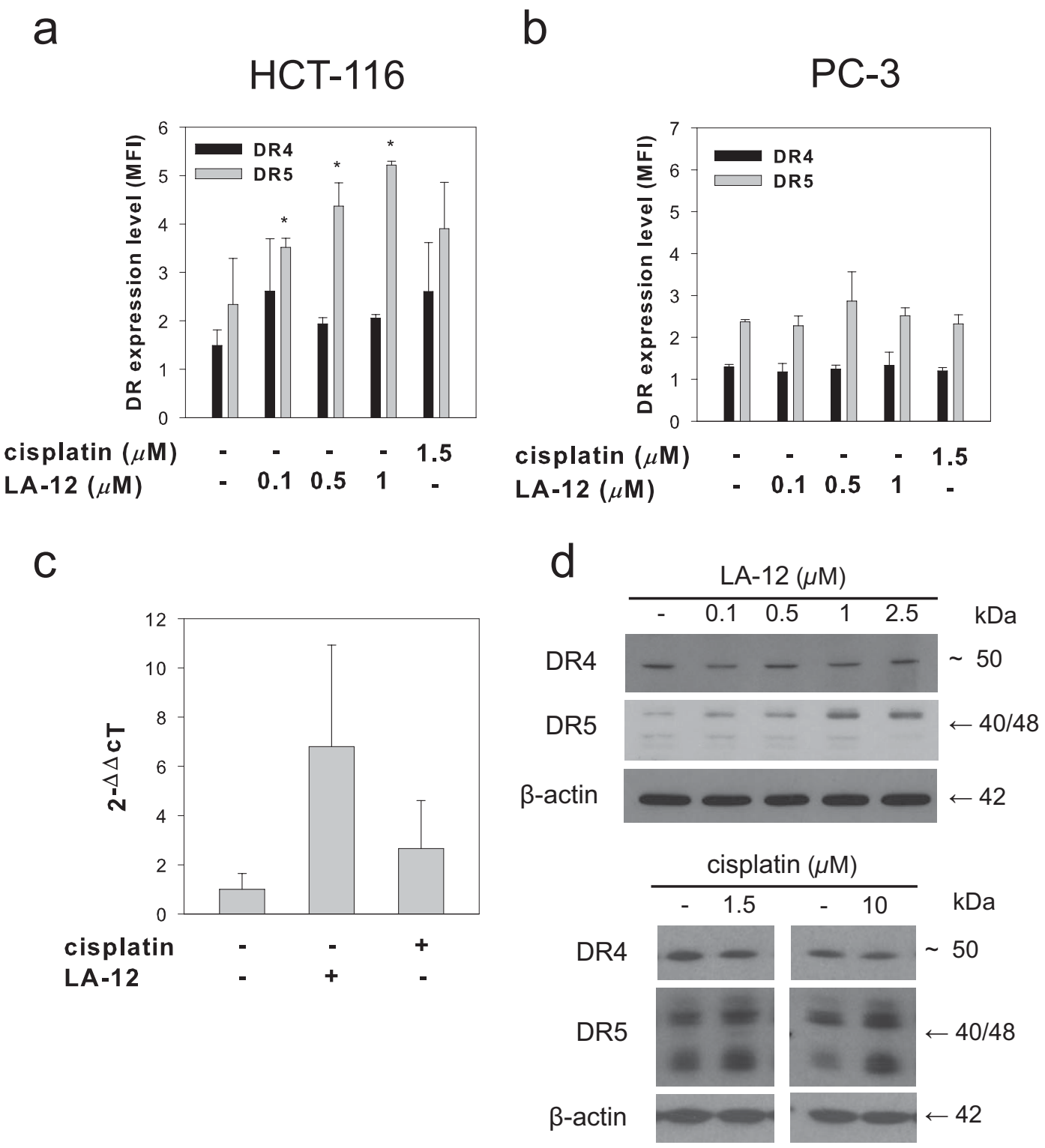


Figure 3

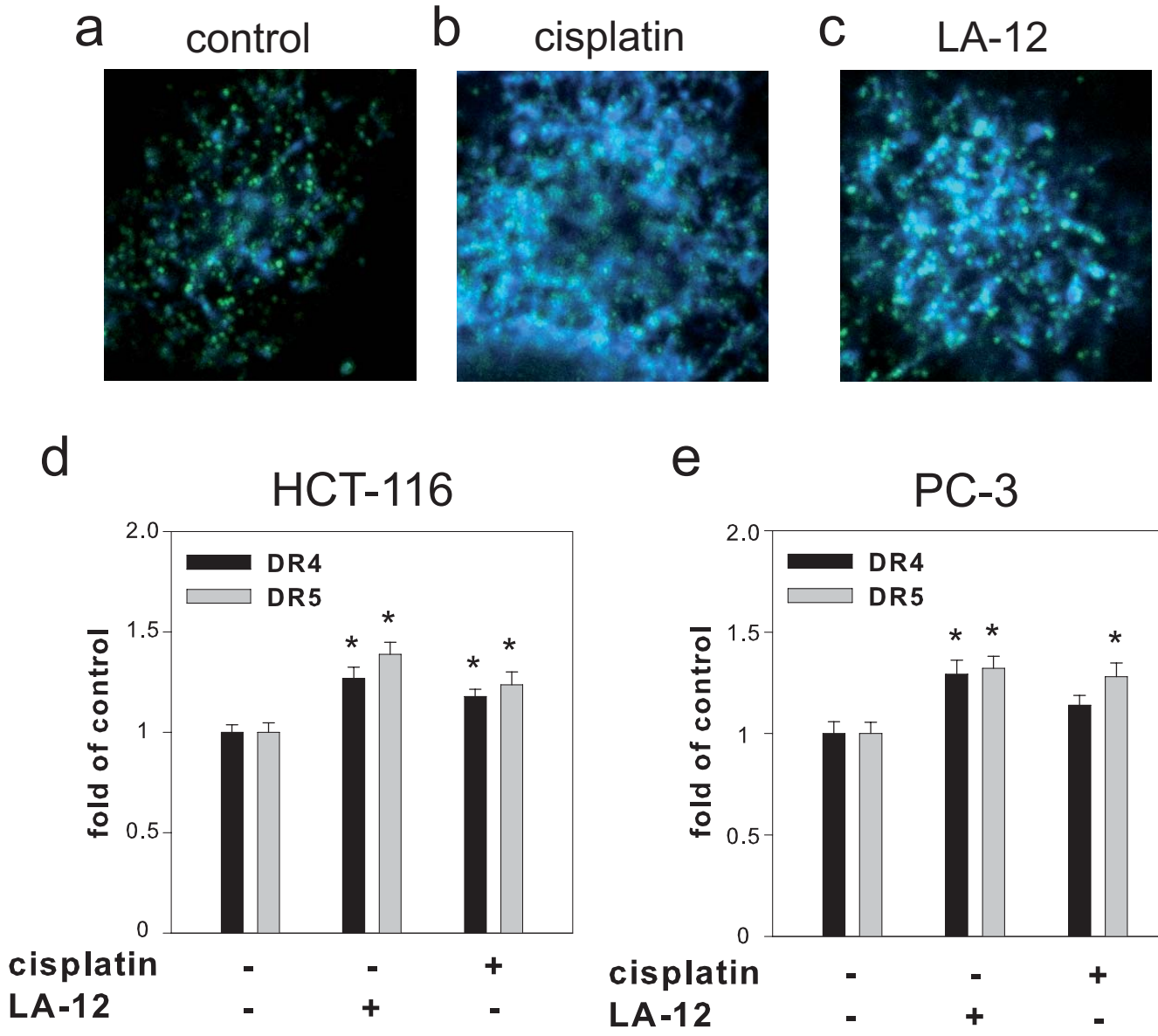
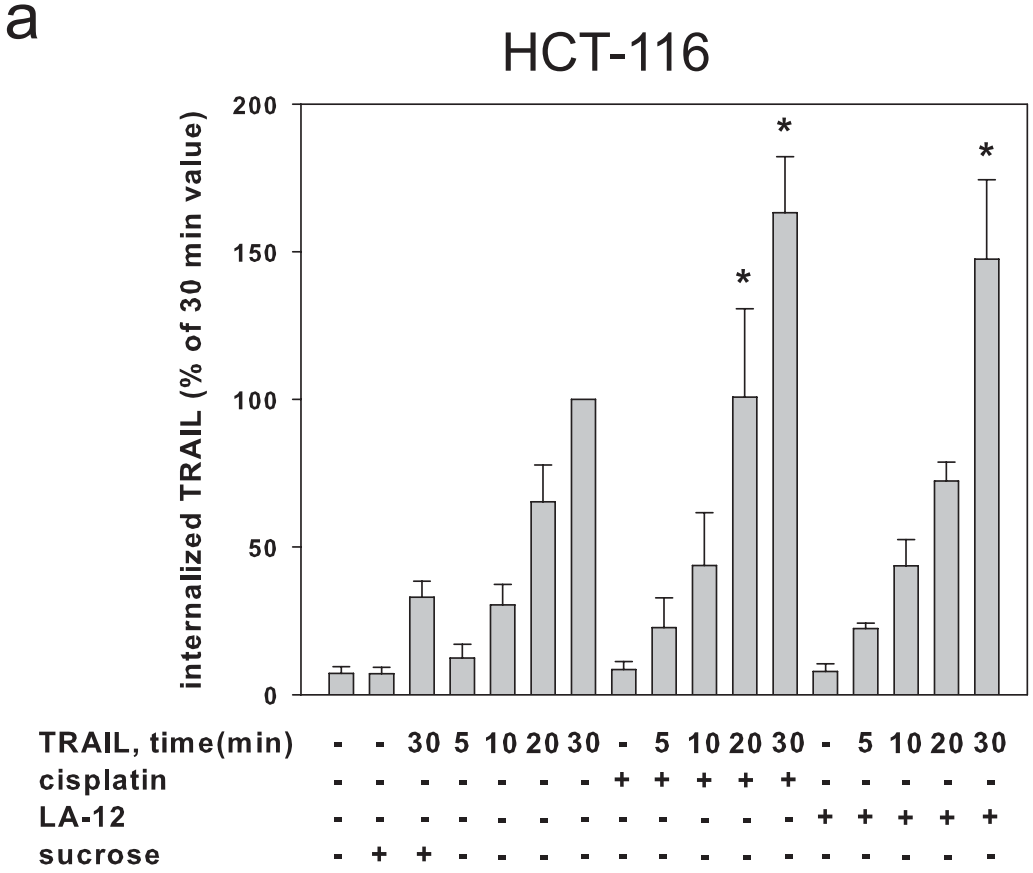


Figure 4

a



b

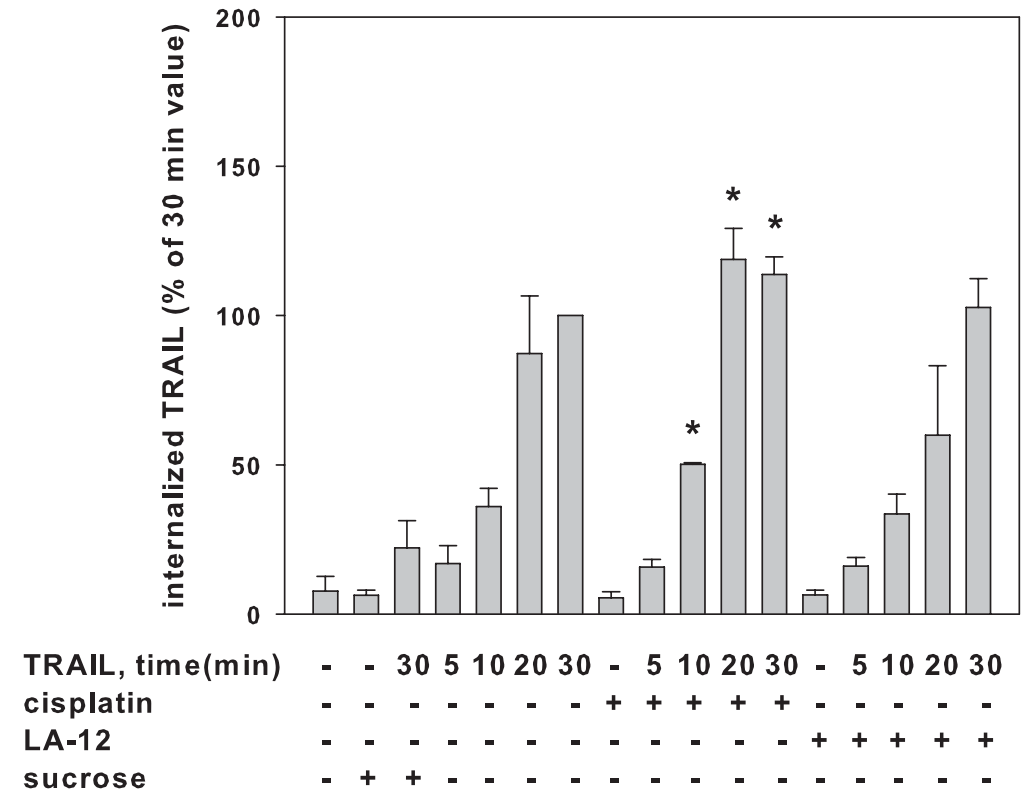


Figure 5

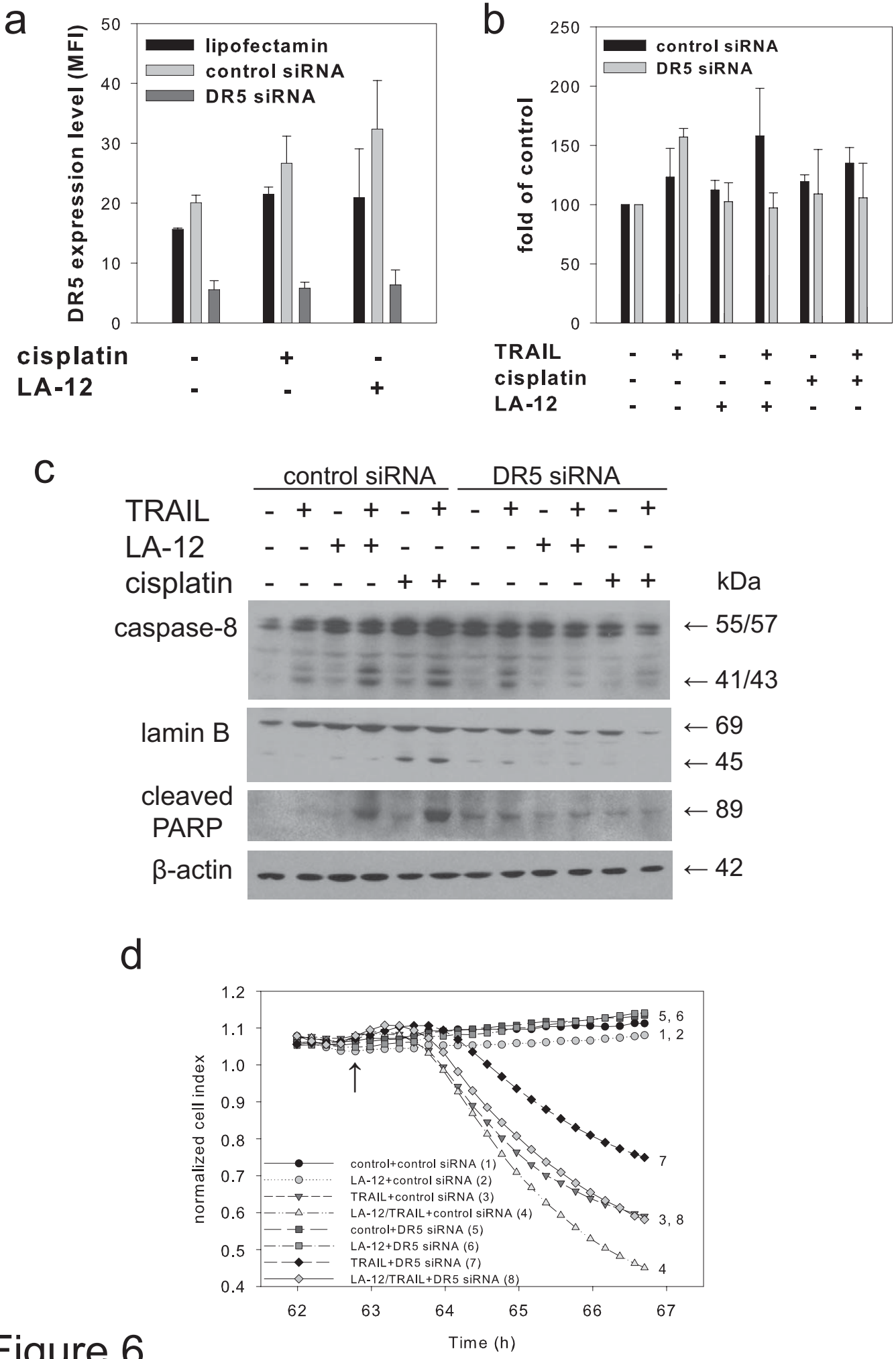


Figure 6