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Synergistic inhibition of GP130 and ERK signaling blocks chemoresistant bladder cancer cell growth

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ABSTRACT

Multidrug resistance is a major treatment obstacle for recurrent and metastatic bladder cancer, which often leads to disease progression and poor clinical outcome. Although overexpression of interleukin-6 (IL-6) appears to play a critical role in the development of chemotherapy resistance, inhibitors for IL-6 alone have not improved clinical outcomes. Since the IL-6/IL-6R/GP130 complex is involved in multidrug resistance, another strategy would be to focus on glycoprotein-130 (GP130) since it dimerizes with IL-6R/CD26 as a membrane-bound signaling transducer receptor and initiates subsequent signaling activation and may be a potential therapeutic target. Currently, the role of GP130 in chemoresistant bladder cancer is unknown. In the present study, we demonstrate that GP130 is over-expressed in cisplatin and gemcitabine-resistant bladder cancer cells, and that the inhibition of GP130 expression significantly reduces cell viability, survival and migration. Downstream of GP130 is PI3K/AKT/mTOR signaling, which is inactivated by SC144, a GP130 inhibitor. However, Raf/MEK/ERK signaling, which also is downstream of GP130 is activated by SC144. This activation is likely based on a mTOR/S6K1/PI3K/ERK negative feedback loop, which is presumed to counteract the inhibitory effect of SC144 on tumor aggressiveness. Blocking both GP130 and pERK resulted in synergistic inhibition of cytotoxicity, clonal survival rates and cell migration in our chemotherapy resistant bladder cancer cells. This vertical inhibition offers a novel therapeutic strategy for targeting human chemoresistant bladder cancer.

1. Introduction

The most frequently used systemic chemotherapy for patients with metastatic urothelial bladder cancer is a combination of gemcitabine and cisplatin (GC) [1]. Despite a consistent rate of initial response, multiple cycles of GC often result in the development of chemotherapy resistance, leading to therapeutic failure [2]. Treatment failure results in a poor median survival of 12–14 months [3]. Unfortunately, there is no approved second-line therapy in the US after cisplatin-based treatment. This remains a major challenge for patients with progressively unresponsive disease. Therefore, new agents are needed to improve patient outcome.

Interleukin-6 (IL-6) plays a critical role in a number of biological and pathobiological events, including cellular proliferation, differentiation, and apoptosis [4–6]. IL-6 binds to domains on the IL-6 receptor (IL-6R), thereby inducing dimerization of the transmembrane glycoprotein-130 (GP130; CD130 or IL6ST) and activation of downstream signaling pathways including JAK/STAT, Raf/MEK/ERK, and PI3K/AKT/mTOR [4]. It has been reported that overexpression of the IL-6/IL-6R/GP130 complex is involved in the occurrence of multidrug resistance in a few cancers including prostate, ovarian and breast. This suggests a new window for clinical applications of the IL-6/IL-6R/GP130 complex, and targeting this complex may be a promising option for progressive human chemoresistant bladder cancer. The use of IL-6 inhibitors as anticancer agents has been reported in lung cancer [7], multiple myeloma [8], prostate cancer [9], renal cell carcinoma [10], and ovarian cancer [11]; however, there has been very little clinical benefit. Based on these reports, GP130, a transmembrane protein located at a central point for a number of oncogenic signaling cascades, may be a better target. Recently, we demonstrated that high GP130 expression was linked to advanced bladder cancer [12]. However, targeting GP130 expression in drug-resistant bladder cancer has not been investigated.

In this study, we demonstrate a key role of GP130 in the development of chemoresistance in bladder cancer cells. We measured the effect of GP130 inhibition on tumorigenic properties as well as on

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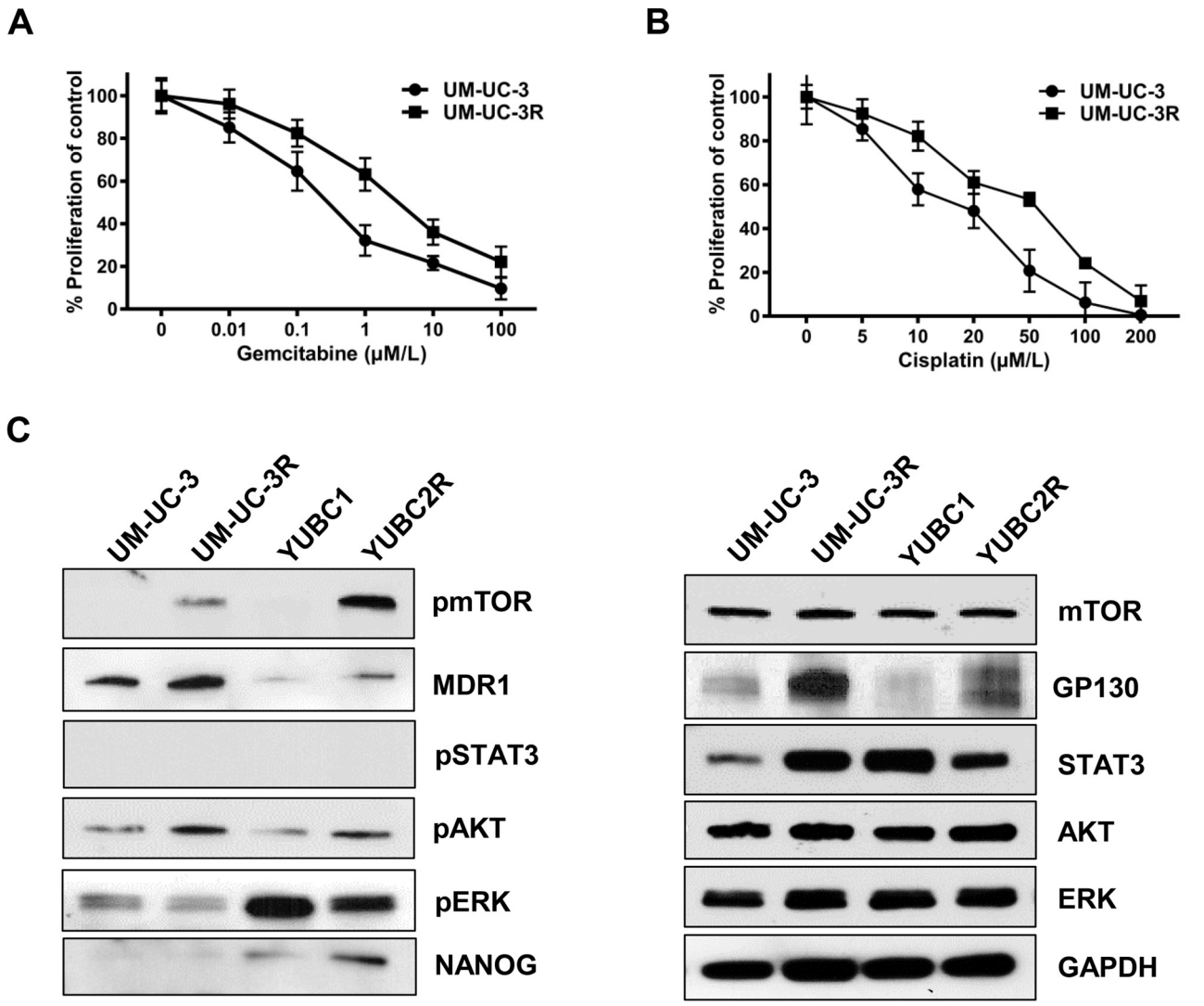

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Fig. 1. Cytotoxicity and biomarker expression of bladder cancer cells exposed to chemotherapeutics. Cytotoxicity assays were performed on UM-UC-3 and UM-UC-3R bladder cancer cells exposed to increasing concentrations of gemcitabine (0.01, 0.1, 1, 10 and 100 μM) **(A)** or cisplatin (2, 5, 10, 20, 50, 100 and 200 μM) **(B)**. Western blot was performed on UM-UC-3, UM-UC-3R, YUBC1 and YUBC2R human bladder cancer cells to determine the expression of downstream GP130 signaling, multidrug resistant genes and cancer stemness associated proteins **(C)**. Representative blots are shown. GAPDH was used for loading equivalency. Quantification of biomarker expression is shown **(D)**. Data are shown as the mean ± SD, $n = 3$.

downstream signaling through the PI3K/AKT/mTOR, JAK/STAT and Raf/MEK/ERK pathways. Our results demonstrate that monotargeted inhibition of GP130 may not provide satisfactory efficacy due to a complex cross-talk and/or feedback mechanism between GP130 and its downstream signaling pathways. Nevertheless, we demonstrate that a synergistic vertical inhibition of GP130 and the Raf/MEK/ERK pathway provides a promising approach for preclinical research and future clinical application in the treatment of chemoresistant bladder cancer.

2. Materials and methods

2.1. Reagents and antibodies

Gemcitabine and Cisplatin were purchased from Santa Cruz Biotechnology (Santa Cruz, Dallas, Texas, USA) and aliquots of stock solutions (10 mM) were made in DMSO and stored at -20 °C. GP130 antibody was purchased from Novus Biologicals (Littleton, CO, USA) whereas phospho-mTOR (pmTOR, Ser2448), mTOR, phospho-STAT3 (pSTAT3, Tyr705), STAT3, phospho-p44/p42 MAPK (pERK), p44/p42 MAPK (ERK), phospho-AKT (pAKT, Ser473), AKT, MDR1, BCL-XL, NANOG and GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA). U0126 (MEK1/2) and SC144 (GP130) inhibitors also were purchased from Cell Signaling Technology.

2.2. Cell culture

UM-UC-3 human bladder cancer cells were obtained directly from the American Type Culture Collection. The resistant subline UM-UC-3R (IC50: gemcitabine 3.5 μM, IC50: cisplatin 39 μM) was established by continuous exposure to increasing gemcitabine and cisplatin concentrations. Primary urothelium bladder cancer (YUBC1) cells were derived from a de-identified bladder tumor collected from a cystectomy specimen in which the patient was chemotherapy näive, whereas the primary urothelium recurrent bladder cancer (YUBC2R) cells were collected from a cystectomy specimen in which the patient had failed Gemzar plus Carboplatin treatment. All patients were provided informed consent and offered enrollment into a biospecimen repository approved by the Yale University Institutional Review Board. UM-UC-3, UM-UC-3R, YUBC1, YUBC2R, and TCCsup cells were maintained in Minimum Essential Medium Eagle (MEME) (Sigma-Aldrich, Inc.St. Louis, MO), in a humidified atmosphere containing 5% CO2 in air, supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich, Inc. St. Louis, MO) and 1% glutamine (Sigma-Aldrich, St. Louis, MO). For each experiment, the cells were used in the exponential growth phase. Each cell line was used within 10 passages from its archival passage number and the cells were routinely tested for mycoplasma (MycoAlert™, Lonza Biologics Inc., Portsmouth, NH).

2.3. Clonogenic survival

UM-UC-3 and UM-UC-3R cells were treated for 72 h with and without SC144 (3.3 μM). The cells were then plated at a density of 18 viable cells / cm^2. After 3 weeks, the colonies were formed, fixed with 4% paraformaldehyde for 10 min, stained with crystal violet dye and washed for 5 min, 3 times in water. The colonies (> 50 cells) were imaged and counted using ImageJ software (NIH), which allowed the determination of the effectiveness of our agents on cell survival.

2.4. Cell growth assay

TCCsup human bladder cancer cells were treated with 5 μM SC144, 21.7 μM U0126 or SC144 + U0126 and platted at 1.45×10^5 cells per well (6-well plate). After 72 h, the cells were fixed with ice-cold methanol for 10 min and stained with 0.5% crystal violet dye for 10 min [13]. The bladder cancer cells were washed three times with deionized water for 5 min before being dried and photographed.

2.5. Migration assay

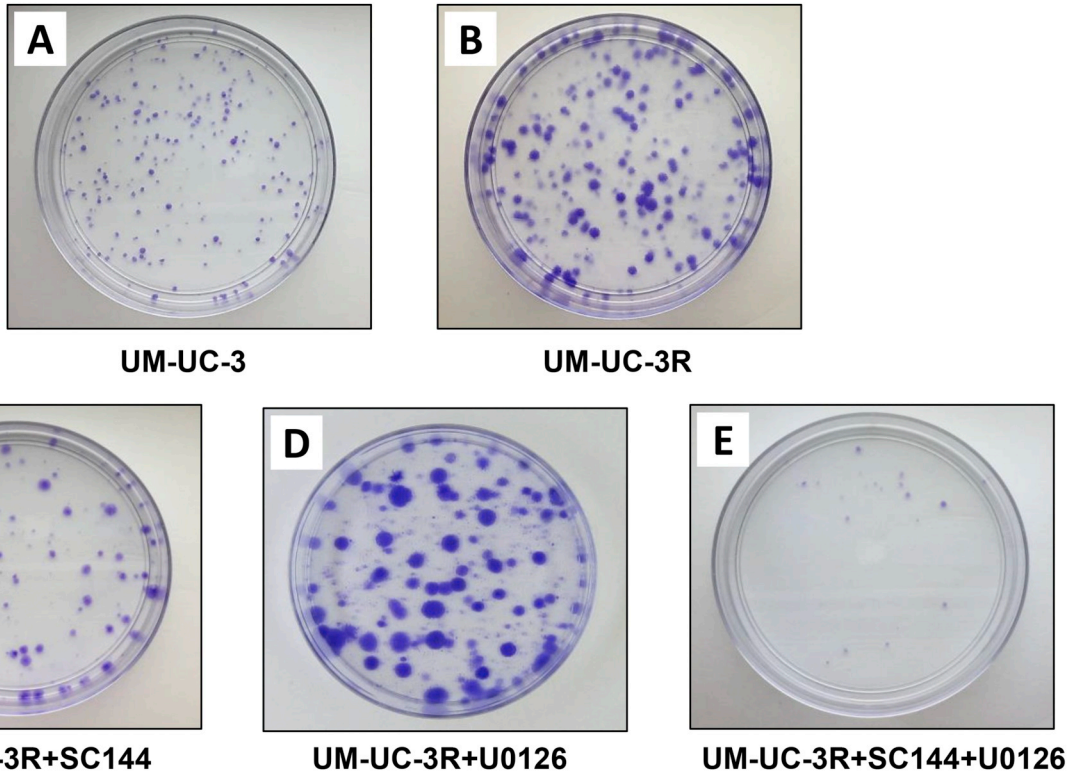
UM-UC-3 and UM-UC-3R cells were treated for 72 h with or without SC144 (3.3 μM). The cells were then seeded in triplicate in a 24-well plate and grown to confluency. A scratch was created using a P200 tip in a confluent monolayer of cells, and the cells were washed three times with PBS++ (Sigma Aldrich, Inc. St. Louis, MO). The cells were maintained in culture medium with 1% FBS. The number of cells that migrated into the scratch area was counted at 12 and 24 h, and quantified using ImageJ software (NIH).

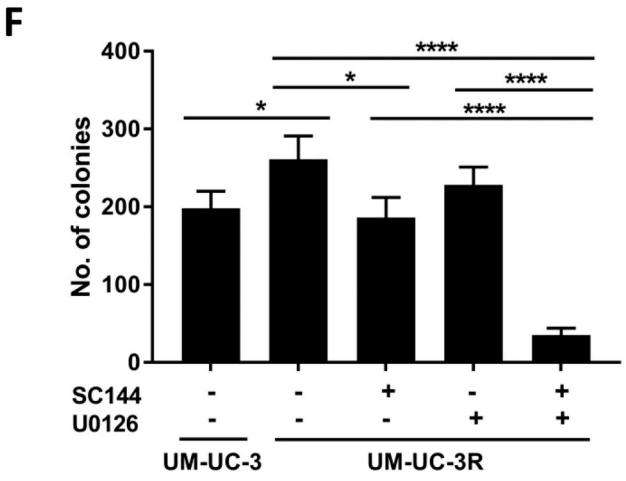
2.6. Cytotoxicity assay

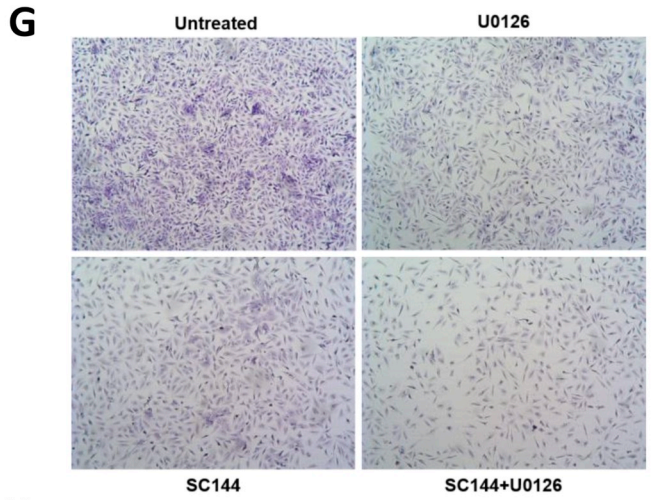
Five thousand UM-UC-3 and UM-UC-3R cells were seeded in a 96-well plate and exposed to an increasing dose of gemcitabine (5 to 200 μM) or cisplatin (0.01 to 100 μM), respectively. Control cells were incubated with 0.1% Dimethyl Sulfoxide (DMSO). To test the synergistic effect of SC144 and U0126 on UM-UC-3R cells, the cells were cultured with SC144 (2, 5, 10, 20, or 50 μM), U0126 (10, 25, 50, 100, or 250 μM), or combined concentrations of the two compounds (2/10, 5/25, 10/50, 20/100, or 50/250 μM/μM), respectively. Cells were incubated for 72 h before adding WST-1 (Takara Bio, Inc., CA, USA). Each drug dose was performed in sextuplicate, and three independent experiments were performed. IC50 was calculated from the dose-response curve using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). Combination Index (CI) was calculated with CalcuSyn software (Biosoft, Cambridge, UK), and values between 0.3 and 0.7 indicated synergism [14].

2.7. Western blotting

Cells were washed with cold TBS (25 mM Tris-HCl, pH 7.8, 150 mM NaCl) twice and lysed with ice-cold Radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA) supplemented with a cOmplete™, mini, EDTA-free protease inhibitor cocktail (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin (protease inhibitor), and 1 mM sodium fluoride prior to quantification with the Bradford assay [15]. Proteins were quantified using Bradford Protein Assay (Bio-Rad, Laboratories, Inc., CA, USA) before being separated on 4–15% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk in TBST (1× TBS and 0.05% Tween 20) for 1 h at RT before being exposed to a primary antibody at 4 °C overnight. The membranes were subsequently washed with TBST and incubated with HRP conjugated donkey anti-rabbit or donkey anti-mouse IgG secondary antibodies (Cell Signaling Technology). A chemiluminescence system (Thermo Scientific, Rockford IL) was used to detect protein signal. Samples were normalized based on GAPDH quantification, and band density was determined using ImageJ software (NIH).







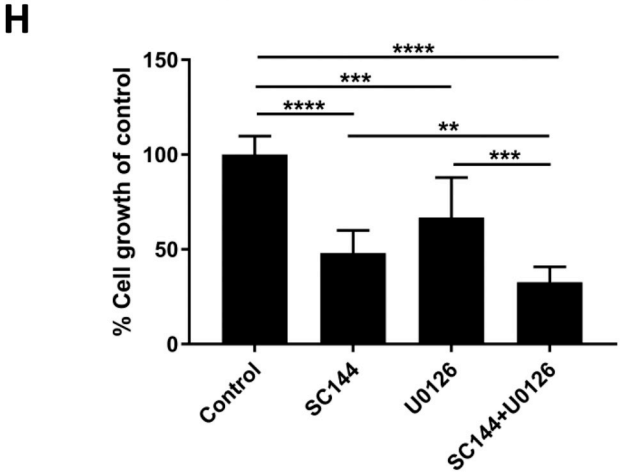


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Fig. 2. Effect of SC144 and U0126 on bladder cancer cell survival. UM-UC-3 **(A)** and UM-UC-3R **(B)** bladder cancer cells were compared for colony formation. UM-UC-3R cells were treated with SC144 **(C)**, U0126 **(D)**, or SC144 plus U0126 **(E)** in cell survival studies. Colonies were counted and quantified **(F)**. TCCsup bladder cancer cells were treated with SC144, U0126 or SC144 plus U0126 **(G)**, and cell growth was quantified upon treatment **(H)**. Data are shown as the mean ± SD (n = 3) in which *, $p < .05$; **, $p < .01$; ***, $p < .001$; ****, $p < .0001$.

2.8. Statistical analysis

Data are expressed as mean ± standard deviation (SD) based on at least three independent experiments. Statistical analysis was accomplished by Student's *t*-test and repeated-measures ANOVA analysis. Drug interactions were analyzed using the median effect method of Chou and Talalay [16–18]. All statistical tests were two-sided and were considered to be statistically significant at $P < .05$. Results are presented as mean +/− SD in which * represents $p < .05$, ** represents $p < .01$, *** represents $p < .001$, and **** represents $p < .0001$ unless indicated differently. Statistical analysis was carried out using the GraphPad Prism 7.0 (GraphPad Software, Inc., Avenue, CA, USA) and CalcuSyn software (Biosoft, Cambridge, UK).

3. Results

3.1. GP130 expression in bladder cancer cells exposed to chemotherapeutic agents

We treated UM-UC-3 and UM-UC-3R bladder cancer cells with either gemcitabine or cisplatin to determine IC50s. The IC50 for gemcitabine was increased by 10-fold in the UM-UC-3R bladder cancer cells (3.5 μM) compared to the parental UM-UC-3 cells (0.35 μM) (Fig. 1A). Similarly, the IC50 for cisplatin was increased by 2.5-fold in the UM-UC-3R bladder cancer cells (39 μM) compared to the parental UM-UC-3 cells (16 μM) (Fig. 1B). Then, we assessed GP130 levels as well as downstream signaling pathways by western blot (Fig. 1C, D). There was a statistically significant upregulation of GP130 levels in the UM-UC-3R (7-fold, $p = .0004$) and YUBC2R (16-fold, $p < .0001$) chemotherapy resistant cells compared to the näive UM-UC-3 and YUBC1 cells, respectively. Downstream we observed a statistically significant increase of pmTOR levels (66-fold, $p = .0002$; 13-fold, $p < .0001$), and a statistically significant decrease of pERK levels (1.5-fold, $p = .02$; 11-fold, $p < .0001$) in UM-UC-3R and YUBC2R bladder cancer cells compared with UM-UC-3 and YUBC1 bladder cancer cells. The level of pSTAT3 was below the level of detection for all cell types. Furthermore, we showed that MDR1 (multidrug resistant protein) was upregulated in UM-UC-3R (3-fold, $p = .03$) and YUBC2R (3-fold, $p = .001$) compared to UM-UC-3 and YUBC1 bladder cancer cells, respectively, whereas NANOG (cancer stemness associated protein) was upregulated in UM-UC-3R (1.5-fold, $p = .0007$) and YUBC2R (2.3-fold, $p < .0001$) compared to UM-UC-3 and YUBC1 bladder cancer cells, respectively (Fig. 1C, D).

3.2. Blocking GP130 expression effects tumorigenic properties of resistant bladder cancer cells

To address the critical role of GP130 expression in the treatment of resistant bladder cancer cells, we measured the tumorigenic properties by cell survival and migration potential in UM-UC-3 and UM-UC-3R bladder cancer cells.

A clonogenic survival showed that there was an increase in the number of colonies from 198 to 261 when comparing UM-UC-3 cells (Fig. 2A) to UM-UC-3R cells (Fig. 2B). When UM-UC-3R cells were pretreated with 3.3 μM SC144 there was a significant 29% decrease in survival (186 colonies; Fig. 2C and F). However, U0126 treatment alone did not affect the number of colonies present (Fig. 2D and F). When the UM-UC-3R cells were treated with a combination of SC144 plus U0126 there was a significant 87% decrease (35 colonies) in cell survival (Fig. 2E and F). These results suggest that SC144 alone or SC144 plus U0126 suppressed cell survival in resistant bladder cancer cells. Then we tested the effect of SC144 plus U0126 on TCCsup human bladder cancer cells. We found that the SC144 + U0126 combination had a 67% growth inhibition ($p < .0001$) when compared to control cells. In addition, we demonstrated that the SC144 + U0126 combination had a 15% ($p < .01$) and 34% ($p < .001$) decrease in growth when compared to SC144 alone and U0126 alone, respectively (Fig. 2G and H).

UM-UC-3R bladder cancer cells were used to assess migration and we showed that on average 59 and 94 cells at 12 and 24 h, respectively moved into the scratch (Fig. 3C, D and I) whereas the number of UM-UC-3 bladder cancer cells that migrated into the scratch at 12 and 24 h was on average 37 and 61, respectively (Fig. 3A, B and I). When the UM-UC-3R cells were pretreated with the SC144 inhibitor, there was on average 38 and 57 cells that had migrated into the scratch area at 12 and 24 h post-scratch, respectively (Fig. 3E, F and I). However, when the UM-UC-3R cells were pretreated with a combination of SC144 and U0126, there was a significant decrease in the number of cells that had migrated. At 12 and 24 h there was only 19 and 28 cells that had migrated into the scratch area, respectively (Fig. 3G, H and I). These results suggest that SC144 plus U0126 had a significant effect on cell migration of resistant bladder cancer cells.

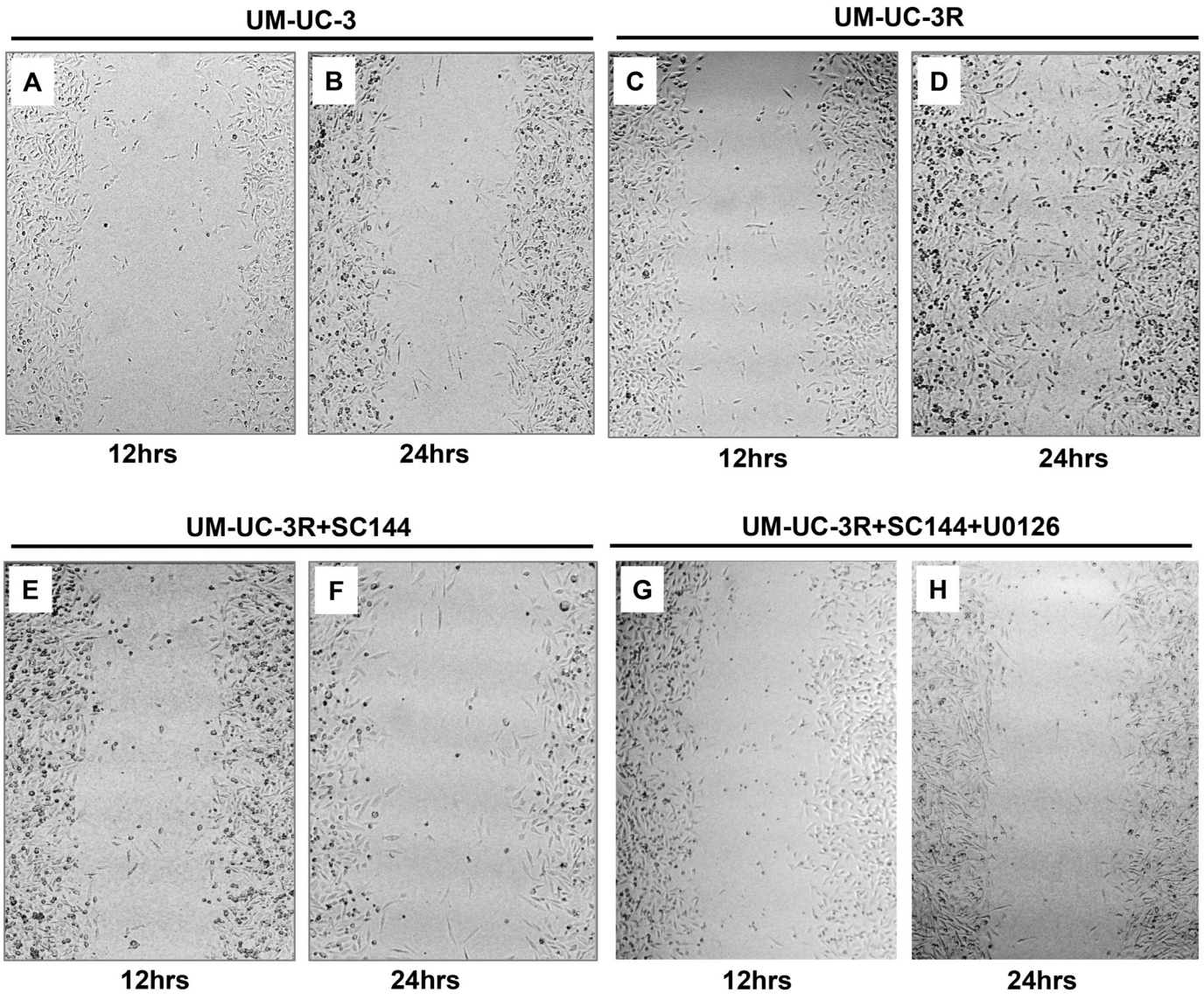
3.3. Blocking GP130 expression levels inactivated pmTOR, but elevated pERK

We analyzed the effect of blocking GP130 levels on downstream signaling. UM-UC-3R cells were treated with 3.3 μM SC144 for 1, 2 or 3 days as well as 3 days with SC144 followed by a washout (3 days without SC144) for a total of 6 days (Fig. 4A and B). The levels of GP130 were progressively inhibited by SC144 in a time dependent manner with little detection after 3 days of treatment, but then restored after 3 days upon SC144 washout.

Downstream signaling of GP130 revealed that pmTOR expression parallels the changes in GP130, whereas the expression of pERK was increased by 6.7-fold, 6.1-fold, 7.8-fold and 2.8-fold at the four different timepoints compared to control (Fig. 4A and B). Additionally, activation of a multidrug resistant protein (MDR1; Fig. 4C and D) and an apoptosis inhibitory protein (BCL-XL; Fig. 5A and B) were reduced by the GP130 inhibitor further supporting that GP130 has a role in chemotherapy resistance bladder cancer signaling.

3.4. Inhibition of proliferating chemoreistance bladder cancer cells by a synergistic verticial blockage of GP130 and pERK signaling

It is well known that activation of the Raf/MEK/ERK pathway leads to oncogenesis and chemoresistance. In addition, we demonstrated an increase in pERK upon GP130 inhibition on UM-UC-3R bladder cancer cells. We evaluated the effect of blocking pERK or GP130 signaling with U0126 or SC144, respectively on the proliferation of UM-UC-3R bladder cancer cells (Fig. 6). The proliferation of UM-UC-3R cells was inhibited by SC144 or U0126 in a dose-dependent manner. The IC50 of SC144 and U0126 were 3.3 μM and 21.7 μM, respectively. Then, we analyzed the combination of SC144 plus U0126 on the proliferation of UM-UC-3R cells (Fig. 6). A combination of SC144 plus U0126 synergistically inhibited proliferation of UM-UC-3R at 25, 50, 75 and 95 percentage of fractions affected (Fa), as indicated by the corresponding CI-values (Table 1).



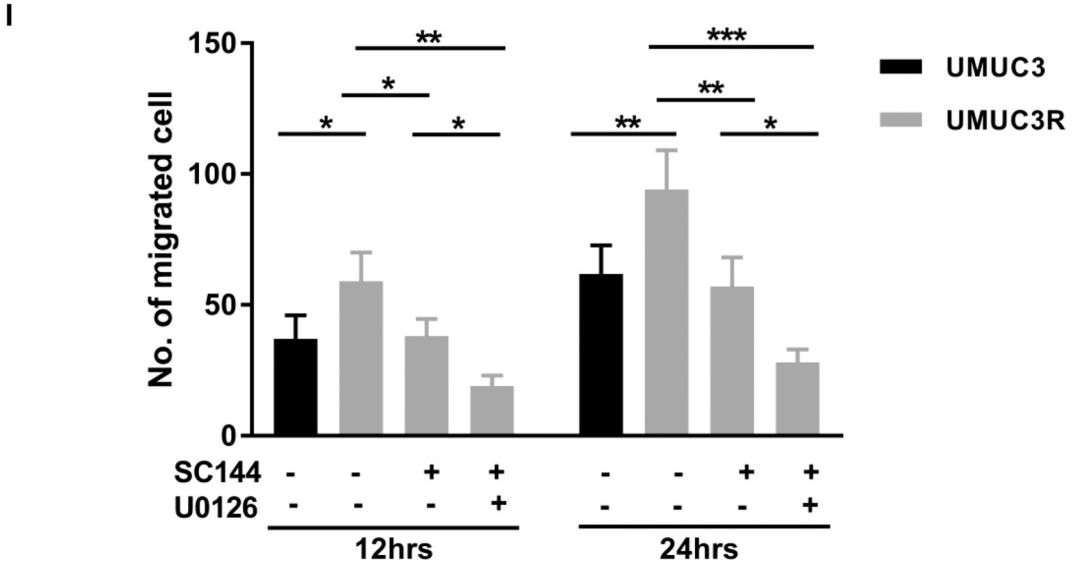


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Fig. 3. Cell migration of bladder cancer cells treated with SC144. The migration of UM-UC-3 and UM-UC-3R bladder cancer cells was determined at 12 h (A, C) and 24 h (B, D), respectively. In addition, migration potential was determined when UM-UC-3R bladder cancer cells were treated with SC144 (1 μM) or SC144 (1 μM) plus U0126 (4.6 μM) at 12 h **(E, G)** and 24 h **(F, H)**, respectively. Quantitative analysis for migration potential was performed on untreated or treated (SC144 and SC144 + U0126) UM-UC-3R bladder cancer cells **(I)**. Data are shown as the mean ± SD (n = 3) in which *, $p < .05$; **, $p < .01$; ***, $p < .005$.

4. Discussion

Preclinical and clinical studies have shown that continuous activation of IL-6/IL-6R signaling plays a critical role in acquired chemotherapy resistance, indicating that blockade of IL-6 signaling may be a promising strategy in the treatment of chemoresistant cancer. IL-6 inhibitors sensitized multidrug resistant cancer cells to chemotherapy in renal cell carcinoma [10], ovarian cancer [11], prostate carcinoma [19] and breast cancer [20]. However, IL-6 antibodies have failed to completely inhibit the IL-6/IL-6R/GP130 complex, and downstream signaling pathways. As demonstrated by a Phase II clinical trial for castration-resistant prostate cancer with one prior chemotherapy, CNTO328, an IL-6 monoclonal antibody, increased the levels of IL-6 > 250-fold, lessening its clinical therapeutic efficiency despite CNTO328 demonstrating biological activity [21]. These findings suggest that a change in targeting from IL-6 to GP130, which is a transmembrane protein that is located at a central point for a number of oncogenic signaling cascades, may improve the treatment of chemoresistant bladder cancer. Previously, our group demonstrated that GP130 expression was linked to advanced bladder cancer [12]. In addition, GP130 over-expression is present in cancers including brain tumors [22], kidney tumors [23], myeloma [8] and lymphoma [24], and has been correlated with a poor clinical prognosis.

Constitutive activation of GP130 signaling can induce chemotherapy and radiation resistance in oral [25], ovarian [26], lung [7] and prostate carcinoma cell lines [9] by inhibiting cellular apoptosis proteins (BCL-2, BCL-XL, and XIAP) and elevating expression of multidrug resistance-proteins (MDR1 and GSTpi) [11,27]. In the present study, we analyzed the expression GP130 expression in bladder cancer cells and tumors that were chemotherapy resistant or that had failed chemotherapy treatment, respectively. We found GP130 to be over-expressed and pmTOR signaling, which is known to play an essential role in multidrug resistance, to be increased in chemotherapy resistant cells. In addition, multidrug-resistant genes (*i.e.*, MDR1) and cancer stemness associated proteins (*i.e.*, NANOG), which are mechanistically important for chemoresistant bladder cancers, were over-expressed in our chemotherapy resistant cell models, suggesting that GP130 may be a potential target in treating multidrug resistant bladder cancer cells.

The Jak/STAT3 pathway is tightly associated with GP130 activation, and the activation of GP130/Jak/STAT3 promotes the expression of multiple genes [28], including BCL-2 [29], MMP-7 [30], Cyclin D1 and survivin [31], which are involved in cell proliferation, metastasis and drug-resistance [32,33]. Multiple lines of evidence place STAT3 at a central node in the progression of human tumors and the development of chemoresistance, suggesting STAT3 as an anticancer target. However, GP130 has been documented to stimulate the proliferation of prostate cancer 22Rv1 cells through the PI3-kinase/Akt pathway without activating Stat3 [34]. We noted a similar STAT-independent finding in our study. This diminished or impaired pSTAT3 signaling in our cells also has been noted in some other drug-resistant cancer cells [25]. Alternatively, pSTAT3 levels are below our level of detection. Furthermore, the mTORC1 inhibitor, rapamycin, induces phosphorylation of both AKT and Raf/ERK in metastatic breast cancer [35,36]. These data support an anticancer therapeutic effect with vertical targeting of AKT and mTOR or the combined targeting of AKT and mTOR as well as ERK signaling in *in vitro* and *in vivo* models [14,37–39].

Genetic ablation of ERK2 leads to a strong downregulation of GP130 expression by binding to the GP130 promoter, interacting with the mediator complex or remodeling the chromatin [6,40]. Similarly, other downstream GP130 pathways, Raf/MEK/ERK and PI3K/AKT/mTOR, are under complex control with negative feedback regulation and crosstalk, which has been shown in normal and malignant cells. As major regulators of cell proliferation, metabolism and chemotherapeutic cytotoxicity, the Raf/MEK/ERK and PI3K/AKT/mTOR pathways are commonly activated during oncogenesis and chemoresistance, supporting the notion of using mTOR or ERK inhibitors for therapeutic clinical intervention [2]. Unfortunately, the blockade of either Raf/MEK/ERK or PI3K/AKT/mTOR pathways alone in the clinical trial setting has proven unsuccessful, which may be due to complicated cross-inhibition and feedback regulation.

For the first time, we have shown that drug resistant bladder cancer cells significantly overexpress GP130, which supports targeting GP130 expression as a therapeutic intervention. As downstream signaling of pmTOR was shown to be up-regulated while pERK was down-regulated in UM-UC-3R and YUBC2R drug resistant cells, the blockade of GP130 by SC144 inactivated pmTOR signaling, but up-regulated pERK expression. Based on these findings, we hypothesized that if GP130 inhibition induces activation of pERK, as a result of a feedback or crosstalk mechanism, with the resultant restriction of the inhibitory action of SC144 in the drug resistant bladder cancer cells, then a combined blockade of both PI3K/AKT/mTOR and Raf/MEK/ERK pathways may be necessary in order to facilitate the efficacy of GP130 inhibition in the treatment of chemoresistant bladder cancer. To confirm this theory, we applied a dual vertical blockade of GP130 and pERK by SC144 and U0126, respectively. A resultant synergistic effect was observed on the cell survival and migration potential of UM-UC-3R cells. Also, we performed sequential drug treatment using our *in vitro* bladder cancer models and found that the order of the addition of SC144 or U0126 alone did not influence the degree of inhibition of cell growth and did not have the maximum impact on cell growth. We treated human bladder cancer UM-UC-3R cells for a total of 72 h with (A) SC144 for 24 h followed by U0126 for 48 h; (2) U0126 for 24 h followed by SC144 for 48 h; or (3) SC144 plus U0126 for 72 h. Then we tested cell viability at 0.5 to 3 h post-treatment using the WST assay. We found that the most significant impact on cell viability was when both drugs were administered simultaneously for the duration of the treatment (Fig. S1). As was shown in Figs. 2 and 3, a combined inhibition of GP130 plus pERK significantly reduced the number of migrated cells at 12 and 24 h, as well as the number of colonies compared to blocking GP130 alone. In addition, we showed that this combined vertical inhibition had an effect on the inhibitory apoptosis protein BCL-XL in the UM-UC-3R cells. The potentiated inhibition of proliferation and induction of apoptosis resulting from combining SC144 and U0126 suggests the potential efficacy of such combined therapy in multidrug resistant bladder cancer. A previous study has reported the complex feedback regulation and crosstalk between pmTOR and pERK signaling, indicating that the up-regulation of pmTOR by blocking pERK signaling relies on a mTOR/S6K/PI3K/MAPK negative feedback loop [29]. We noted that the effect of the combined inhibitors or the GP130 inhibitor alone has similar outcomes for pmTOR (Fig. 5). Also, in line with GP130 having a role in chemotherapy resistance, we were able to downregulate MDR1 and BCL-XL expression in the resistant sublines with SC144 alone. However, we did note a greater inhibition when SC144 was combined with the ERK inhibitor, U0126.

5. Conclusion

Given the fact that there is a limited benefit in using IL-6 inhibitors alone in the clinical setting, our findings identify the importance of a shift in the targeting from IL-6 to GP130, and the further use of a dual

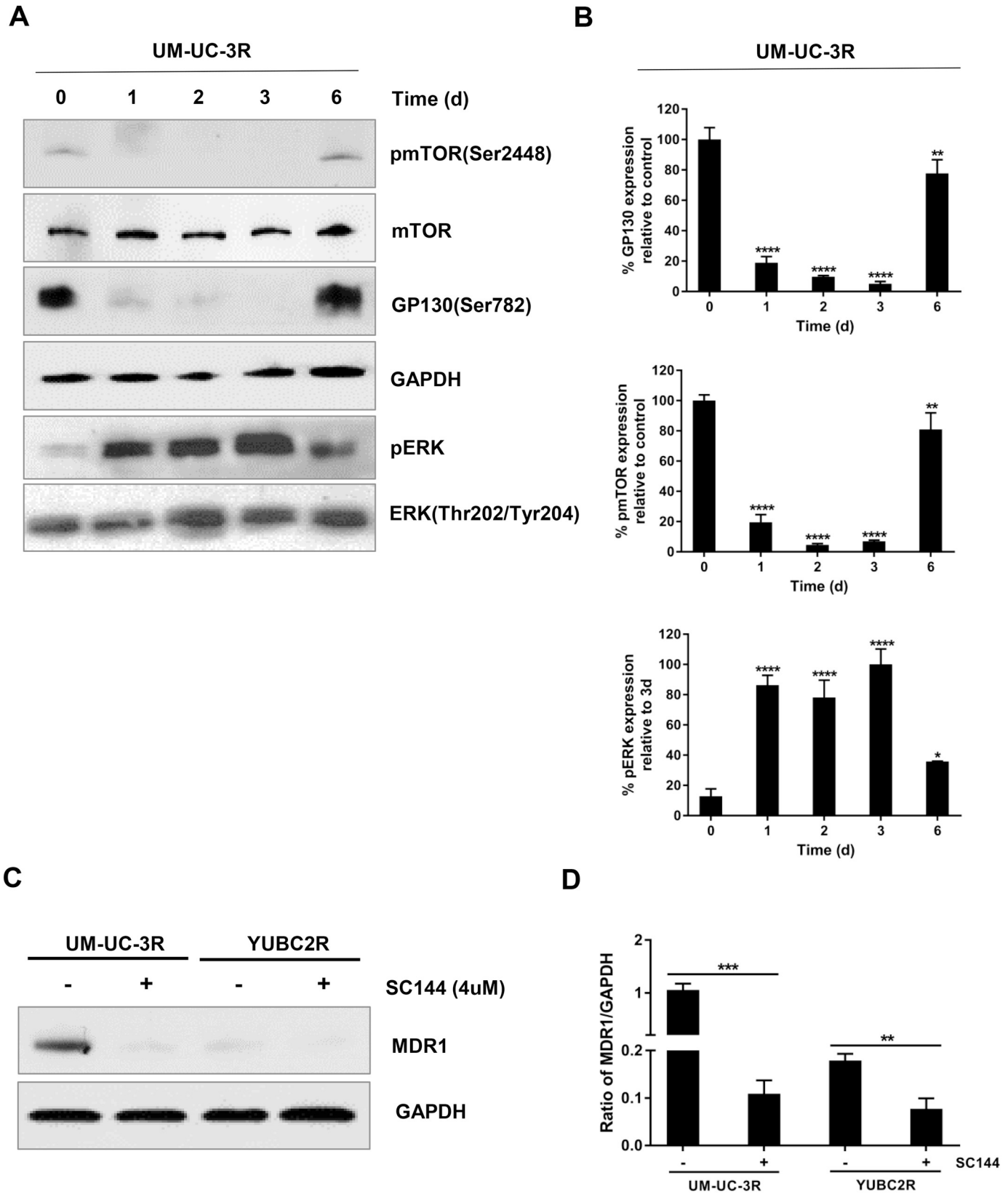

Fig. 4. Inhibition of GP130 affects downstream signaling pathways as well as multidrug resistant genes in chemotherapy treated bladder cancer cells. GP130 and downstream signaling proteins such as mTOR, pmTOR, pERK, and ERK **(A, B)** were assessed in UM-UC-3R bladder cancer cells. In addition, to assess the role of GP130 in bladder cancer cells treated with chemotherapy (UM-UC-3R and YUBC2R), cells were treated with or without SC144 and western blots were performed for MDR1 **(C, D)**. Data are shown as the mean ± SD, n = 3.

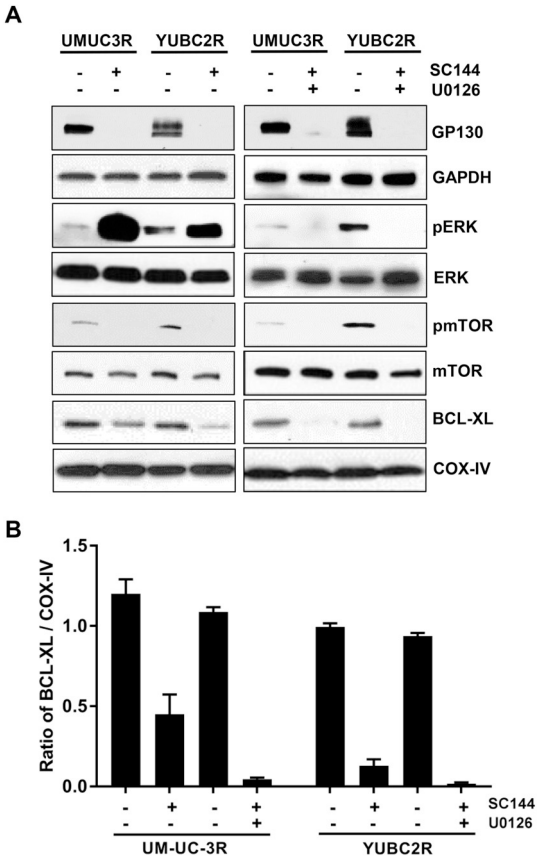

Fig. 5. GP130 and pERK inhibition resulted in an inactivation of anti-apoptosis protein, BCL-XL. UM-UC-3R bladder cancer cells were incubated with SC144 alone or with a combination of SC144 plus U0126, and protein signaling was assessed. Data are shown as the mean ± SD, n = 3.

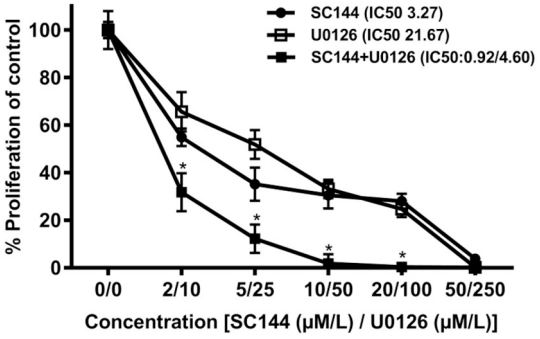

Fig. 6. Combined treatment with increasing concentrations of SC144 and U0126 synergistically suppressed proliferation of chemoresistant bladder cancer cells. Cytotoxicity assays were performed on UM-UC-3R bladder cancer cells exposed to increasing concentrations of SC144, U0126 or a combination of SC144 plus U0126. Data are shown as the mean ± SD (n = 3) in which *, $p < .05$.

blockade to combat a feedback loop between ERK, mTOR and AKT. These findings may provide a more effective treatment strategy for future preclinical and clinical investigations of multidrug-resistant bladder cancer.

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Table 1
Dual blockage of GP130 (SC144) and pERK (U0126) has synergistic inhibitory effects on proliferation of bladder cancer cells.

Fa %	SC144 (μM/L)	U0126 (μM/L)	SC144 (μM/L) / U0126 (μM/L)	CI
5	0.12	3.10	0.12/0.60	1.164
25	0.96	10.50	0.43/2.14	0.651 *
50	3.27	21.67	0.92/4.60	0.494 *
75	11.15	44.74	1.98/9.89	0.398 *
95	87.56	151.26	7.15/35.73	0.318 *

Chemotherapy resistant bladder cancer cells were treated with various concentrations of the indicated inhibitors. Combinatorial indices (CI) were measured at different percentages of fractions affected (Fa). CI values from 0.3 to 0.7 indicate synergism * [14].

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Conflict of Interest

The authors declare no potential conflicts of interest.

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