

Tumor necrosis factor-related apoptosis-inducing ligand as a therapeutic option in urothelial cancer cells with acquired resistance against first-line chemotherapy

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Abstract. Patients with urothelial carcinoma frequently fail to respond to first-line chemotherapy using cisplatin and gemcitabine due to development of resistant tumor cells. The aim of the present study was to investigate whether an alternative treatment with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) that induces tumor cell death via the extrinsic apoptotic pathway may be effective against chemotherapy-resistant urothelial cancer cell lines. The viability of the urothelial cancer cell line RT112 and its chemotherapy-adapted sublines was investigated by MTT assay. The expression of anti-apoptotic proteins was determined by western blotting and the individual roles of cellular inhibitor of apoptosis protein (cIAP)1, cIAP2, x-linked inhibitor of apoptosis protein (XIAP) and induced myeloid leukemia cell differentiation protein (Mcl-1) were investigated by siRNA-mediated depletion. In particular, the bladder cancer sublines that were resistant to gemcitabine and cisplatin were cross-resistant to TRAIL. Resistant cells displayed upregulation of anti-apoptotic molecules compared with the parental cell line. Treatment with the second mitochondrial activator of caspases (SMAC) mimetic LCL-161 that antagonizes cIAP1, cIAP2 and XIAP resensitized chemoresistant cells to TRAIL. The resensitization of tumor cells to TRAIL was confirmed by depletion of antiapoptotic proteins with siRNA. Collectively, the findings of the present study demonstrated that SMAC mimetic LCL-161 increased the sensitivity of the parental cell

line RT112 and chemotherapy-resistant sublines to TRAIL, suggesting that inhibiting anti-apoptotic molecules renders TRAIL therapy highly effective for chemotherapy-sensitive and -resistant urothelial cancer cells.

Introduction

Patients with metastatic urothelial cancer of the bladder have limited therapeutic options. First-line cisplatin-based chemotherapy, usually consisting of gemcitabine and cisplatin, is only associated with a median survival of 12-14 month (1,2). In addition, available second-line therapies with the vinca alkaloid vinflunine or immune checkpoint inhibitors, such as pembrolizumab (PD-1), nivolumab (PD-1), or atezolizumab (PD-L1) show only limited success in terms of prolongation of survival (3,4). Therefore, improved novel therapies are urgently needed.

It has long been known that tumor necrosis factor (TNF) can induce selective death in tumor cells (5). Unfortunately, several studies have reported that TNF treatment induced a lethal inflammatory shock syndrome, which markedly limits its applicability as a selective cancer treatment (6). TNF α -related apoptosis-inducing ligand (TRAIL) induces tumor cell death via the extrinsic apoptotic pathway without causing a lethal inflammatory shock syndrome (7-9). However, only a small proportion of patients in clinical trials responded to various drugs that targeted TRAIL death receptors (10,11). In addition, several urothelial cancer cell lines appear to be resistant to TRAIL therapy (12). A possible reason for this resistance to TRAIL treatment is the presence of alterations in the apoptotic pathways (13). Restoring the integrity of apoptotic pathways in resistant cancer cells may be a promising new approach to overcoming TRAIL and chemotherapy resistance. Inhibitor of apoptosis proteins (IAP), including cellular inhibitor of apoptosis protein (cIAP)1, cIAP2 and X-linked inhibitor of apoptosis protein (XIAP), have been emerging as anticancer drug targets (14). The expression of IAPs contributes to drug resistance in bladder cancer (15). A possible strategy to resensitize tumor cells that are resistant to TRAIL therapy may be

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inhibition of IAPs with the second mitochondrial activator of caspases (SMAC) mimetic, LCL-161, which antagonizes cIAP1, cIAP2 and XIAP (16). SMAC mimetics are currently under preclinical and clinical development as anticancer drugs (17). LCL-161 is an orally bioavailable SMAC mimetic that was shown to be well-tolerated in phase I/II clinical trials, exhibiting no dose-limiting toxicity in the observed dosage range (18,19).

The objective of the present study was to investigate the antitumoral activity of TRAIL and to explore a possible way of circumventing TRAIL resistance in a model of acquired chemotherapy resistance in urothelial carcinoma.

Materials and methods

Drugs. Cisplatin was purchased from Gry-Pharma and gemcitabine was obtained from Lilly Deutschland GmbH. LCL-161 was purchased from Selleckchem. TRAIL (SuperKillerTRAIL™) was purchased from EnzoLifeSciences.

Cells. The RT112 cell line was obtained from the Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. Mycoplasma testing was performed for the cell lines used. The cell lines have been authenticated by STR profiling. The drug-resistant sublines were established by continuous exposure to increasing drug concentrations, as described previously (20). Drug-resistant sublines were considered to be cross-resistant to another drug if the ratio IC_{50} drug-resistant subline/ IC_{50} respective parental cell line for this drug was >2 . The drug-resistant sublines RT112^{CDDP}¹⁰⁰⁰ (cisplatin-resistant) and RT112^{GEMCI}²⁰ (gemcitabine-resistant), were derived from the Resistant Cancer Cell Line collection (<https://research.kent.ac.uk/ibc/the-resistant-cancer-cell-line-rccl-collection>). All cell lines were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS; Gibco, Thermo Fisher Scientific Inc.), 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C.

Viability assay. Cell viability was determined by the MTT dye reduction assay after 120 h of incubation modified as described previously (20). MTT assays were repeated 3 times. IC_{50} values were defined as the drug concentration producing 50% growth inhibition relative to untreated controls. Calculations were performed with CalcuSyn software, version 1.1 (Biosoft).

RNA interference. Synthetic siRNA oligonucleotides targeting cIAP1, cIAP2 or XIAP (ON-TARGET plus SMART pool siRNAs) were purchased from Dharmacon via Thermo Fisher Scientific, Inc. The non-targeting siRNA ON-TARGET plus SMART pool (Dharmacon) was used as negative control. For transfection, cells were grown to ~60–80% confluence, trypsinised, and 4×10^5 cells were resuspended in 2 ml cell culture medium containing 50 nM siRNA and 8 µl DharmaFECT 2 (Dharmacon). Experiments were repeated 3 times.

Western blotting. Cells were lysed in Triton X sample buffer and separated by SDS-PAGE. Proteins were detected using specific antibodies against β-actin (Sigma-Aldrich, Merck KGaA, cat. no. A2228, 1:5,000), TRAILR1 (DR4, EnzoLifeSciences,

cat. no. ALX-804-912-0100, 1:5,000), TRAILR2 (DR5, EnzoLifeSciences, cat. no. ALX-210-743-C200, 1:1,000), TRAILR3 (DCR1, EnzoLifeSciences, cat. no. ALX-804-667-C100, 1:500), TRAILR4 (DCR2, EnzoLifeSciences, cat. no. ADI-AAP-371-E, 1:1,000), XIAP (Cell Signaling via New England Biolabs, cat. no. 14334, 1:1,000), cIAP1 (Cell Signaling via New England Biolabs, cat. no. 7065, 1:1,000), cIAP2 (Cell Signaling via New England Biolabs, cat. no. 3130, 1:1,000), and Mcl-1 (Cell Signaling via New England Biolabs, cat. no. 94296, 1:1,000) and were visualized by enhanced chemiluminescence using a commercially available kit (GE Healthcare). Experiments were repeated 3 times.

Statistical analysis. Statistical data analysis was performed with GraphPad Prism 5.0 (GraphPad Software, Inc.). Results are expressed as mean \pm standard deviation of at least three experiments. Comparisons between two groups were performed using Student's t-test. Comparisons among three or more groups were performed by ANOVA followed by the Student-Newman-Keuls test. P-values <0.05 were considered to indicate statistically significant differences.

Results

Effects of TRAIL monotherapy on urothelial cancer cell viability, baseline protein expression of TRAIL receptors and anti-apoptotic proteins in urothelial cancer cell lines, and effects of LCL-161 on tumor cell viability. The chemotherapy-naïve cell line RT112 was more sensitive to TRAIL treatment compared with its chemoresistant sublines (IC_{50} : 17.19 ± 0.28 ng/ml in RT112 vs. 42.79 ± 2.82 ng/ml in RT112^{GEMCI}²⁰ vs. 40.16 ± 1.92 ng/ml in RT112^{CDDP}¹⁰⁰⁰) (Table I).

The sensitivity of tumor cells may depend on the expression of TRAIL receptors and on the expression of anti-apoptotic proteins, including IAPs (cIAP1, cIAP2 and XIAP) and Bcl2 family members (Mcl-1) (11,21–24). Western blotting revealed upregulated expression of TRAILR1 in the chemoresistant sublines RT112^{GEMCI}²⁰ and RT112^{CDDP}¹⁰⁰⁰ compared with RT112 cells, but the expression of TRAILR2, TRAILR3 and TRAILR4 was not significantly different (Fig. 1). The expression of IAPs (cIAP1, cIAP2 and XIAP) was uniformly elevated in the chemoresistant sublines of RT112. Mcl-1 expression was also elevated in the resistant sublines of RT112 compared with that in parental cells (Fig. 1).

LCL-161 treatment was slightly more effective in chemotherapy-naïve RT112 cells compared with RT112^{CDDP}¹⁰⁰⁰ cells (IC_{50} : 3.69 ± 0.15 µmol/ml in RT112 vs. 4.21 ± 0.09 µmol/ml in RT112^{CDDP}¹⁰⁰⁰). Gemcitabine resistance did not affect LCL-161 sensitivity (IC_{50} : 3.69 ± 0.16 µmol/ml in RT112^{GEMCI}²⁰) (Table I).

The combination of TRAIL with LCL-161 achieved a significant enhancement of the antitumor effects in all tested cell lines (Table I).

Effects of TRAIL treatment on protein expression of TRAIL receptors and anti-apoptotic proteins in urothelial cancer cell lines. The expression of IAPs (cIAP1, cIAP2 and XIAP) was increased following incubation with TRAIL for 24 h in RT112 parental cells and drug-resistant sublines. Mcl-1

Table I. IC₅₀ values following treatment of urothelial cancer cells with TRAIL and LCL-161.

| Cell line | TRAIL (ng/ml) | LCL-161 (μ M) | IC ₅₀ TRAIL (ng/ml) + 0.6 μ M LCL-161 |
|---|------------------|--------------------|--|
| RT112 | 17.19 \pm 0.28 | 3.69 \pm 0.15 | 0.79 \pm 0.01 ^a |
| RT112 ^r GEMCI ²⁰ | 42.79 \pm 2.82 | 3.69 \pm 0.16 | 4.01 \pm 0.20 ^a |
| RT112 ^r CDDP ¹⁰⁰⁰ | 40.16 \pm 1.92 | 4.21 \pm 0.09 | 3.03 \pm 0.23 ^a |

^aSignificant differences compared with TRAIL monotherapy. The IC₅₀ values of each drug were determined by MTT assay after 120 h of incubation. The values represent means of three independent experiments performed in triplicate \pm standard error of the mean. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

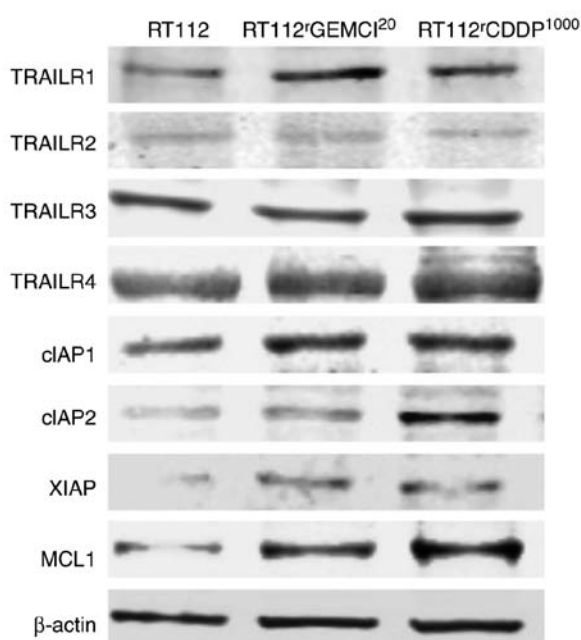


Figure 1. Evaluation of baseline protein expression. Western blotting showing protein expression of TRAIL receptors, IAPs and Mcl-1. The results shown are representative of three independent experiments. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IAPs, inhibitors of apoptosis; cIAP, cellular inhibitor of apoptosis protein; XIAP, x-linked inhibitor of apoptosis protein; Mcl-1, myeloid leukemia cell differentiation protein 1; RT112^rGEMCI²⁰, gemcitabine-resistant RT112 subline; RT112^rCDDP¹⁰⁰⁰, cisplatin-resistant RT112 subline.

expression did not increase following TRAIL treatment in chemotherapy-naïve and chemoresistant RT112 cells (Fig. 2).

Effects of combination therapy with TRAIL and LCL-161 on protein expression. LCL-161 treatment at a dosage far below the IC₅₀ value (see Table I) did not change the protein expression of cIAP1, cIAP2, XIAP and Mcl-1 in RT112 parental cells and chemoresistant sublines. TRAIL treatment increased the expression of cIAP1, cIAP2 and XIAP in RT112 parental cells and chemoresistant sublines and the combination of LCL-161 with TRAIL again reduced the protein expression (Fig. 3).

Effects of RNAi-mediated depletion of cIAP1, cIAP2, XIAP and Mcl-1 on TRAIL sensitivity. cIAP1, cIAP2, XIAP and Mcl-1 were depleted using siRNA in RT112, RT112^rGEMCI²⁰ and RT112^rCDDP¹⁰⁰⁰ cells. Downregulation of cIAP1, XIAP

and Mcl-1 resulted in resensitization to TRAIL therapy. However, depletion of cIAP2 did not exert a significant effect on cell viability compared with TRAIL monotherapy (Fig. 4).

Discussion

The standard therapy for locally advanced or metastatic urothelial carcinoma of the bladder consists of the combination of gemcitabine and cisplatin (1,2). Over the previous years, newly developed therapies, such as immune checkpoint inhibitors, were approved as second-line therapy following initial failure of cisplatin-based chemotherapy or for patients who are not eligible for cisplatin-based chemotherapy as a first-line therapy (4,25). However, the success of the new therapeutic options has also been very limited and further antitumor therapies are needed (4,25). The prognosis is mostly affected by development of acquired resistance.

TRAIL is known to induce tumor cell death without causing a lethal inflammatory shock syndrome, as was reported for TNF treatment (7-9). Using TRAIL as an antitumor therapy for bladder cancer had already been suggested (24,26), but its role had not yet been investigated in an acquired chemotherapy resistance model. Cancer cell lines with acquired resistance to anticancer drugs were demonstrated to reflect the characteristics of clinical acquired drug resistance (27-29). In the present study, a well-established urothelial cancer cell model of acquired chemotherapy resistance was used to study the role of TRAIL as a pro-apoptotic antitumor drug in chemosensitive and chemoresistant bladder cancer (30,31).

The sensitivity of urothelial cancer cell lines to TRAIL-induced cell death varies among different cell lines (32). In the present study, the sensitivity of urothelial cancer cell lines to TRAIL-induced cell death was similar to that reported previously (33).

Li *et al* analyzed the correlation between the expression of the TRAIL receptors TRAILR1 (DR4) and TRAILR2 (DR5) and its effects on prognosis. They reported an expression rate of TRAILR1 in 75.1% and of TRAILR2 in 74.2% of the bladder cancer specimens (24). The expression of TRAIL receptors was shown to be a resistance factor in different tumor entities (21-23). In the present study, an elevated expression of TRAILR1 was observed in chemotherapy-resistant and TRAIL cross-resistant RT112^rGEMCI²⁰ and RT112^rCDDP¹⁰⁰⁰ sublines compared with chemotherapy-naïve RT112 cells. However, receptor expression does not appear to be the only resistance factor. Furthermore, IAP expression was similarly

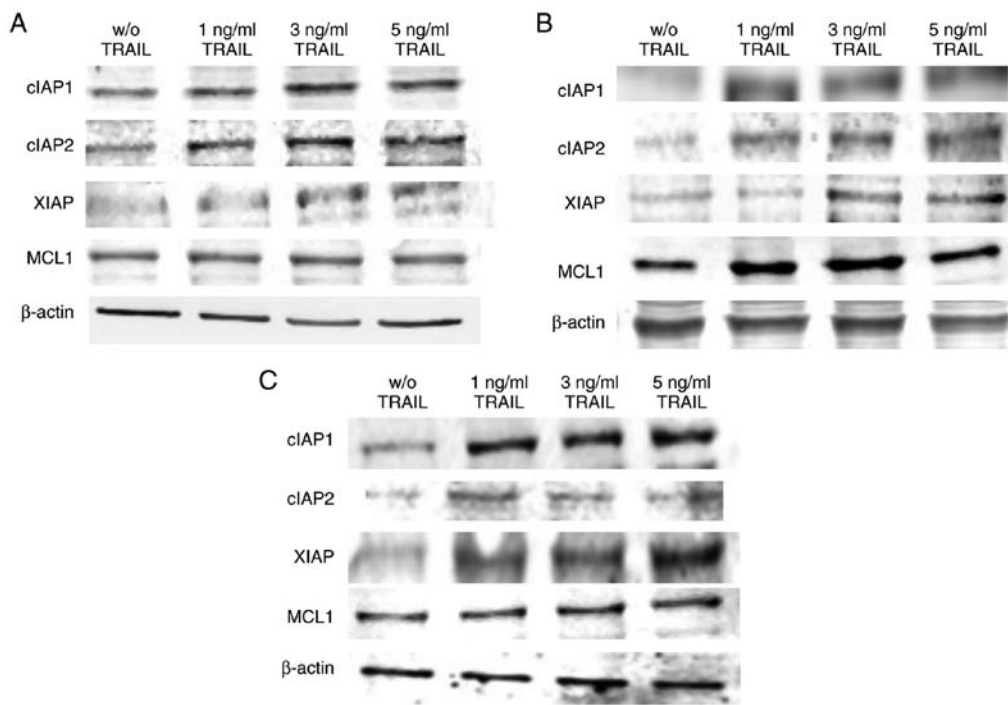


Figure 2. Protein expression following treatment with TRAIL. Western blotting following incubation for 24 h with increasing concentrations of TRAIL in (A) RT112, (B) RT112^{GEMC1²⁰} and (C) RT112^{CDDP¹⁰⁰⁰} cells. The results shown are representative of three independent experiments. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; cIAP, cellular inhibitor of apoptosis protein; XIAP, x-linked inhibitor of apoptosis protein; Mcl-1, myeloid leukemia cell differentiation protein 1; RT112^{GEMC1²⁰}, gemcitabine-resistant RT112 subline; RT112^{CDDP¹⁰⁰⁰}, cisplatin-resistant RT112 subline.

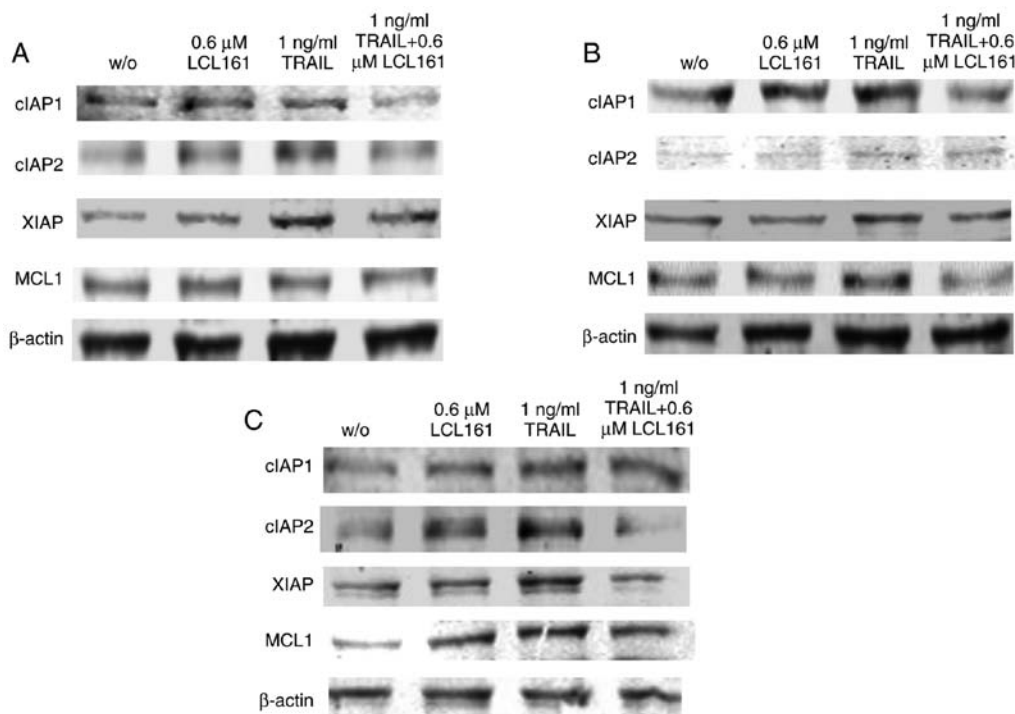


Figure 3. Protein expression following combination therapy with TRAIL and LCL-161. Western blotting following incubation for 24 h with LCL-161, TRAIL, and the combination of both compounds in (A) RT112, (B) RT112^{GEMC1²⁰} and (C) RT112^{CDDP¹⁰⁰⁰} cells. The results shown are representative of three independent experiments. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; cIAP, cellular inhibitor of apoptosis protein; XIAP, x-linked inhibitor of apoptosis protein; Mcl-1, myeloid leukemia cell differentiation protein 1; RT112^{GEMC1²⁰}, gemcitabine-resistant RT112 subline; RT112^{CDDP¹⁰⁰⁰}, cisplatin-resistant RT112 subline.

enhanced in both RT112 sublines with acquired chemotherapy resistance and TRAIL cross-resistance (RT112^{GEMC1²⁰} and RT112^{CDDP¹⁰⁰⁰}) compared with parental chemotherapy-naïve and TRAIL-sensitive RT112 cells. Overexpression of IAPs,

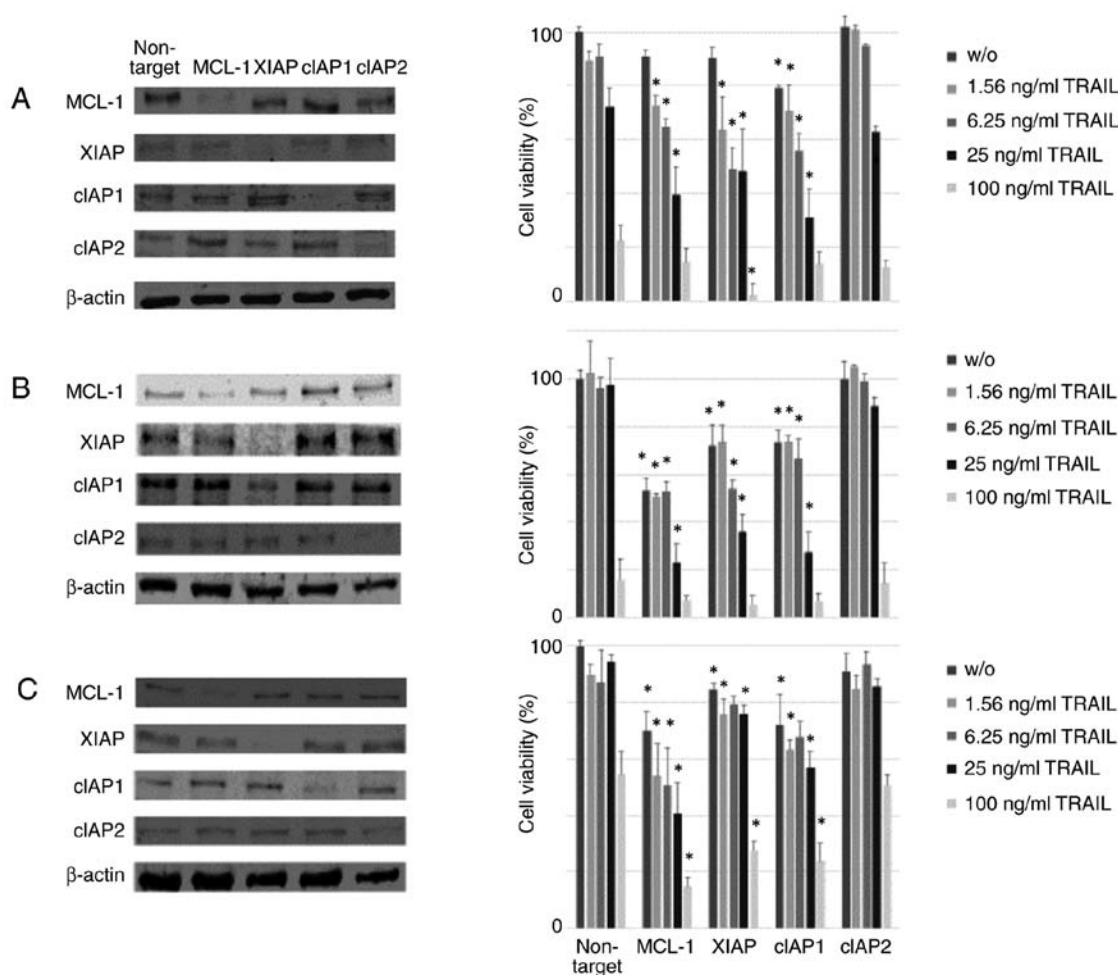


Figure 4. Effects of selective depletion of IAPs and Mcl-1 on cancer cell viability. Effects of siRNA-mediated depletion of cIAP1, cIAP2, XIAP and Mcl-1 on TRAIL sensitivity in (A) RT112, (B) RT112^{GEMC1} and (C) RT112^{CDDP} cells following incubation for 48 h. Western blots (left) showing depletion of the knockdown protein. Cell viability was measured via the MTT assay (right), demonstrating the effect of siRNA depletion of the proteins cIAP1, cIAP2, XIAP and Mcl-1 on sensitivity to TRAIL treatment. The results shown are representative of three independent experiments. *P<0.05 relative to control. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IAPs, inhibitors of apoptosis; cIAP, cellular inhibitor of apoptosis protein; XIAP, x-linked inhibitor of apoptosis protein; Mcl-1, myeloid leukemia cell differentiation protein 1; RT112^{GEMC1}, gemcitabine-resistant RT112 subline; RT112^{CDDP}, cisplatin-resistant RT112 subline.

such as XIAP, and pro-survival Bcl-2 members, such as Mcl-1, have been shown to be an important cause of TRAIL resistance in bladder cancer and other cancer entities (32,34-37). IAP expression has also been shown to predict prognosis in human urothelial carcinoma patients in an intrinsic resistance setting (38). In the present study, a dose-dependent upregulation of the IAPs cIAP1, cIAP2 and XIAP was detected as a possible response of cancer cells to the pro-apoptotic TRAIL stimulus (Fig. 2).

Different research groups demonstrated that SMAC mimetics exerted anticancer effects and enhanced the efficacy of TRAIL and cytotoxic anticancer drugs in chemosensitive or intrinsically drug-resistant urothelial cancer cells (32,39-41). Combinations of LCL-161 and cytotoxic chemotherapies are currently being developed (41). In the present study, the SMAC mimetic LCL-161 that targets IAPs, including cIAP1, cIAP2 and XIAP, affected the viability of chemosensitive and chemoresistant urothelial cancer cells at low micromolar concentrations (Table I). In a study of the Pediatric Preclinical Testing Program, only 3 of 23 pediatric cancer cell lines demonstrated effects in a similar concentration range (42). Moreover,

the urothelial cancer cell lines from our panel exhibited a higher LCL-161 sensitivity compared with hepatocellular carcinoma cells in a recently published report (16).

Since IAP and Mcl-1 expression was enhanced in cell lines with acquired chemotherapy resistance and TRAIL cross-resistance in the present study, we further sought to evaluate whether the combination of TRAIL with the SMAC mimetic LCL-161 could improve the efficacy of TRAIL treatment. Following treatment with a sublethal dose of LCL-161, no changes in the expression of IAPs or Mcl-1 were detected (Fig. 3). Of note, the combination of low-dose LCL-161 with TRAIL was able to reverse the aforementioned upregulation of pro-survival molecules, such as cIAP1, cIAP2 and XIAP (Fig. 3). Moreover, TRAIL sensitivity was markedly increased in chemotherapy-naïve as well as in chemoresistant cancer cell lines when combined with LCL-161 (Table I). Therefore, the data of the present study suggest that TRAIL, particularly in combination with the SMAC mimetic LCL-161, may be a treatment option for urothelial cancer cells, including those with acquired resistance to standard gemcitabine and cisplatin combination chemotherapy after failure of first-line chemotherapy.

Finally, we aimed to evaluate the effects of RNAi-mediated depletion of cIAP1, cIAP2, XIAP and Mcl-1 on TRAIL sensitivity. The resensitization of tumor cells to TRAIL was confirmed by depletion of antiapoptotic proteins with siRNA. Following specific depletion of the antiapoptotic proteins and treatment with TRAIL, a dose-dependent effect on cell survival was observed for cIAP1, XIAP and Mcl-1. Depletion of cIAP2 was not associated with survival (Fig. 4).

To the best of our knowledge, the present study is the first to examine the role of TRAIL as a drug against urothelial cancer cells with acquired resistance to standard chemotherapy with gemcitabine and cisplatin. It was demonstrated that TRAIL and, in case of acquired chemotherapy resistance, TRAIL in combination with the SMAC mimetic LCL-161, may represent a promising treatment option for urothelial cancer. Therefore, further clinical evaluation is warranted.

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Availability of data and materials

The original datasets of the present study are available from the corresponding author on reasonable request.

Authors' contributions

SV substantially contributed to the conception and design, acquisition of data and analysis, drafting of the manuscript and revising it critically for important intellectual content and final approval of the version to be published. HS substantially contributed to the conception and design, acquisition of data and analysis. MB acquired data and analysis. MM drafted the manuscript and revised it critically for important intellectual content. RW drafted the manuscript and revised it critically for important intellectual content. FR drafted the manuscript and revised it critically for important intellectual content. JC substantially contributed to the conception and design, drafting of the manuscript and revising it critically for important intellectual content and final approval of the version to be published. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

All the authors declare that they have no competing interests.

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