Combination therapy using TGF-β1 and STI-571 can induce apoptosis in BCR-ABL oncogene-expressing cells

Abstract: The BCR-ABL oncogene is a tyrosine kinase gene that is over-expressed in CML. It inhibits the TGF-β1 signaling pathway. Due to resistance of cells to the tyrosine kinase inhibitor, STI-571, the combined effect of STI-571 and TGF-β1 on K562 cells was studied in the present research. Results revealed that the TGF-β1 cell signaling pathway, which is activated in K562 cells treated with TGF-β1, activates collective cell signaling pathways involved in survival and apoptosis.

It is noteworthy that treating K562 cells with STI-571 triggered apoptotic pathways, accompanied by a reduction in proteins such as Bcl-xL, Bcl-2, p-AKT, p-Stat5, p-FOXO3, and Mcl-1 and an increase in the pro-apoptotic proteins PARP cleavage, and p27, leading to an increase in sub-G1 phase-arrested and Annexin-positive cells.

Interestingly, the proliferation behavior of TGF-β1-induced cells was changed with the combination therapy, and STI-571-induced apoptosis was also prompted by this combination. Thus, combination treatment appears to promote sub-G1 cell cycle arrest compared to individually treated cells. Furthermore, it strongly triggered apoptotic signaling. In conclusion, TGF-β1 did not negatively impact the effect of STI-571, based on positive annexin cells, and AKT protein phosphorylation remains effective in apoptosis.

Introduction

In 1970, Rowley identified the Philadelphia (Ph) chromosome, a shortened chromosome obtained from cross-transfer between the long arms of chromosomes 9 and 22 that leads to the formation of the BCR-ABL oncoprotein [1]. Chronic myeloid leukemia (CML) is associated with the BCR-ABL tyrosine kinase gene in the Ph chromosome, as well as with uncontrolled proliferation of progenitor cells [2-4]. The activated BCR/ABL kinase is vital for the survival and growth of leukemia cells; it governs pathogenesis and malignancy by activating multiple intracellular signal transduction pathways [5-7], such as the JAK-STAT pathway, the P13K-mTOR pathway, and the RAS/RAF/MEK/ERK pathway [8, 9]. In addition, anti-apoptotic Bcl-2 family members, like bcl-xL and Mcl-1, are expressed in CML cells [10, 11].

Since the BCR-ABL oncoprotein is a tyrosine kinase, STI-571 mesylate, a tyrosine kinase inhibitor (TKI), has drawn recent attention for inhibiting BCR-ABL in CML patients, displaying an effective role against CML, and marking a major milestone in cancer drug discovery [12, 13]. It induces apoptosis, thereby inhibiting the kinase activity of BCR-ABL [14]. However, a major problem with current CML therapies is resistance to STI-571, which can lead to heterogeneous molecular responses [15-17] such as over-expression of LYN to increase p27 proteolysis, thereby increasing cancer cell proliferation [18]. To overcome this obstacle, scientists are working on combination therapies with other agents, which could prevent the development of multiple drug resistance (MDR) and provide synergistic effects in CML treatment. To achieve this goal, an investigation of cell signaling proteins, especially TGF-β1, could be valuable. It is noteworthy that TGF-β1 has both

Keywords: Leukemia; Myelogenous; Chronic; BCR-ABL Positive; transforming growth factor beta1; Drug resistance; Apoptosis.
inhibitory and stimulatory effects on leukemia cells [19], and its signaling pathway is initiated by the formation of a heteromeric complex with transmembrane serine/threonine kinase receptors [20]. It acts as an inhibitor of Cdk2 and cyclin E activity via promotion of p27 [19, 21]; BCR-ABL can completely block TGF-β1-induced expression of p27 [3]. BCR-ABL may play an effective suppressive role by blocking the function of SMAD3 in the TGF-β1/SMAD signaling pathway, thereby promoting carcinogenesis. Based on this finding, it seems that a combination of TGF-β1 and a TKI could increase apoptosis by inhibiting the blocking effect of BCR-ABL on the TGF-β1 signaling pathway. Evidence indicates that the interaction of AKT with unphosphorylated SMAD3, the subsequent inhibition of PI3K/AKT signaling by TGF-β1 [22, 23], and activation of SMAD2 and SMAD3 [24], serve as anti-proliferative mechanisms. In fact, the TGF-β1/SMAD signaling pathway is critical for CML cells; it supports growth inhibition, differentiation, and apoptosis in leukemia cells [3]. On the other hand, unlike TGF-β1, serine/threonine kinase MAPK (mitogen-activated protein kinase) causes AKT activation and suppresses both activity of forehead O (FOXO) transcription factors and degradation of p27, thereby enhancing the survival and proliferation of cancer cells [17]. Interestingly, Naka et al. investigated the effect of a TGF-β1 inhibitor on leukemia-initiating cells (LICs) and revealed that this treatment damaged colony-forming ability in CML, while TGF-β1 affects LICs in a complicated manner [25]. Based on the aforementioned information, the combined effect of STI-571 and TGF-β1 on K562 cells was investigated for the first time in the present investigation, with the goal of determining whether TGF-β1 signaling pathways trigger apoptotic signals in single and combination therapy and whether p-FOXO3 levels are more important than FOXO3 level, in influencing the roles of p27 as an apoptotic protein and of p-Stat 5 as a survival protein.

Cell cycle analysis

Cell cycle analysis was performed to investigate the effect of the TKI, STI-571 (Sigma-Aldrich), and TGF-β1 (Biolegend) alone and in combination on different stages of the K562 cell cycle and on the sub-G1 percentage as a marker of dead K562 cells. Of note, STI-571 and TGF-β1 stock solutions were prepared in DMSO and PBS-BSA, respectively. K562 cells were treated with 2 ng/ml TGF-β1 (concentration was optimized based on previous work [26] and incubations were also performed for 24 h at 2 ng/mL, data not shown) and different concentrations of STI-571 (0.125, 0.25, and 0.5 µM) for 48 h. Also, to investigate the influence of combination therapy on sub-G1 K562 cells, a combination of 2 ng/mL TGF-β1 with 0.125, 0.25, and 0.5 µM concentrations of STI-571 was assessed. K562 cells were washed and centrifuged when the incubation period was complete. Then, ice-cold 70% ethanol was used to fix K562 cells overnight at −20°C. First, cells were washed using phosphate-buffered saline (PBS). In the dark, 1 mg/mL propidium iodide solution (Sigma) and 50 µg/mL RNase A (Roche, Germany) were applied to stained cells for 30 min at room temperature. Cell cycle data was collected by FACScan cytometer (Becton Dickinson) and analyzed using Flowjo software (v7.6.1). Experiments were repeated in triplicate, and mean values with positive and negative standard deviations (±SD) were estimated across triplicate experiments.

Annexin-PI Assay

To evaluate the relationship between the apoptosis potential of K562 cells and treatment with STI-571, TGF-β1, or a combination, Annexin V-FITC/PI flow-cytometry was performed after 48 h. Briefly, K562 cells were seeded overnight in a 24-well plate at a concentration of 1 × 10^5 cells/well before being treated with 2 ng/mL TGF-β1 and STI-571 at concentrations of 0.125, 0.25, or 0.5 µM at 37 °C with 95% humidity and 5% CO₂. To investigate the effect of combination therapy, leukemia cells were treated with a combination of 2 ng/mL TGF-β1 and STI-571 at concentrations of 0.125, 0.25, and 0.5 µM. After the incubation time, cells were washed twice with cold PBS. Then, cells were suspended in 100 µL binding buffer, 5 µL Annexin V, and 5 µL PI. Samples were kept in the dark for 15 min, and fluorescent intensities (n = 10000 cells) were evaluated by flow cytometer (FACScan; BD Biosciences). FACS data were analyzed using Flowjo software (v7.6.1). The experiment was repeated three times, and the mean ± SD was calculated.

Materials / Methods

Cell culture

Human bone marrow K562 cells were purchased from ATCC and cultured at 37 °C with 95% humidity and 5% CO₂ in 25 cm² flasks in RPMI 1640 with 10% (v/v) heat-inactivated FBS (BioSera), 100 U/mL penicillin, 100 U/mL streptomycin (Sigma), and 20 mM HEPES. Cells were in the logarithmic growth phase during the performance of all experiments.
Western blotting

To determine levels of proteins related to apoptotic and anti-apoptotic functions and assess regulation of signaling biomolecules, western blotting was performed according to previously published methods [26]. Briefly, cells (3 × 10⁶ cells) were seeded for 75 cm² flask culture overnight and then treated with TGF-β1 (2 ng/mL), STI-571 (0.5 µM), or a combination for 48 h. After the incubation time, cells were lysed in RIPA lysis buffer (0.5 M NaF, 1 M Tris, 0.1% Triton-X100, 1% NP40, 0.1% NaN3, 1% TBP, 100 µL/mL Cocktail Protease Inhibitor, 200 mM PMSF, 0.1M NaVO4) and centrifuged at 13000 rpm, after which the supernatant was transferred to a fresh microtube. Protein concentration was measured by Bradford assay (Bio-Rad kit). For each sample, 40 µg protein was boiled with 5x loading buffer (Fermentase) and resolved on a 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gel, then transferred to a nitrocellulose membrane.

Blots were blocked with 5% non-fat dry milk-TBST for 2 h and incubated overnight with primary antibodies. Primary antibodies consisted of apoptotic antibodies for monoclonal rabbit p27 and cleaved PARP; anti-apoptotic antibodies for monoclonal rabbit MCL-1, bcl-xL, and Bcl2; and cell signaling antibodies for AKT, p-AKT, Stat5, p-Stat5, FOXO3, and p-FOXO3, all of which were diluted 1:1000 in 5% non-fat dry milk-TBST (Cell Signalling Technology). β actin was used as a reference protein. After the incubation, blots were washed three times with TBST and incubated with secondary goat anti-rabbit and horseradish peroxidase (HRP) conjugate for 1 h (Santacruze Biotechnology). The blots were visualized by the chemiluminescent method using the Amersham ECL kit. The experiment was repeated thrice, and the mean ± SD was calculated.

Quantitative gene assessment via Real-Time PCR

To quantitate the fold change in BCL2, bcl-xL, and SMAD3 gene expression, qRT-PCR was performed. Briefly, 1 × 10⁶ cells were seeded for 25 cm² flask culture overnight and then treated with TGF-β1 (2ng/mL), STI-571 (0.5 µM), or a combination for 48 h. After the incubation, the cells were centrifuged. Total RNA was isolated using a Thripure isolation kit (Roche) as indicated in the instruction manual, and samples were treated with DNase I. A QuantiNova reverse transcription Kit (Qiagen) was used to synthesize 1µg cDNA, which was used as input for 40 cycles of PCR using a Rotor-gene Q real-time analyzer (Corbett, Australia) and QuantiNova SYBR green PCR kit (Qiagen). The following primers were used: SMAD3: F: 5'GCGGAGTACAGGAGACGAC-3', R:5'ACACTGGACGCGATGC-3', BCL2:5'-ATCGCCCTGTGATGACTGAG-3', R:5'CAGCCAGGAAATCA AACAGAGG-3', Bcl-xL:5'TGCATTTCCCATAGAGTTTCA-3', R:5'-CTCTGATGACACCTAGACCTT-3' and reference gene GAPDH: F:5'-GAAGATGGTGATGGGATTTC-3', R:5'-GAAGGCTGTTC-3'. The reaction was performed in two steps, with cycling for 120, 5, and 15 sec at 95°C, 95°C, and 60°C, respectively. Each experiment was repeated three times, and the relative fold change gene expression ΔΔCT technique was used for quantification.

Results

TGF-β1 enhances STI-571-induced apoptosis in K562 cells

The apoptosis potential of TGF-β1 and STI-571 at different concentrations (0.125, 0.25, and 0.5 µM) in the cells was investigated by Annexin V/PI staining. Our results show that the number of Annexin-positive cells significantly increased in a dose-dependent manner from 0.125 to 0.5 µM. Although TGF-β1 (6.78% ± 3.9%) did not induce significant apoptosis compared to the control (8.12% ± 1.2%) (P > 0.05), its combination with STI-571 increased cell apoptosis. Interestingly, the cell apoptosis potential of 0.25 µM STI-571 at a concentration of (22.11% ± 5.17%) was significantly higher than that of TGF-β1 combined with 0.125 µM STI-571 (15.43% ± 4.6%) (P < 0.01), whereas the cell apoptosis potential of 0.5 µM STI-571 (30.48% ± 6.7%) was significantly less than that of TGF-β1 combined with 0.25 or 0.5 µM STI-571 (50.91% and 65.45%, respectively) (P < 0.01) (Fig. 1a,b).

TGF-β1 increases STI-571-induced growth of sub-G1 cell population

To investigate the effect of STI-571, TGF-β1, and their combination using the sub-G1 cell population as a marker of cell death, K562 cells were treated with STI-571 at the concentrations of 0.125, 0.25, and 0.5 µM in the presence or absence of 2 ng/mL TGF-β1 for 48 h (Fig. 2a,b). The results derived from the sub-G1 cell cycle subset showed that, although TGF-β1 did not significantly increase the sub-G1 subset compared to the control group (2.75% and 3.87%, respectively), the influence of 0.125 and 0.25 µM STI-571 as a kinase inhibitor (9.3% ± 1.7%,14.98% ±
Figure 1: a) Histogram of Annexin-PI-positive K562 cells treated with 2 ng/mL of TGF-β1 and different concentrations of STI-571, individually or in combination, for 48 hours. The graph on the left represents different concentrations of STI-571, while the one on the right represents their combination. b) Graph of Annexin-PI-positive K562 cells treated with 2 ng/mL of TGF-β1 and different concentrations of STI-571, individually or in combination, for 48 hours. The number of apoptotic cells gradually increased with increasing STI-571 concentrations and with the combination therapy. The data are represented as the mean ± SD. *** indicates $P < 0.001$. 
1.68%, respectively) was significantly less than that of the combination therapy (23.9% ± 3.25%, 41.51% ± 3.39%, respectively) (p < 0.001). However, the effect of 0.5 µM STI-571 (25.83 ± 0.76) was less than that of the combination of 0.25 µM STI-571 and 2 ng/mL TGF-β1 (Fig2b) (p < 0.001). Interestingly, the combination therapy had a significantly stronger effect on sub-G1 subset cell mortality (62.03% ± 1.37%) (p < 0.001) (Fig. 2a).

**TGF-β1 reduces phosphorylation of AKT in STI-571-treated K562 cells**

To evaluate the role of survival-associated proteins, K562 cells were exposed to STI-571, TGF-β1, or a combination for 48 hours. Based on regulation of anti-apoptotic proteins, western blot data showed that phospho-AKT protein levels were reduced in STI-571-treated and combination-treated cells as compared with the TGF-β1-treated or control groups (Fig. 3a). The decrease in phospho-AKT protein was more prominent in the combination therapy group. Also, levels of phospho-STAT5 and phospho-FOXO3 were decreased in the STI-571-treated and combination-treated groups as compared with the TGF-β1-treated or control groups. However, the levels of these proteins were decreased in combination-treated cells, which indicates that TGF-β1 could have an effect on phospho-STAT5 and phospho-FOXO3 proteins (Fig. 3a,b).
Protein expression and phosphorylation levels of AKT, p-AKT, Stat5, p-Stat5, FOXO3, and p-FOXO3 proteins in K562 cells treated with 2 ng/mL of TGF-β1 and 0.5 µM STI-571, individually and in combination, for 48 h. Combination therapy with TGF-β1 was associated with a lower phosphorylation level than treatment with TGF-β1 individually. Data represented as mean ± SD, and quantitative expression of proteins was normalized to the level of β-actin. The results showed a significant difference in treated groups compared to other groups (P < 0.05).

Figure 3a) Protein expression and phosphorylation levels of AKT, p-AKT, Stat5, p-Stat5, FOXO3, and p-FOXO3 proteins in K562 cells treated with 2 ng/mL of TGF-β1 and 0.5 µM STI-571, individually and in combination, for 48 h. Combination therapy with TGF-β1 was associated with a lower phosphorylation level than treatment with TGF-β1 individually. Data represented as mean ± SD, and quantitative expression of proteins was normalized to the level of β-actin. The results showed a significant difference in treated groups compared to other groups (P < 0.05).
bcl-xL and Mcl-1 levels decrease after combination treatment compared to TGF-β1 alone

It is noteworthy that bcl-xL and Mcl-1 levels in K562 cells treated with TGF-β1 alone were significantly higher than those of cells treated with the combination therapy. Anti-apoptotic proteins bcl-xL and Mcl-1 were reduced in cells treated with the combination of TGF-β1 and STI-571 compared to their levels in the other groups (Fig. 4a,c). Cleavage of the PARP protein is a hallmark of apoptosis. Therefore, levels of cleaved PARP, which is involved in programmed cell death and DNA repair, were evaluated. The results showed that more PARP cleavage was seen in combination-treated cells compared with the TGF-β1 group. However, TGF-β1 induced PARP cleavage significantly in the combination therapy group compared with the control (Fig. 4b,c).

Discussion

Combination therapy represents a potential solution for cancer therapy; it is beneficial, because two or more drugs can overcome the problem of MDR. In combination therapy, two or more drugs that act in different ways and affect distinct cytotoxic mechanisms are chosen. CML involves upregulation of the tyrosine kinase, BCR-ABL, which inhibits drug-induced apoptosis; it is considered one of the most critical cancer mechanisms [27]. Thus, complete destruction of BCR-ABL-expressing leukemia cells may be achieved by a combination of BCR-ABL inhibitors, such as the TKI STI-571 mesylate, with other agents that affect cell survival pathways. Since the pleiotropic molecule TGF-β1 is suppressed by BCR-ABL through a special cell transformation mechanism [19, 20], the present investigation studied the effects of the combination of STI-571 and TGF-β1 on K562 cells. Although cell cycle results revealed that STI-571 induces a higher level of Sub-G1 arrest and leads to the increase and reduction, respectively, of p27 and p-AKT, combination therapy was shown to lead to a marked increase in the number of apoptotic cells and a reduction in the signaling biomolecules p-AKT, p-FOXO3, and p-STAT5, subsequently causing an increase in pro-apoptotic proteins and PARP cleavage and a decrease in the anti-apoptotic proteins bcl-xL and Mcl-1.

One of the mechanisms of cancer therapy is arrest of the cell cycle [28]; p27 plays a critical role in cell cycle arrest through its involvement with cyclin-dependent kinases. Furthermore, oncogenes that are activated during cancer induce p27 proteolysis, inhibiting cell cycle arrest. In fact, stable p27 helps maintain the G0 phase in cells [29, 30]. Based on these facts, an increase in p27 would be consistent with G1 and S phase arrest, as corroborated in the present study. Combination therapy enhanced the G1 arrest induced by STI-571 by increasing p27 and reducing p-FOXO3, ultimately leading to down-regulation of cyclin D1 and D2 and the Stat5/Bcl-6 pathway [31]. Kumatsu et al., [32] demonstrated that STI-571 inhibits phosphorylation of FOXO3, leading to p27 enhancement, cyclin D1 and D2 downregulation, and ultimately, cell cycle arrest. Interestingly, there was no notable difference
Figure 4: a, b) Protein expression of the anti-apoptotic proteins Mcl-1 and bcl-xL and the pro-apoptotic proteins p27 and cleaved PARP in K562 cells treated with 2 ng/mL of TGF-β1 and 0.5 µM STI-571, individually and in combination, for 48 h. The results for all groups were significantly different. c) Data is represented as mean ± SD, and quantitative expression of proteins was normalized to the level of β-actin. The results showed significant differences between treated groups and other groups (P < 0.05).
in the sub-G1 phase between the TGF-β1-treated group and the control group; this is consistent with the expression pattern of p27. Since p27Kip1 over-expression reduces the proliferation rate of BCR-ABL-expressing cells, the suppressive effect of the TKI, STI-571, on BCR-ABL increases p27Kip1 levels and induces apoptosis [33]. p27 downregulation in response to TGF-β1 is associated with inhibition of Cdk2 [19]. Sub-G1- and Annexin-positive cells serve as markers of cell death and apoptotic cells, respectively, and both fractions were significantly higher in the combination therapy group than in cells treated with STI-571 or TGF-β1 individually, presumably due to the modulation of pro- and anti-apoptotic proteins. Although the impact of TGF-β1 in combination therapy on mitosis and inhibition of cell proliferation was not greater than that of STI-571, combination therapy can be proposed to induce cell death by triggering cell signaling cascades leading to apoptosis. However, its weak inhibitory effect on proliferation remains obscure. At low concentrations of STI-571 (0.25 µg/mL) in combination therapy, this covert effect became apparent, and the number of apoptotic cells was greater than when 0.5 µg/mL of STI-571 was used individually.

It has been demonstrated that BCR-ABL eventually leads to apoptosis resistance in leukemia cells through activation of the Stat5, PI3K/AKT, and Ras pathways [15, 34]. To further study the apoptosis signaling cascade in combination therapy, other biomolecules, including AKT, Stat5, FOXO3, and their phosphorylated states, were investigated. Interestingly, STI-571 and its combination with TGF-β1 at 0.125, 0.25, and 0.5 µg/mL concentrations led to a greater number of apoptotic cells and a lower number of necrotic cells compared to the control and TGF-β1 groups. This effect is due to the triggering of apoptosis signaling pathways by a reduction in the phosphorylated states of AKT and Stat5 and an increase in pro-apoptotic proteins, p27 and PARP cleavage. The results show that, although the combination therapy induced a decrease in p-Stat5 and p-FOXO3, unlike when STI-571 or TGF-β1 were used individually, TGF-β1 increased p-Stat5 and p-FOXO3 to levels similar to the control group. Remarkably, it seems that TGF-β1 does not directly affect cell death pathways, but functions through other signaling pathways via the activation of AKT and its phosphorylated state and the modulation of pro-apoptotic proteins, PARP cleavage, and the anti-apoptotic proteins Mcl-1 and bcl-xL. Komatsu et al. demonstrated that activated AKT directly
phosphorylates FOXO3 [32]. Although this finding is consistent with the effects of TGF-β1 and STI-571, the level of p-FOXO3 in the combination therapy group was related to the decrease in p-AKT. It seems that other signaling pathways are triggered to inhibit phosphorylation of FOXO3 and translocate it from the cytoplasm into the nucleus to function as a transcription factor. Naka et al. [25] reported that administration of FOXO3a- in combination with STI-571 increases survival in CML mice, unlike administration of FOXO3a+. FOXO3a negatively regulates the survival of non-leukemia initiating cells [18] and LICs. Komatsu et al. reported that, although STI-571 inhibits phosphorylation of FOXO3 (p-FOXO3 is an inactive form of FOXO3), it does not change FOXO3 expression [32]. This finding is consistent with the effects of combination therapy and STI-571, in that FOXO3 enhancement did not occur in parallel with FOXO3 phosphorylation. However, it seems that the level of FOXO3 influences the level of its phosphorylation in TGF-β1 therapy. It has been demonstrated that dephosphorylated FOXO3, which functions downstream of the BCR-ABL signaling pathway, positively regulates p27 expression [32]. In the present investigation, it was shown that p-FOXO3 regulates p27 in the context of TGF-β1 and STI-571 treatment. To follow up on these results, the kinase AKT, which is present upstream of the p-FOXO pathway and is directly involved in FOXO3 phosphorylation, was selected for further investigation. The results showed that the direct involvement of PI3K with the TGF-β1 intracellular signaling receptor is correlated with rapid TGF-β1-mediated AKT phosphorylation, and TGF-β1 decreased the level of AKT phosphorylation. Furthermore, treatment of K562 cells with the TKI, STI-571, suppressed the ability of BCR-ABL to stimulate upregulation of p-AKT and p-FOXO3; some other studies have confirmed this finding [1, 23]. An interesting observation related to the combination therapy with TGF-β1 and STI-571 is that the reduction in phosphorylated AKT levels under these conditions was higher than in the case of STI-571 therapy alone, in which either lesser phosphorylation or greater dephosphorylation takes place. This is a valuable piece of information and can be further confirmed by blocking the phosphorylated sites in AKT.

Another survival signaling pathway that influences cancer therapy is the Stat5 pathway. In vitro studies indicated that tyrosine phosphorylation and Stat5 dimerization through JAK2 are stimulated by BCR-ABL. This allows Stat5 dimers to translocate the nucleus, where they promote expression of pro-survival genes. A reduction in Stat5 levels is associated with increased TKI-mediated killing [35]. The present investigation revealed that high levels of phosphorylated Stat5 in control and TGF-β1-treated groups were associated with the absence of TKI; in other words, TGF-β1 has the potential to partially support cell proliferation by increasing p-Stat5, increasing the inactive phosphorylated state of FOXO3. However, when combined with STI-571, TGF-β1 changed its behavior and converted to a cell survival suppressor protein, functioning to increase cell mortality and arrest cells in the sub-G1 phase by reduction of p-Stat5, p-AKT, and p-FOXO3.

In addition, expression of bcl-xL is stimulated by BCR-ABL through constitutive activation of Stat5, a transcription factor that binds to the Stat5-binding element in the bcl-xL promoter [33]. It also promotes expression of the Bcl-2 family member Mcl-1 in leukemia cells. Mcl-1 is known to be an upstream regulator that controls both anti- and pro-apoptotic proteins [9, 24]. The target genes of Stat5, such as Bcl-2 and bcl-xL, form a cytotoxic and apoptotic barrier. The TKI, STI-571, decreased expression of Mcl-1 and bcl-xL [8, 14], as seen in the current study; when it was combined with TGF-β1, levels of anti-apoptotic proteins, such as Mcl-1 and bcl-xL, were further reduced, concomitant with a significant reduction in p-Stat5.

In conclusion, the arrested TGF-β1 cell signaling pathway, which can be activated in BCR-ABL-expressing cells with TGF-β1, can be proposed to activate a variety of cell signaling pathways involved in mortality and survival. On one hand, survival pathways were triggered, associated with increased p-Stat5 and p-FOXO. On the other hand, apoptotic pathways were activated via decreased p-AKT and bcl-xL and increased PARP cleavage. Hence, the final result of this phenomenon remains unclear. In cells treated with the TKI, STI-571, all studied apoptotic pathways were triggered, causing a reduction in the levels of proteins involved in cell survival, such as p-AKT, p-Stat5, p-FOXO3, Mcl-1, bcl-xL, and Bcl-2, as well as the enhancement of pro-apoptotic proteins, PARP cleavage, and p27, ultimately leading to an increase in cells in the sub-G1 phase of the cell cycle and an increase in the number of Annexin-positive cells in a leukemia model.

Conclusion

Remarkably, when STI-571 and TGF-β1 are used in combination, TGF-β1 behavior is altered. Apoptosis-signaling pathways are triggered by the action of STI-571, leading to reductions in p-AKT, p-Stat5, p-FOXO3, Mcl-1, and bcl-xL, as well as to increased PARP cleavage, ultimately resulting in an increase in the number of Annexin-positive leukemia cells. Overall, combination
therapy using STI-571 and TGF-β1 could serve as a promising method for overcoming the MDR effect, which is the result of the BCR-ABL protein.

Statistical Analysis

Calculation and percentage analysis of Annexin and PI positive cells, Sub-G1 cells, protein levels, and relative fold changes in gene expression for treated cells was performed using Graphpad software. Experiments were performed as mean ±SD. One way ANOVA with multiple comparisons and statistical analysis was performed using the Tukey post test. Statistical significance was determined by a P value less than 0.05.

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Conflict of interest: Authors state no conflict of interest.

Data Availability Statement: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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