Combination of ERK2 and STAT3 Inhibitors Promotes Anticancer Effects on Acute Lymphoblastic Leukemia Cells

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Abstract. Background/Aim: Deregulated activation of signaling through the RAS/RAF/mitogen-activated protein kinase/extracellular signal-regulated kinase (RAS/RAF/MEK/ERK) and signal transducer and activator of transcription (STAT) pathways is involved in numerous hematological malignancies, making it an attractive therapeutic target. This study aimed to assess the effect of the combination of ERK2 inhibitor VX-11e and STAT3 inhibitor STA-21 on acute lymphoblastic leukemia cell lines REH and MOLT-4. Materials and Methods: REH and MOLT-4 cell lines were cultured with each drug alone and in combination. Cell viability, ERK activity, cell cycle distribution, apoptosis and oxidative stress induction were assessed by flow cytometry. Protein levels of STAT3, phospho-STAT3, protein tyrosine phosphatase 4A3 (PTP4A3), survivin, p53 and p21 were determined by western blotting. Results: VX-11e in combination with STA-21 significantly inhibited cell viability, induced G0/G1 cell-cycle arrest, enhanced production of reactive oxygen species, and induced apoptosis. These effects were associated with an increased level of p21 protein in REH cells and with reduced levels of phospho-STAT3, survivin and PTP4A3 proteins in MOLT-4 cells. Conclusion: Our findings provide a rationale for combined inhibition of RAS/RAF/MEK/ERK and STAT3 pathways in order to enhance anticancer effects against acute lymphoblastic leukemia cells.

Acute lymphoblastic leukemia (ALL) is the most common hematological malignancy in children and adolescents, and includes B-lineage acute lymphocytic leukemia (B-ALL), which constitutes 25% of all cancer in children, and T-lineage acute lymphoblastic leukemia (T-ALL), which occurs at a lower frequency and is associated with a worse prognosis (1, 2). ALL cells display dysregulation of several intracellular signaling pathways including RAS/RAF/mitogen-activated protein kinase/extracellular signal-regulated kinase (RAS/RAF/MEK/ERK) and signal transducer and activator of transcription (STAT) pathways, which are relatively common (3).

Protein kinases ERK1/2 are central signaling kinases that control cell proliferation, survival, differentiation and apoptosis. ERK1/2 phosphorylates multiple substrates localized in different subcellular compartments and activates the expression of many regulatory proteins involved in the cell cycle and apoptosis, including p21 and survivin (4-7).

Recently, the RAS/RAF signal transduction pathway has become an important target in cancer treatment research. VX-11e, a selective ERK2 inhibitor, has been shown to reduce growth of melanoma xenografts and to reduce survival of various human cancer cell lines (8, 9). It has also been found to induce cell-cycle arrest and apoptosis in leukemia cell lines (10).

STAT3 is an important transcription factor. Once phosphorylated, it regulates the expression of numerous transcriptional targets, such as p53, p21, protein tyrosine phosphatase 4A3 (PTP4A3) and survivin (11, 12). Dysregulation of STAT3 contributes to oncogenesis by changing the expression of genes regulating the cell cycle and cell growth, leading to disruption of these processes. Moreover, abnormal expression of activated STAT3 can also prevent apoptosis through up-regulation of anti-apoptotic proteins (13). Since STAT3 is frequently activated in ALL, it represents a promising target for the development of a new approach in the treatment of ALL (14).

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STA-21 is a small molecule that inhibits STAT3 DNA-binding activity and STAT3–STAT3 dimerization. It has been shown to reduce the survival of breast cancer cells that express constitutively active STAT3 (15). Moreover, STA-21 was found to induce a dose-dependent increase in apoptotic cells in T-cell large granular lymphocyte leukemia (16).

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To enhance therapeutic response in ALL, new strategies have recently been proposed, including combinations of drugs affecting the RAS/RAF/MEK/ERK pathway and other molecular targets. Accordingly, a synergistic effect was observed for ERK2 inhibitor in combination with megestrol in T-cell prolymphocytic leukemia cells (17). In our previous studies, we also demonstrated that MEK1/2 and ERK2 inhibitors in combination with voreloxin increased anti-proliferative and pro-apoptotic effects in leukemia cells (10, 18). Recently, it was reported that combination of MEK and STAT3 inhibitors enhanced therapeutic response in pancreatic ductal adenocarcinoma (19).

The aim of this study was to determine the effect of ERK2 inhibitor VX-11e in combination with STAT3 inhibitor STA-21 on viability, cell-cycle distribution and apoptosis of human lymphoblastic leukemia cells.

Materials and Methods

**Inhibitors.** STA-21 and VX-11e were obtained from Selleck Chemicals (Selleckchem, Houston, TX, USA) and dissolved in dimethyl sulfoxide (DMSO; Merck Millipore, Billerica, MA, USA). Stock solutions were kept at −20˚C until use. In drug combination experiments, the final concentration of DMSO, which was used as vehicle control, did not exceed 0.1%.

**Cell lines and cell culture.** The human cell lines REH (B-ALL) and MOLT-4 (T-ALL) were purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and grown in RPMI-1640 GlutaMax medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 10 μg/ml streptomycin (all reagents were obtained from Life Technologies, Carlsbad, CA, USA). The cells were cultured at 37˚C in a humidified atmosphere with 5% CO2.

**Cell viability assay.** REH and MOLT-4 cells were seeded in triplicate at a density of 2×10^5 cells/well in a 96-well plate. Cells were treated with either DMSO (control) or different concentrations of STA-21 (2.5, 5, 10, 20 or 40 μM) for 24 and 48 h. For combination drug treatment, cells were simultaneously exposed to 4 μM of VX-11e with 5 μM of STA-21 for 48 h. The Muse Count and Viability Assay Kit (Merck Millipore) was used to assess cell viability according to the manufacturer’s instructions. Briefly, cells were incubated with the Muse Count and Viability Reagent for 5 min at room temperature and quantified using Muse Cell Analyzer (Merck Millipore). The relative cell viability was expressed as the percentage relative to that of control cells.

**ERK activity assay.** REH and MOLT-4 cells were seeded in triplicate in a 96-well plate at a density of 2×10^4 cells/well and treated with vehicle or with each agent alone or in combination (4 μM of VX-11e/5 μM of STA-21) for 48 h. The cell-cycle distribution was evaluated using the Muse Cell Cycle Kit (Merck Millipore) according to the manufacturer’s protocol with some modifications. Briefly, 10^5 control and treated cells were fixed overnight with 70% ice-cold ethanol at −20˚C and then incubated with Cell Cycle Reagent for 20 min at room temperature in the dark. All samples were analyzed on a Muse Cell Analyzer.

**Apoptosis assay.** REH and MOLT-4 cells were seeded at a density of 10^5 cells/well in triplicate, in a 24-well plate and incubated with vehicle or with each inhibitor alone or in combination (4 μM of VX-11e/5 μM of STA-21) for 48 h. For assessment of apoptotic cells, the Muse Annexin V and Dead Cell Kit (Merck Millipore) was used according to the manufacturer’s instructions. This assay utilizes Annexin V to detect apoptotic cells and a dead cell marker, 7-aminoactinomycin D as an indicator of cell membrane integrity. In brief, 105 control and treated cells were re-suspended in phosphate-buffered saline supplemented with 1% FBS and Muse Annexin V and Dead Cell Reagent for 20 min at room temperature in the dark. The cells were quantified using Muse Cell Analyzer.

**Oxidative stress assay.** REH and MOLT-4 were seeded in triplicate in a 96-well plate (2×10^5 cells/well) and treated with vehicle alone or with 4 μM of VX-11e/5 μM of STA-21 for 48 h. The cell population undergoing oxidative stress was measured using the Muse Oxidative Stress Kit (Merck Millipore) according to the manufacturer’s protocol. Briefly, cells were re-suspended in Muse Oxidative Stress working solution containing dihydroethidium (DHE) and incubated for 30 min at 37˚C. The cells were quantified using Muse Cell Analyzer and the relative percentage of reactive oxygen species (ROS) positive- and ROS-negative cells was estimated by Muse analysis software.

**Western blotting.** Total protein was extracted from control and treated cells using RIPA buffer (Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with 1X Protease Inhibitor Cocktail (Roche Diagnostic, Basel, Switzerland) followed by centrifugation at 20,000 × g for 15 min at 4˚C. Protein concentrations were determined by bicinchoninic acid assay (Thermo Scientific/Pierce Biotechnology, Rockford, IL, USA). Protein samples (25 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis using 12% sodium dodecyl sulfate, and then electrottransferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% non-fat milk and incubated overnight at 4˚C with primary antibodies (Cell Signaling Technology, Danvers, MA, USA): Mouse monoclonal anti-STAT3 (#9139, 1:1000); mouse monoclonal anti-phospho-STAT3 (pSTAT3), (#4113, 1:800); rabbit polyclonal anti-PTP4A3 (#6484, 1:800); rabbit monoclonal anti-survivin (#2808, 1:800); rabbit monoclonal anti-p21 Waf1/Cip21 (#2947, 1:1000); and rabbit monoclonal anti-β-actin (#4970, 1:1000). After washing, the membranes were incubated with goat anti-rabbit/ anti-mouse secondary antibodies conjugated with alkaline phosphatase (1:2000; Proteintech Group, Inc., Rosemont, IL, USA). Bands were developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Roche Diagnostic) as substrate. The
optical density of the bands was quantified with ChemiDoc MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). β-Actin was used for normalization.

Statistical analysis. GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA) was used for the statistical analysis. The results are presented as the mean±standard deviation (SD) of three independent experiments. Statistical differences were analyzed by Mann–Whitney or Student’s t-test with values of \( p<0.05 \) as statistically significant.

Results

**STA-21 alone and in combination with VX-11e inhibits cell viability.** REH and MOLT-4 cells were treated with increasing concentrations of STA-21 (2.5 to 40 μM) for 24 and 48 h. The cell viability decreased in a dose- and time-dependent manner in both cell lines (Figure 1A and B). A concentration of 5 μM STA-21 was selected for all subsequent experiments, as the concentration exhibiting relatively low toxicity to treated cells after 48 h (~80% viability). A concentration of 4 μM VX-11e was chosen on the basis of our previous study (18). The combination of non-toxic concentration of 4 μM VX-11e and 5 μM STA-21 significantly inhibited cell viability of REH and MOLT-4 cells as compared with the effects of each drug used alone (Figure 1C).

**VX-11e and STA-21 reduce ERK activity.** The use of VX-11e alone and in combination with STA-21 for 48 h significantly reduced the percentage of ERK-activated cells in both cell lines, reaching the lowest level in REH cells treated with both inhibitors (22.3%±7.2%). STA-21 alone significantly reduced ERK activity in the MOLT-4 cell line compared to control cells (Figure 2). These results therefore indicate that the combination of VX-11e and STA-21 is highly effective in the inhibition of ERK activity in REH and MOLT-4 cells.

Figure 1. Signal transducer and activator of transcription-3 (STAT3) inhibitor STA-21, alone and in combination with extracellular-regulated kinase (ERK) 2 inhibitor VX-11e inhibited acute lymphoblastic leukemia cell viability. REH (A) and MOLT-4 cells (B) were exposed to STA-21 alone at increasing concentrations for 24 and 48 h and to a combination of 4 μM VX11e with 5 μM STA-21 for 48 h (C). The cell viability was determined by Muse Count and Viability Kit. Each value is the mean±SD of three independent experiments. **Significantly different at \( p<0.01 \) vs. control.
Combined treatment with VX-11e and STA-21 induces cell-cycle arrest and apoptosis. VX-11e and STA-21 alone did not significantly affect the cell cycle, whereas combined treatment significantly increased the proportion of REH and MOLT-4 cells in the G₀/G₁ phase, with a corresponding decrease of the percentage of cells in the G₂/M phase (Figure 3).

VX-11e significantly increased the total apoptotic rate (early and late apoptosis) to approximately 20% of both cell lines, whereas STA-21 alone induced this process in about
10% of MOLT-4 cells and 30% of REH cells (Figure 4). As compared to the effect of each drug alone, the combined treatment resulted in further increase in the percentage of apoptotic cells from 50.9%±9.7% in MOLT-4 cells to 65.9%±9.6% in REH cells. These results demonstrate that the combined treatment with VX-11e and STA-21 enhances apoptosis in REH and MOLT-4 cells and this can be attributed to cell-cycle perturbations.

STA-21 alone and in combination with VX-11e increases ROS production. VX-11e reduced the percentage of ROS-positive REH cells and had no significant effect on oxidative stress induction in MOLT-4 cells. In both cell lines, a significant increase in the percentage of ROS-positive cells (25.9%±2.3% in MOLT-4 cells and 72.5±1.6% in REH cells) was observed after treatment with STA-21 and this effect was markedly potentiated in MOLT-4 cells after combination of STA-21 with VX-11e (60.5%±7.6%) (Figure 5). The results suggest that STA-21 promotes the generation of intracellular ROS, leading to oxidative stress.

Combination of VX-11e and STA-21 alters levels of some apoptosis-related proteins in ALL cell lines. To further confirm the pro-apoptotic effect of VX-11e and STA-21 in
Figure 4. Combination of extracellular-regulated kinase (ERK) 2 inhibitor VX-11e and signal transducer and activator of transcription-3 (STAT3) inhibitor STA-21 enhanced apoptosis in acute lymphoblastic leukemia cells. REH and MOLT-4 cells were incubated for 48 h with 4 μM VX-11e and 5 μM STA-21, alone and in combination. A: Representative dot plots of annexin V/7-aminoactinomycin D apoptotic assay. B: Graph showing the percentage of apoptotic cells. Each value is the mean±SD of three independent experiments. Significantly different at: *p<0.05, **p<0.01 vs. control; #p<0.05, and ## p<0.01 vs. VX-11e alone; $$ p<0.01 vs. STA-21 alone.
combination, we investigated related proteins. Treatment with these compounds alone and in combination did not affect the level of STAT3 in either cell line. VX-11e and STA-21 alone also had no effect on pSTAT3 protein, whereas their combination significantly reduced the pSTAT3 level in MOLT-4 cells. VX-11e and STA-21 alone and in combination also did not influence the level of p53 protein. The amount of p21 in REH cells was markedly increased upon combined treatment, whereas expression of this protein was undetectable in MOLT-4 cells, irrespective of the absence or presence of VX-11e and STA-21. The levels of survivin and PTP4A3 were significantly decreased in MOLT-4 cells upon combined treatment when compared to control cells and cells treated with either inhibitor alone (Figure 6). These results suggest that the combination of VX-11e and STA-21 exerts its pro-apoptotic effect by up-regulating p21 in a p53-independent manner.

**Figure 5.** Combination of extracellular-regulated kinase (ERK) 2 inhibitor VX-11e and signal transducer and activator of transcription-3 (STAT3) inhibitor STA-21 enhanced reactive oxygen species (ROS) production in acute lymphoblastic leukemia cells. REH and MOLT-4 cells were incubated for 48 hours with 4 μM VX-11e and 5 μM STA-21, alone and in combination. A: Representative histograms of ROS-negative (M1) and ROS-positive (M2) cells. B: Graph showing the percentage of ROS-positive cells. Each value is the mean±SD of three independent experiments. Significantly different at: **p<0.01 vs. control; ## p<0.01 vs. VX-11e alone; $$ p<0.01 vs. STA-21 alone.
Figure 6. Combination of extracellular-regulated kinase (ERK) 2 inhibitor VX-11e and signal transducer and activator of transcription-3 (STAT3) inhibitor STA-21 affected levels of phospho (p)STAT3, p21, survivin and protein tyrosine phosphatase 4A3 (PTP4A3). REH and MOLT-4 cells were incubated for 48 hours with 4 µM VX-11e and 5 µM STA-21, alone and in combination. A: The level of STAT3, pSTAT3, p53, p21, survivin and PTP4A3 proteins was detected by western blot. β-Actin was used as a loading control. B: Quantification of proteins was performed by densitometric analysis and normalized to the internal loading control. Each value is the mean±SD of three independent experiments. ND: Not detected. Significantly different at: *p<0.05, and **p<0.01 vs. control; #p<0.05, and ## p<0.01 vs. VX-11e alone; $$ p<0.01 vs. STA-21 alone.
way in REH cells and by down-regulating pSTAT3, survivin and PTP4A3 in MOLT-4 cells.

Discussion

The combination of drugs that target different signaling pathways involved in the induction and progression of cancer may provide encouraging leads for anticancer therapy. In the present study, we have shown that a combination treatment using ERK2 inhibitor VX-11e and STAT3 inhibitor STA-21 enhanced anticancer effects in ALL cells by reducing viability, and inducing cell-cycle arrest, ROS production and apoptosis.

We have demonstrated that VX-11e alone significantly inhibited ERK activity in both cell lines and this effect was maintained after combination with STA-21, especially in REH cells. Interestingly, STA-21 slightly but significantly reduced ERK activity in MOLT-4 cells. It was recently reported that STAT3 inhibitor HJC0152 affected ERK activity in gastric cancer cells (20). Moreover, we found that STA-21 alone had no effect on levels of total and phosphorylated STAT3 protein in either cell line. A possible explanation for our observations might be associated with the mechanism of action of STA-21. It has been demonstrated that a direct STAT3 inhibitor galitellalactone reduced STAT3 binding to DNA in prostate cancer cells, blocking the STAT3 signaling pathway without altering its phosphorylation (21). On the basis of these results, it is possible that in our study STA-21 inhibited STAT3 activation without affecting its phosphorylation status. However, VX-11e in combination with STA-21 reduced the level of phosphorylated STAT3 in MOLT-4 cells but not in REH cells. The drug response may vary depending on the cell line tested. Moreover, the different effect of VX-11e and STA-21 in combination on the level of pSTAT3 in REH and MOLT-4 cells might be due to expression of erythroblastosis variant gene 6 translocation (ETV6)—runt-related transcription factor 1 (RUNX1) fusion gene in REH cells, which regulates STAT3 phosphorylation (22). Taken together, we conclude that ERK signaling inhibition might influence STA-21 action leading to reduced STAT3 phosphorylation in MOLT-4 cells. However, our results do not exclude the involvement of other mechanisms.

Combined treatment with VX-11e and STA-21 induced G0/G1 cell-cycle arrest with a concomitant decrease in the G2/M population in both cell lines. Moreover, in REH cells this effect was associated with the increased protein level of the cell-cycle inhibitor p21, whereas in MOLT-4 cells, p21 expression was undetectable by western blotting in untreated and treated cells. It has been shown that p21 plays a crucial role in G1/S phase progression and its expression is p53-dependent (23). However, in this study we have found that the increase in p21 protein level might be regulated through a p53-independent mechanism in REH cells. Interestingly, some studies have demonstrated an anti-apoptotic role of p21 in leukemia cells (24-26). In contrast, it was shown that taxol increased p21 expression and induced growth arrest and apoptosis in breast cancer cells (27). Similarly, we have shown previously that combination of VX-11e with voroelixin increased the level of p21 protein in REH cells, leading to apoptosis (18).

Survivin, an inhibitor of apoptosis, plays a significant role in cell proliferation and survival (28), and its deregulated expression has been shown in hematopoietic malignancies, including ALL (29). While survivin has been identified as a gene downstream of STAT3, the inhibition of STAT3 may reduce the expression of survivin and induce apoptosis in hematological malignancies (30-32). Moreover, it was reported that survivin can be regulated by MEK/ERK-dependent signaling (33). In this study we observed a significant reduction in the survivin level in MOLT-4 cells treated with both VX-11e and STA-21. Similarly, we found a decrease in survivin level in leukemia cell lines after combined treatment with VX-11e and voroelixin (18).

PTP4A3 participates in many cellular processes, such as proliferation, survival, motility and invasion (34, 35). PTP4A3 expression has been documented as being significantly elevated in a variety of solid tumor types compared with healthy tissues, as well as in acute myeloid leukemia (AML) and ALL cells (36, 37). In this study, we observed the level of PTP4A3 to be reduced in MOLT-4 cells treated with the combination of both drugs. Moreover, it has been demonstrated that STAT3 functionally regulates PTP4A3 expression in AML and that targeting either STAT3 or PTP4A3 reduced cell viability (35). It has also been found that knockdown of PTP4A3 reduced cell proliferation through enhanced production of reactive oxygen species in colon cancer cells (38).

ROS are considered to be essential in the regulation of proliferation, cell-cycle progression, differentiation, migration and cell death (39). An elevated ROS level plays a significant role in cancer treatment by inducing cell death through apoptosis (40, 41). We observed that apoptosis induced by combinatorial treatment with both VX-11e and STA-21 is related to enhanced ROS production. Interestingly, STA-21 alone significantly increased the ROS level in both cell lines as compared to control and VX-11e-treated cells, and this effect was markedly potentiated after combined treatment with VX-11e in MOLT-4 cells. Similarly, in our previous study, the MEK inhibitor TAK-733 in combination with voroelixin increased ROS production in HL60 leukemia cells (10). Of note, the drug response in cancer cells may depend upon a variety of factors. In combinatorial treatment, one drug can sensitize cells to another drug by increasing ROS production (42, 43).

Apoptotic cell death can occur through different mechanisms depending on the cell type. Thus, it is possible that the pro-apoptotic effect of VX-11e and STA-21 in B-ALL REH cells might be due to an increased level of p21 protein, whereas reduction in pSTAT3, survivin and PTP4A3 protein levels after treatment with both drugs may enhance, at least in part, the...
apoptotic process in T-ALL MOLT-4 cells. It seems therefore possible that different mechanisms may be involved in the pro-apoptotic effects of VX-11e and STA-21 in REH and MOLT-4 cells. This intriguing finding requires further investigations.

**Conclusion**

Targeting cellular signaling pathways constitutes a promising therapeutic approach to the treatment of leukemia. As far as we are aware, this study is the first to demonstrate that ERK2 inhibitor VX-11e in combination with STAT3 inhibitor STA-21 promotes anticancer effects in ALL cell lines. Our findings provide a rationale for combining drugs targeting ERK and STAT3 pathways, potentially useful for the treatment of ALL.

**Conflicts of Interest**

None declared.

**Authors’ Contributions**

EJG designed the research study. EJG and HJ performed the research. EJG, MJ and GJL analyzed the data. EJG wrote the article. JAL edited the article. All Authors read and approved the final article.

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