Cardiac Glycosides Initiate Apo2L/TRAIL-Induced Apoptosis in Non–Small Cell Lung Cancer Cells by Up-regulation of Death Receptors 4 and 5

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Abstract

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (Apo2L/TRAIL) belongs to the TNF family known to transduce their death signals via cell membrane receptors. Because it has been shown that Apo2L/TRAIL induces apoptosis in tumor cells without or little toxicity to normal cells, this cytokine became of special interest for cancer research. Unfortunately, cancer cells are often resistant to Apo2L/TRAIL-induced apoptosis; however, this can be at least partially negotiated by parallel treatment with other substances, such as chemotherapeutic agents. Here, we report that cardiac glycosides, which have been used for the treatment of cardiac failure for many years, sensitize lung cancer cells but not normal human peripheral blood mononuclear cells to Apo2L/TRAIL-induced apoptosis. Sensitization to Apo2L/TRAIL mediated by cardiac glycosides was accompanied by up-regulation of death receptors 4 (DR4) and 5 (DR5) on both RNA and protein levels. The use of small interfering RNA revealed that up-regulation of death receptors is essential for the demonstrated augmentation of apoptosis. Blocking of up-regulation of DR4 and DR5 alone significantly reduced cell death after combined treatment with cardiac glycosides and Apo2L/TRAIL. Combined silencing of DR4 and DR5 abrogated the ability of cardiac glycosides and Apo2L/TRAIL to induce apoptosis in an additive manner. To our knowledge, this is the first demonstration that glycosides up-regulate DR4 and DR5, thereby reverting the resistance of lung cancer cells to Apo2/TRAIL-induced apoptosis. Our data suggest that the combination of Apo2L/TRAIL and cardiac glycosides may be a new interesting anticancer treatment strategy. (Cancer Res 2006; 66(11): 5867-74)

Introduction

Lung cancer is the leading cause of cancer death in the United States among both men and women. The projected number of lung cancer in 2005 in the United States is 172,570, accounting for 13% of all new cancer cases and 29% of all cancer deaths (1). In fact, more people die each year from lung cancer than from breast, colorectal, prostate, and ovarian malignancies combined. Therefore, new treatment strategies are needed for this disease.

A potential new anticancer drug is the recently discovered tumor necrosis factor (TNF)–related apoptosis-inducing ligand (Apo2L/TRAIL). Apo2L/TRAIL is a cytokine that is closely related to TNF-α and Fas ligand (FasL), members of the TNF family (2). Apo2L/TRAIL induces apoptosis via interacting with death receptor 4 (DR4; TRAIL-R1) and death receptor 5 (DR5; TRAIL-R2) leading to the formation of the death-inducing signaling complex (DISC) with binding of caspase-8 (FLICE; refs. 3, 4). Recruitment of caspase-8 to the DISC activates its proteolytic properties, which initiates a cascade of protease activation involving enzymes, such as caspase-3, promoting subsequent cleavage of death substrates and finally resulting in apoptosis (3). Apo2L/TRAIL can also bind to three other receptors [i.e., TRAIL-R3 (DcR1 or TRID), TRAIL-R4 (DcR2 or TRUNDD), and the osteoprotegerin receptor OPG]. Because these receptors contain no functional cytoplasmic death domain, they are presumed to primarily operate as competitive decoys for Apo2L/TRAIL (5). Although the ratios of death and decoy receptors are instrumental in the regulation of the apoptotic pathway, it is unclear whether they are also responsible for the resistance to Apo2L/TRAIL-induced apoptosis. Whereas different studies have shown that Apo2L/TRAIL induce apoptosis only in tumor but not normal cells (6, 7), other reports showed cytotoxic effects of Apo2L/TRAIL against certain types of normal cells (8, 9), which might be caused by different Apo2L/TRAIL preparations (10). Tumor cells that are resistant to Apo2L/TRAIL can be sensitized to apoptosis by chemotherapeutic drugs (11, 12) and other agents (13, 14).

In the present study, we show that different cardiac glycosides sensitize lung cancer cells but not normal human peripheral blood mononuclear cells (PBMC) to Apo2L/TRAIL-induced apoptosis. Cardiac glycosides are commonly used for the treatment of cardiac congestion and some types of cardiac arrhythmias for >200 years (15). The action of cardiac glycosides are explained by inhibition of Na^+/K^+ ATPase leading to an increase of intracellular Ca^{2+}, which leads to a better interaction between actin and myosin filaments in cardiac myocytes. Cardiac glycosides were also suggested to have some anticancer activity (16, 17), however, by a mechanism different from targeting the Na^+/K^+ ion pump (18). Our data show for the first time that cardiac glycosides are able to increase Apo2L/TRAIL receptor expression, which accounts for the demonstrated sensitization to Apo2L/TRAIL-induced apoptosis. Although the intracellular mechanism of Apo2L/TRAIL receptor up-regulation remains unclear and requires further investigation, the combination of cardiac glycosides and Apo2L/TRAIL might be of clinical importance as a new strategy for the treatment of lung cancer.

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Materials and Methods

Reagents. Soluble, nontrimerized Apo2L/TRAIL was kindly provided by Genentech (South San Francisco, CA). U0126, SB203580, and SP600125 were obtained from Alexis (San Diego, CA). Oleandrin was from Phytochem (Ichenhausen, Germany). Digitoxin, digoxin, oubain, lanatoside C, and bufalin were purchased from Sigma (St. Louis, MO).

Cell culture. The human lung cancer cell lines A549, NCI-H358, Calu1, and SkLu1 (American Type Culture Collection, Manassas, VA) were cultured at 37°C and 5% CO_2 in DMEM supplemented with 10% FCS and antibiotics. If not stated otherwise, cells were seeded at 1×10^5 per well in 24-well plates and allowed to attach overnight. After stimulation for the indicated times and concentrations, cells were harvested and prepared for further procedure.

Isolation of normal human PBMCs. Heparinized blood was obtained from healthy volunteers. Whole blood was transferred into cell separation tubes (BD Vacutainer CPT, Becton Dickinson, Franklin Lakes, NJ). Blood samples were then centrifuged at $1{,}600 \times g$ for 15 minutes at room temperature. The PBMC interface was collected and washed with sterile PBS. The PBMCs were pelleted by centrifugation at $400 \times g$ for 15 minutes. The pellet was then resuspended in RPMI containing 10% FCS and seeded at 1×10^5 per well in 48-well plates.

Apoptosis assays. Cell death was evaluated by assessment of propidium iodide (PI) uptake. Trypsinized cells were resuspended in ice-cold PBS, PI was added to a final concentration of 10 μg/mL, and probes were immediately analyzed by FACScan (BD Biosciences, San Jose, CA). Caspase-3 activity was determined as described previously (14) with slight modifications. Briefly, cells were lysed in a buffer containing 20 mmol/L HEPES (pH 7.5), 120 mmol/L NaCl, 0.2 mmol/L EDTA, and 1% Triton X-100. Protein (100 μg) from cell lysates in a total of 20 μL was combined with 80 μL of a mix of 32 μL caspase assay buffer [312.5 mmol/L HEPES (pH 7.5), 0.3% CHAPS, 3.1% sucrose], 2 μL DMSO, 1 μL of 1 mol/L DTT, 1 μL DEVD-amc caspase-3 substrate (100 μmol/L stock solution in DMSO; Calbiochem, La Jolla, CA), and 44 μL H_2O. The mixture was transferred to a black microwell plate (Nunc, Roskilde, Denmark) and relative fluorescence was measured at 30°C over a 50-minute period using a Spectramax Gemini Fluorometer (λ_{ex}, 360 nm; λ_{em}, 460 nm; Molecular Devices, Sunnyvale, CA).

Immunoblot analysis. Proteins (50 μg) were separated by PAGE under reducing conditions and transferred onto nylon membranes (Bio-Rad, Hercules, CA) as described previously (19). Protein detection was done using the Immunoblot Chemiluminescence Reagent Plus (New England Nuclear, Life Science Products, Boston, MA). The following antibodies were used: caspase-3 (CM1), caspase-7 (clone 10-1-62; BD Biosciences), caspase-9 (clone 96-2-22; Biolegend, San Diego, CA), caspase-8 (clone C-15), poly(ADP-ribose) polymerase (PARP; clone C-2-10; Alexis), cleaved lamin A (Cell Signaling Technology, Inc., Beverly, MA), and α-tubulin. Secondary horseradish peroxidase–conjugated goat anti-rabbit (Bio-Rad) and goat anti-mouse (Sigma) antibodies were used for detection. For stripping, membranes were incubated for 30 minutes at 50°C in a buffer containing 62.5 mmol/L Tris-HCl (pH 6.7), 2% SDS, and 100 mmol/L β-mercaptoethanol. Subsequently blots were washed, blocked, and reprobed again.

Determination of Apo2L/TRAIL receptor expression. Cells were harvested by short trypsinization, washed once with ice-cold PBS containing 1% bovine serum albumin (BSA), and resuspended in 100 μL PBS with 1% BSA. Then, 5 μg primary anti-Apo2L/TRAIL receptor antibody (DR4-M271, DR5-M413, DcR1-M430, and DcR2-M444; a gift from Immunex Corp., Seattle, WA) was added. Control IgG isotypes (Immunotech, Marseilles, France) were applied to assess nonspecific staining. After 30-minute incubation on ice, cells were washed twice and incubated with FITC-labeled secondary antibody (Immunotech). At least 2×10^4 cells were analyzed by FACScan.

PCR. Total RNA was isolated using the GeneElute Mammalian Total RNA Miniprep kit (Sigma). After DNase digestion using the DNase I kit (Sigma), cDNA was synthesized by standard methods using reverse transcriptase and oligo(dT) primer from Roche (Rotkreuz, Switzerland). For the semiquantitative PCR, 5 μL cDNA-template was mixed with 2.5 μL of 10× PCR buffer, 0.5 μL of 10 mmol/L deoxynucleotide triphosphates, 0.25 μL Taq polymerase, and 0.25 μL of each primer (50 μmol/L; Invitrogen Custom Primers, Basel, Switzerland) in a total volume of 25 μL for each probe. PCR was carried out in a Eppendorf Mastercycler (Vaudaux-Eppendorf, Schönenbuch, Switzerland) using the following primers (sense and antisense, respectively): DR4 5′-TTGTGTCCACCAGGATCTCA-3′ and 5′-GTCACTCCAGGGCGTACAAT-3′ (20) and DR5 5′-ACTCCTGGAATGACTACCTG-3′ and 5′-ATCCCAAGTGAACTTGAGCC-3′. Amplification of 28S rRNA served as internal control. The 28S rRNA primers were 5′-GTGGAATGCGAGTGCCTA-3′ and 5′-GTTGATTCGGCAGGTGAGTT-3′. Negative controls were done for each set of primers. After amplification, PCR products were separated by electrophoresis on 1.5% agarose gels containing ethidium bromide and visualized by UV light illumination. PCR conditions were as follows: 1 cycle for 3 minutes at 95°C and 22 to 26 cycles for 30 seconds at 95°C, 30 seconds at 58°C, and 1 minute at 72°C.

Transfection with small interfering RNA. Calu1 cells were seeded into 24-well plates at 0.5×10^5 per well. After 24 hours, cells were transfected with 1 μg TRAIL receptor-specific and control small interfering RNA (siRNA) by using 2 μL Dharmafect reagent (Dharmacon, Lafayette, CO) according to the manufacturer's protocols. All the siRNAs were synthesized by Qiagen (Valencia, CA). The sequences of siRNAs used were as follows (sense and antisense, respectively): siDR4 5′-r(CAAACUUCAUGAUCAAUCA)dTdT-3′ and 5′-r(UGAUUGAUCAUGAAGUUUG)dAdT-3′, siDR5 5′-r(GACCCUUGUGCUCGUUGUC)dTdT-3′ and 5′-r(GACAACGAGCACAAGGGUC)dTdT-3′ (21), and control siRNA 5′-r(UUCUCCGAACGUGUCACGU)dTdT-3′ and 5′-r(ACGUGACACGUUCGGAGAA)dTdT-3′ (22). Twenty-four hours after transfection, cells were washed with PBS, culture medium was replaced, and cells were stimulated with 160 ng/mL oleandrin and/or 100 ng/mL TRAIL. Cells were harvested either 24 hours after stimulation for determination of mRNA or cell surface receptor expression by PCR or flow cytometry, respectively, or 48 hours after stimulation for PI staining followed by FACScan analysis.

Data analysis. Band intensions of reverse transcription-PCR (RT-PCR) experiments were evaluated densitometrically using Quantity One analysis software (Bio-Rad). For statistical analysis, data were subjected to one-way or two-way ANOVA using GraphPad Prism (GraphPad Software, San Diego, CA). Differences between experimental groups were determined by Bonferroni *post hoc* test. Differences were considered statistically significant at Ps < 0.05.

Results

Oleandrin sensitizes lung cancer cell lines to Apo2L/TRAIL-induced apoptosis. To investigate the mechanisms responsible for cellular resistance to Apo2L/TRAIL-induced apoptosis, we addressed the question whether blocking of death receptor internalization would disrupt or accelerate the death-inducing machinery. Importantly, among all the substances tested that were shown to block receptor internalization (23), only the cardiac glycoside oleandrin had an effect on Apo2L/TRAIL-induced apoptosis in Calu1 lung cancer cells, suggesting that at least in this cell line receptor internalization is not fundamental for susceptibility to Apo2L/TRAIL.

We examined the effect of combined treatment with different concentrations of oleandrin and 100 ng/mL Apo2L/TRAIL for 48 hours in Calu1, a cell line that is highly resistant to Apo2L/TRAIL (12). Neither oleandrin nor Apo2L/TRAIL induced cell death as single agents; however, combined treatment with oleandrin and Apo2L/TRAIL resulted in massive cell death, assuming oleandrin sensitizes Calu1 cells to Apo2L/TRAIL-induced apoptosis (Fig. 1*A*). Similar results were found in NCI-H520, SkLu1, and A549 cells, which also have been shown previously to be resistant to Apo2L/TRAIL (12, 24). However, in the cell line A549, treatment with oleandrin alone resulted in relative high toxicity (61.5% at 160 ng/mL oleandrin), which was not seen in the three other lung cancer cell lines (Fig. 1*A*). Interestingly, when cells were treated with oleandrin alone for 24 hours followed by treatment with Apo2L/

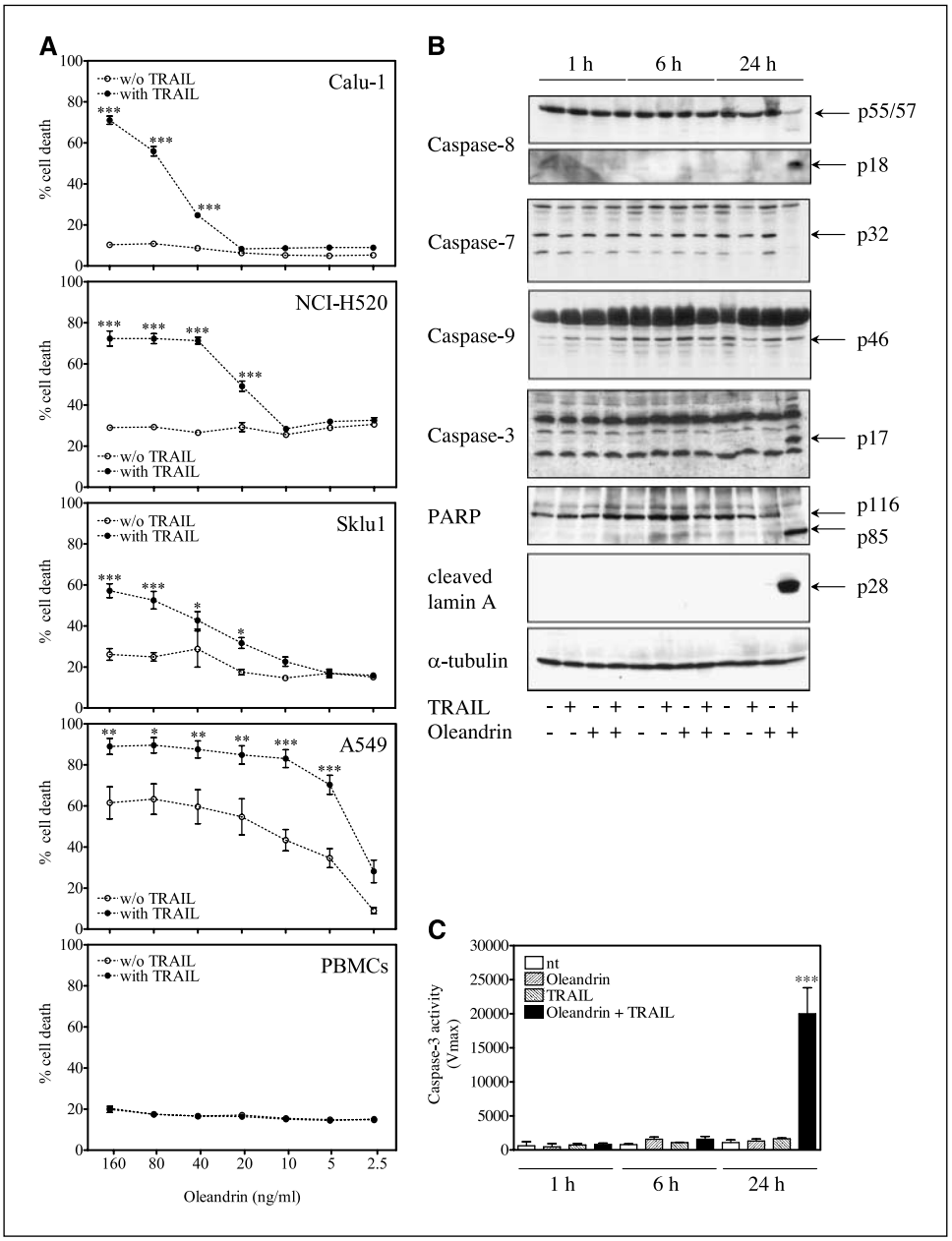

Figure 1. Cell death in lung cancer cells and PBMCs induced by treatment with Apo2L/TRAIL and different concentrations of oleandrin. *A,* cell lines and PBMCs were treated for 48 hours and cell death was assessed by PI staining followed by flow cytometry. *Points,* mean of three independent experiments with duplicates; *bars,* SE. Two-way ANOVA was done to compare the effect of oleandrin concentrations and the effect of absence or presence of Apo2L/TRAIL treatment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. *B,* activation of caspases and cleavage of their substrates as shown by Western blot analysis. *C,* measurement of caspase-3 activity by determination of hydrolysis of DEVD-amc. *Columns,* mean of two independent experiments; *bars,* SE. ***, $P < 0.001$ (two-way ANOVA).

TRAIL alone for additional 24 hours (sequential treatment), less cell death was observed compared with combined treatment for 48 hours (data not shown), suggesting that permanent stimulation by the glycoside is required for sensitization of lung cancer cells to Apo2L/TRAIL-induced apoptosis.

To test whether normal cells are susceptible to the combination of oleandrin and Apo2L/TRAIL, we used normal human PBMCs. Our data in Fig. 1*A* indicate that normal PBMCs are not sensitized by oleandrin to Apo2L/TRAIL-induced apoptosis. Importantly, even when used oleandrin at high concentrations up to 1 μg/mL, the combination with Apo2L/TRAIL was not able to induce apoptosis in normal human PBMCs (data not shown).

We asked next whether the observed cell death after combined treatment with oleandrin and Apo2L/TRAIL reveals typical hallmarks of apoptosis. Western blot analysis showed that cleavage of the proforms into the active fragments of caspase-8, caspase-7, and caspase-3 was only found in cells treated with the combination of oleandrin and Apo2L/TRAIL for 24 hours but not after treatment with each compound alone (Fig. 1*A*). In addition, cleavage of the caspase substrates PARP and lamin A also occurred only after combined treatment (Fig. 1*B*). No cleavage of the proform of caspase-9 into the active fragment was observed, although different caspase-9-specific antibodies have been used. This suggests that the mitochondrial type II apoptotic pathway (25) does not contribute to the demonstrated oleandrin-mediated sensitization to Apo2L/TRAIL-induced apoptosis.

The involvement of apoptosis was also assessed determining the enzymatic activity of caspase-3. Figure 1*C* shows that the treatment of Calu1 cells with a combination of oleandrin and Apo2L/TRAIL resulted in a 20-fold increase of caspase-3 activity when compared with either oleandrin or Apo2L/TRAIL treatment alone. Remarkably, not only cleavage of caspases and caspase substrates but also

enzymatic activation of caspase-3 were undetectable 1 or 6 hours after combined treatment but were pronounced after 24 hours. This fact indicates that metabolic changes in terms of protein expression are likely to precede oleandrin-mediated sensitization of lung cancer cells.

Up-regulation of DR4 and DR5 expression is required for oleandrin-mediated sensitization to Apo2L/TRAIL-induced apoptosis. Sensitization to Apo2L/TRAIL-induced apoptosis has been explained in some studies by up-regulation of DR5 (26, 27), whereas other results show that sensitization can occur without increased TRAIL receptor expression (14). To examine the possible mechanisms of oleandrin-mediated sensitization to Apo2L/TRAIL-induced apoptosis in lung cancer cells, we determined Apo2L/TRAIL receptor expression on the surface of Calu1 cells 24 hours after treatment with oleandrin. As shown in Fig. 2*A*, oleandrin clearly increased cell surface expression of DR4 and DR5, the two Apo2L/TRAIL receptors with functional death domain. In addition, DcR1 was up-regulated by oleandrin, whereas expression of DcR2 was unchanged.

The demonstrated increased surface expression DR4 and DR5 might be caused by two different scenarios. Zhang et al. described a reservoir for Apo2L/TRAIL receptors at the endoplasmic reticulum, where new receptors can be recruited to the cell membrane (28). Alternatively, the increase of receptor proteins may result from *de novo* synthesis and induction of gene expression. A time-course experiment showed a significant increase in mRNA levels of DR4, evident 16 hours after treatment with oleandrin and continuing at least until 24 hours (Fig. 2*B*). Similarly, DR5 mRNA levels started to increase 6 hours after oleandrin treatment and continued rising at least until 24 hours. Our data implicate increased transcription followed by higher rates of protein synthesis in DR4 and DR5 up-regulation, which would also explain the observed delay of >6 hours until cells are sensitized by oleandrin to Apo2L/TRAIL-induced apoptosis.

To analyze whether up-regulation of DR4 and DR5 was just a "side effect" or is in fact necessary for oleandrin-mediated sensitization to Apo2L/TRAIL-induced apoptosis, we blocked up-regulation of the receptors by siRNA against DR4 and DR5. Transfection of Calu1 cells with siRNA targeting the receptors but not with a control siRNA resulted in a marked inhibition of oleandrin-mediated DR4 and DR5 RNA and protein induction (Fig. 3*A* and *B*). The evaluation of cell death after combined treatment with oleandrin and Apo2L/TRAIL in cells transfected

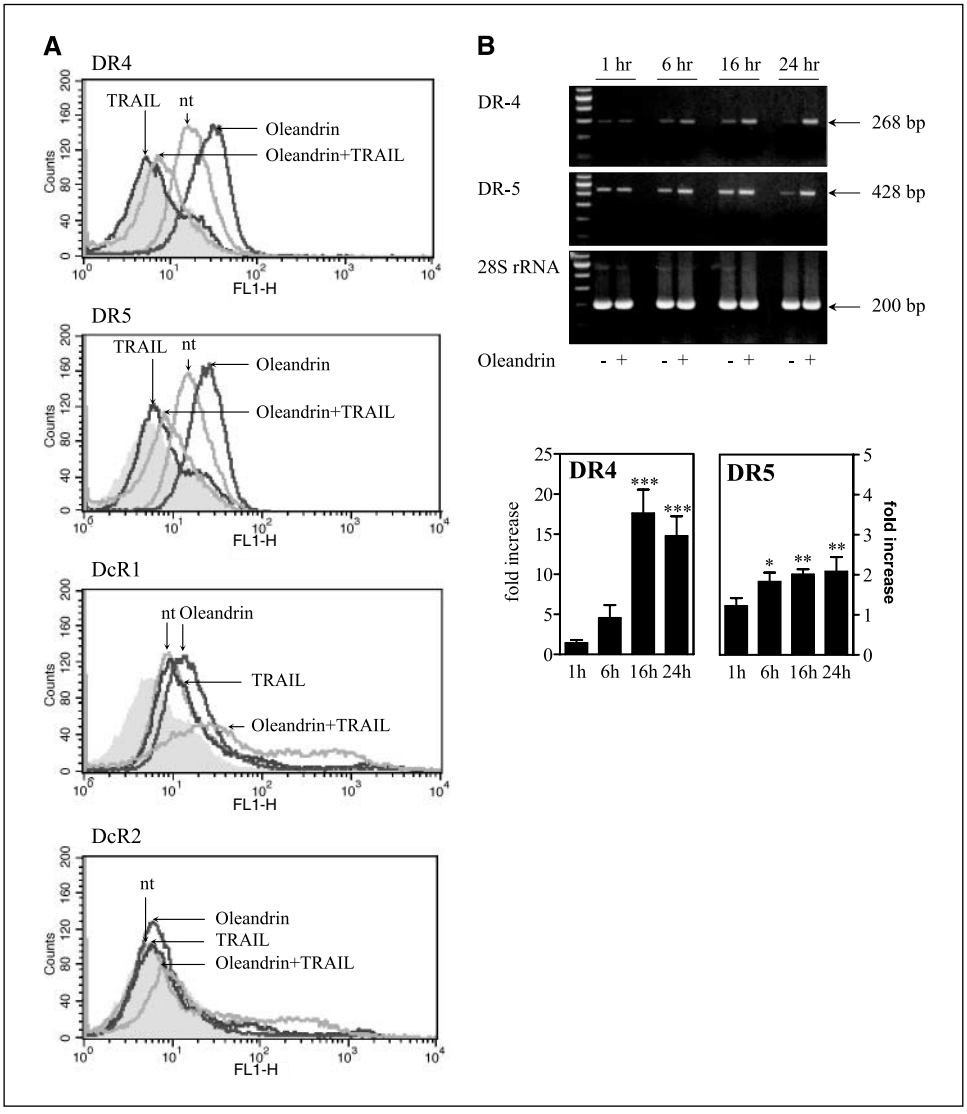

Figure 2. Apo2L/TRAIL receptor surface expression (*A*) and DR4 and DR5 mRNA expression (*B*) in Calu1 cells. *A,* cells were treated with 160 ng/mL oleandrin and 100 ng/mL Apo2L/TRAIL alone or in combination for 24 hours. Subsequently, cells were stained with monoclonal antibodies raised against the extracellular domain of Apo2L/TRAIL receptors DR4, DR5, DcR1, and DcR2. Data were analyzed by flow cytometry. *Gray histogram,* cells stained with isotype control IgG antibody. *B,* cells were treated with 160 ng/mL oleandrin and 100 ng/mL Apo2L/TRAIL alone or in combination for indicated time points and then harvested for extraction of total cellular RNA. DR4 and DR5 mRNA expression was detected by RT-PCR. Amplification of 28S rRNA mRNA was carried out as an internal control. Semiquantitative evaluated DR4 and DR5 mRNA levels of oleandrin-treated samples were expressed as fold increase versus their time-corresponding samples that were not treated with oleandrin. Representative of three independent experiments analyzed using two-way ANOVA.

with siRNA showed that blocking of DR4 and DR5 expression alone significantly reduced the rate of cell death (Fig. 3*C*). The highest inhibition of apoptosis was observed when up-regulation of both receptors was blocked in parallel, thus showing an additive effect of blocking DR4 and DR5 at the same time (Fig. 3*C*). From these observations, we conclude that oleandrin-mediated sensitization of lung cancer cells to Apo2L/TRAIL-induced apoptosis depends on up-regulation of both DR4 and DR5.

Mitogen-activated protein kinases are not responsible for oleandrin-mediated augmentation of Apo2L/TRAIL-induced cell death. Different studies suggest that up-regulation of DR5 as the main player in Apo2L/TRAIL-induced apoptosis is mediated by members of the mitogen-activated protein kinase (MAPK) family (22, 29). To evaluate the involvement of MAPKs in oleandrin-mediated up-regulation of DR4 and DR5, we used specific inhibitors for p38, p42/p44, and c-Jun NH_2-terminal kinase (JNK). Inhibition of p42/p44 by U0126 resulted in a significant reduction of DR4 and DR5 mRNA levels in oleandrin-sensitized cells, whereas inhibition of p38 and JNK had no effect (Fig. 4*A*). However, at the protein level, a reduction of expression was only seen for DR4 in cells treated with oleandrin and U0126 (Fig. 4*B*). This indicates that p42/p44 might be involved in the up-regulation of DR4 but not DR5. Because it is known from other studies and supported by Fig. 3*C* that DR5 plays the pivotal role in Apo2L/TRAIL-induced apoptosis, we conclude from these results that MAPKs might be not required for glycoside-mediated sensitization.

Different cardiac glycosides sensitize lung cancer cells to Apo2L/TRAIL-induced apoptosis. Because oleandrin is not widely used in clinical practice thus far, we examined whether other cardiac glycosides are able to sensitize lung cancer cells to Apo2L/TRAIL-induced apoptosis. Two of the five glycosides used in this study, digoxin and digitoxin, are commonly applied in daily clinical practice. As shown in Fig. 5*A*, all five cardiac glycosides sensitized Calu1 cells to Apo2L/TRAIL-induced apoptosis. Moreover, the kinetics of augmented cell death and the dosage dependence were almost similar. Furthermore, all cardiac glycosides used in this study clearly increased cell surface expression of both DR4 and DR5 (Fig. 5*B*). In summary, these results show that at least six different cardiac glycosides are able to sensitize lung cancer cells to Apo2L/TRAIL-induced apoptosis.

Discussion

Resistance of cancer cells to apoptosis still represents a major problem of anticancer therapy. Therefore, sensitization of cancer cells to certain apoptotic pathways might be a useful strategy to define new treatment options for this devastating disease. In this report, we show for the first time that non–small cell lung cancer (NSCLC) cells can be sensitized to Apo2L/TRAIL-induced apoptosis by combined treatment with different cardiac glycosides. Some of

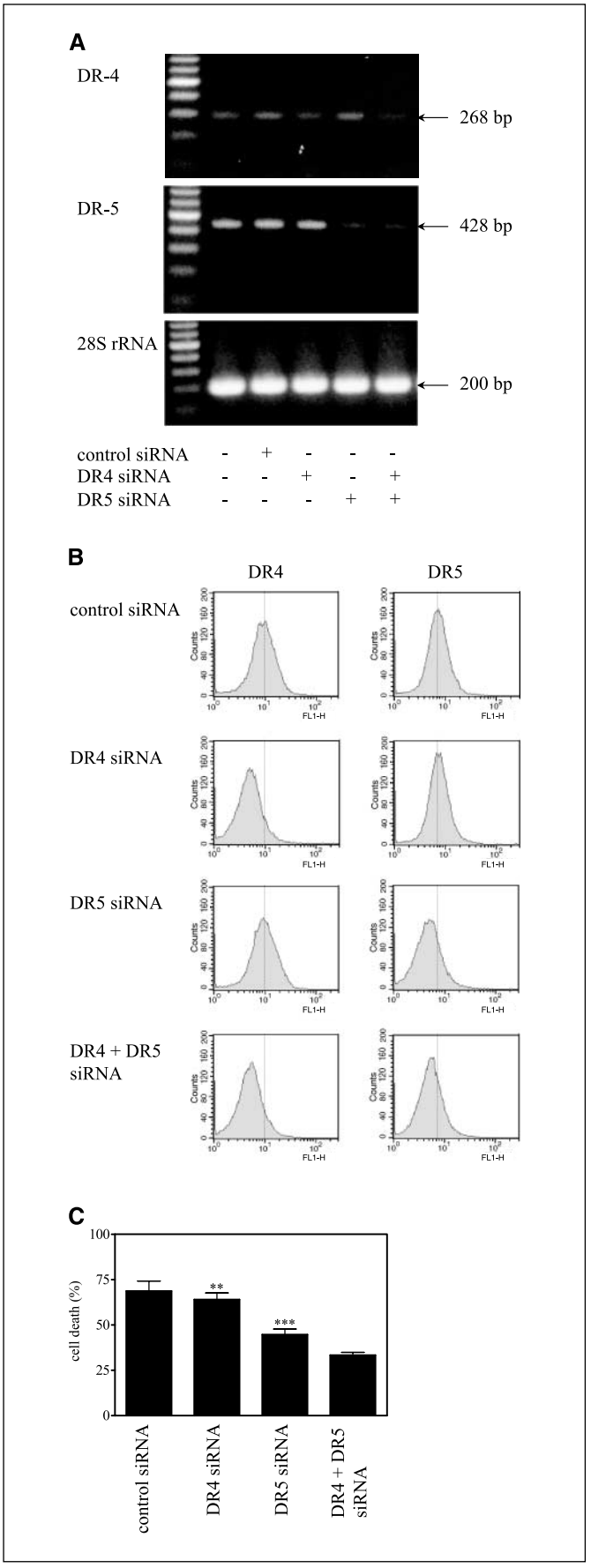

Figure 3. Silencing of oleandrin-induced DR4 and DR5 mRNA expression (*A*) and DR4 and DR5 receptor expression on cell surface (*B*) and its effects on apoptosis induced by combined treatment with Apo2L/TRAIL and oleandrin (*C*). Transfected Calu1 cells were treated with 160 ng/mL oleandrin, and after 24 hours, the cells were either (*A*) harvested for preparation of total RNA and subsequent RT-PCR analysis or (*B*) stained with monoclonal antibodies and analyzed by flow cytometry. *C,* cell death was induced by combined treatment with 160 ng/mL oleandrin and 100 ng/mL Apo2L/TRAIL for 48 hours and assessed by PI staining followed by flow cytometry. *Columns,* mean of three independent experiments in duplicates; *bars,* SD. **, $P < 0.01$; ***, $P < 0.001$, significant difference to cells transfected with control siRNA.

the glycosides used in this study are commonly applied to patients for >200 years.

How cardiac glycosides contribute to the induction of apoptosis is not clear yet. Previous studies reported conflictive findings. Whereas some experimental systems have been described in which cardiac glycosides inhibited apoptosis of normal human prostate and vascular smooth muscle cells (30, 31), other groups reported that cardiac glycoside are able to induce apoptosis of cancer cells *in vitro* (32) and *in vivo* (16). The controversial effects of glycosides on the induction of apoptosis suggest that the apoptotic potential of these compounds is dependent on the cell type.

Thus, it is not astonishing that the mechanism by which cardiac glycosides induce apoptosis remains unclear. Although cardiac glycosides are specific inhibitors of Na^+/K^+-ATPase leading to an intracellular elevation of Ca^{2+}, additional mechanisms other than changes in intracellular ion homeostasis seem to be involved in glycoside-induced signal transduction. Liu et al. showed that the glycoside ouabain induced rapid tyrosine phosphorylation in several proteins, which was not caused by changes in intracellular Ca^{2+} or Na^+ ions (33). In cardiac myocytes, glycoside-mediated tyrosine phosphorylation was shown to activate the Ras/MAPK cascade via activation of Src kinase followed by phosphorylation of epidermal growth factor (34). Signaling events further downstream are controversially discussed. Whereas some studies revealed an induction of the transcription factors c-jun and c-fos and an activation of activator protein-1 (AP-1) after treatment with the glycoside ouabain (35, 36), Manna et al. reported an inhibition of the transcription factors AP-1, nuclear factor-κB (NF-κB), and the MAPK c-Jun by the glycoside oleandrin (37). Our data show that in NSCLC cell lines cardiac glycosides up-regulate DR4 and DR5, which is responsible for sensitization to Apo2L/TRAIL-mediated apoptosis. Up-regulation of DR4 and DR5 by cardiac glycosides was initiated on transcriptional level; however, the participating transcription factors have not been identified. Our results further suggest that MAPKs and their respective transduction pathways might be not crucially involved in glycoside-mediated sensitization to Apo2L/TRAIL-induced apoptosis. In addition, experiments investigating the activity of NF-κB showed no relevant contribution of this transcription factor to glycoside-mediated action at least in Calu1 cells (data not shown).

It might be hypothesized that up-regulation of DR4 and DR5 by cardiac glycosides provides an explanation for the antitumor effects of the glycoside digitoxin observed in a model of chemically induced NSCLC in mice (16) as well as for the reported oleandrin-mediated sensitization of prostate cancer cells to radiation therapy (38). To get more evidence for this hypothesis, it would be important to verify that glycosides are also able to sensitize lung cancer cells *in vivo*, the subject of our ongoing research. In this context, there is some

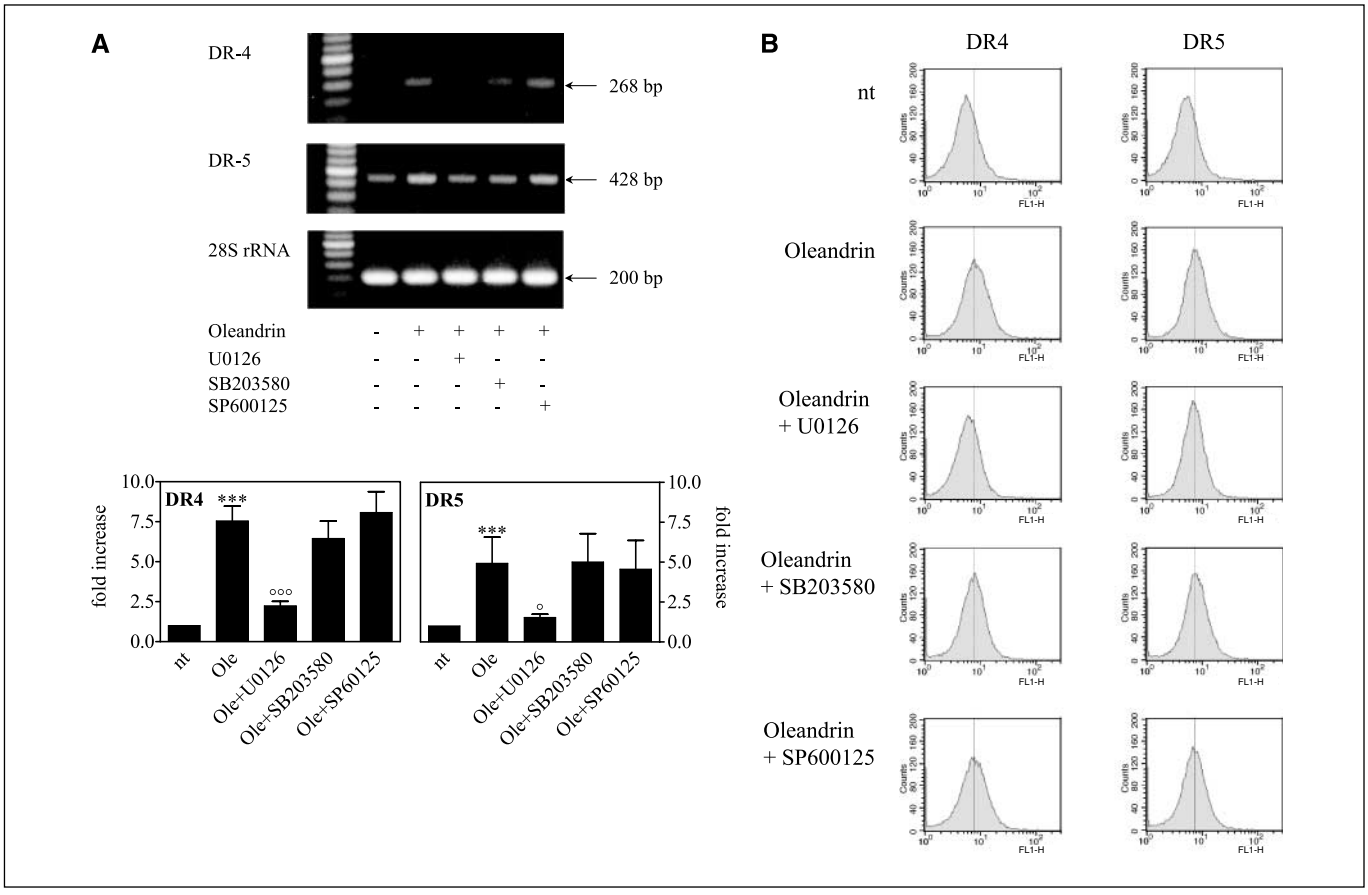

Figure 4. Effect of inhibition of MAPKs [extracellular signal-regulated kinase 1/2 (ERK1/2), JNK, and p38] on expression of mRNA (*A*) and cell surface (*B*) expression of DR4 and DR5. Calu1 cells were preincubated for 30 minutes with 10 μmol/L U0126 (ERK1/2 inhibitor), SP600125 (JNK inhibitor), or SB203580 (p38 kinase inhibitor) and stimulated with 160 ng/mL oleandrin (*Ole*). After 24 hours, cells were either (*A*) harvested for preparation of total RNA extracts and subsequent RT-PCR analysis or (*B*) trypsinized and stained with monoclonal antibodies against DR4 or DR5 followed by flow cytometry. The effect of inhibitors of MAPKs on expression of mRNA expression of DR4 and DR5 receptors was semiquantitatively evaluated and expressed as fold increase relative to nontreated control cells. ***, $P < 0.001$, versus nontreated control cells; °, $P < 0.05$, versus oleandrin-treated cells; °°°, $P < 0.001$, versus oleandrin-treated cells. All experiments were done thrice.

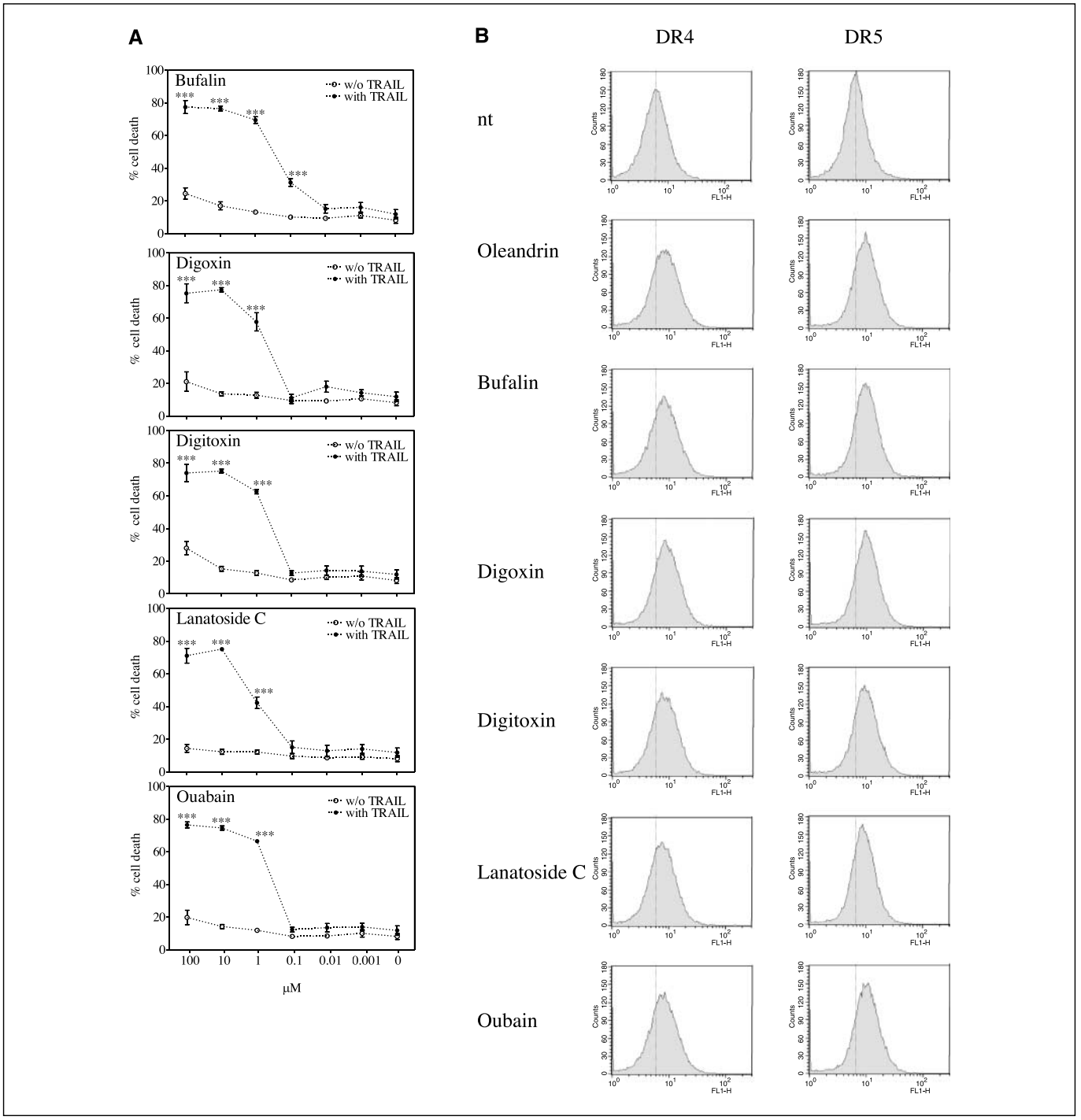

Figure 5. Effect of different cardiac glycosides on Apo2L/TRAIL-induced apoptosis and expression of DR4 and DR5. *A,* Calu1 cells were treated with different concentration of cardiac glycosides alone or in combination with 100 ng/mL Apo2L/TRAIL for 48 hours. Cells were stained with PI for the assessment of cell death followed by flow cytometry analysis. *Points,* mean of three independent experiments with duplicates; *bars,* SE. Two-way ANOVA was done to compare the effect of different concentrations of cardiac glycosides and the effect of absence or presence of Apo2L/TRAIL treatment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. *B,* Calu1 cells were treated with cardiac glycosides for 24 hours and stained with monoclonal antibodies raised against the extracellular domain of DR4 or DR5 followed by flow cytometry analysis.

evidence that cardiac glycosides have influence on the morphology and recurrence rate of human breast carcinoma (39, 40). Thus, it would be of interest to determine whether DR4 and DR5 are up-regulated in patients under digitalis medication, indicating a possible explanation for the suggested antitumor effects.

Another important aspect concerns the question of whether the demonstrated effects of cardiac glycosides on Apo2L/TRAIL-induced apoptosis is restricted to Apo2L/TRAIL or may be extended to other death receptor pathways depending on death domains, such as TNF or FasL/CD95L. Previous studies reported

that the glycoside ouabain potentiates TNF-induced and FasL-induced apoptosis (41, 42). Using the Fas receptor agonistic antibody CH-11 and recombinant TNF in combination with oleandrin, the combination of the glycoside neither with CH-11 nor with TNF promoted cell death as it was seen for Apo2L/TRAIL-induced apoptosis.[3] This implies that glycosides modulate component(s) of the apoptotic machinery, which are specific for the Apo2L/TRAIL death receptor pathway. The identification of these signaling events and the cancer cell type specificity of their mode of action remain important questions to be solved.

In conclusion, we report here that cardiac glycosides sensitize lung cancer cells to Apo2L/TRAIL-induced apoptosis. Glycoside-mediated sensitization is accompanied and requires up-regulation of Apo2L/TRAIL receptors proposing an entire new and before unknown function of cardiac glycosides. Considering the fact that normal blood cells are not sensitized, cardiac glycosides in combination with Apo2L/TRAIL may provide a new treatment strategy for NSCLC.

[3] Unpublished observations.

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