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Pro-Apoptotic Activity of Ruxolitinib Alone and in Combination with Hydroxyurea, Busulphan, and PI3K/mTOR Inhibitors in JAK2-Positive Human Cell Lines

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article; G – other

Abstract

Background. The JAK2V617F mutation plays a crucial role in the pathogenesis of myeloproliferative neoplasms (MPNs). Inhibition of JAK2 activity by ruxolitinib (RX) results in growth inhibition and apoptosis of cells carrying the JAK2V617F mutation however the exact mechanisms regulating apoptosis have not been fully elucidated.

Objectives. This study assessed the potential cytotoxicity of RX against JAK2-positive human cell lines (SET-2 and HEL), either alone or in combination with hydroxyurea, busulphan, rapamycin or LY294002.

Material and Methods. Cell viability, the apoptosis rate (annexin-V staining), drop of mitochondrial transmembrane potential ($\Delta\psi_m$) and caspase activation, were measured using flow cytometry. Additionally, the expression of several apoptosis-regulating proteins was evaluated.

Results. RX showed cytotoxicity against both SET-2 and HEL cell lines. The main mechanism of this action was apoptosis, with significant drop of $\Delta\psi_m$, caspase-3 and -9 activation, and moderate activation of caspase-8 (only for SET-2 cells). Corresponding to enhanced apoptosis, the expression levels of some apoptosis-regulating proteins were changed, the most pronounced in both cell lines being up-regulation of Bax and down-regulation of Bcl-2 proteins. Additionally, up-regulation of Bak and Bad (SET-2) and down-regulation of Mcl-1 (HEL) were observed. Of the studied compounds, a combination of RX + LY294002 induced the greatest cytotoxicity in both SET-2 and HEL cell lines, and rapamycin the least.

Conclusions. This study shows that the combination of RX and a PI3K kinase inhibitor provokes a significant pro-apoptotic effect in JAK2V617F mutated cells, which may justify the beginning of clinical trials based on the combination of these drugs (Adv Clin Exp Med 2015, 24, 2, 195–202).

Key words: ruxolitinib, hydroxyurea, busulphan, rapamycin, LY294002.

The discovery of the JAK2 V617F mutation gave hope for the targeted therapy in myeloproliferative neoplasms (MPNs) [1]. Indeed, in recent years several JAK2 inhibitors have been introduced into clinical trials [2]. Ruxolitinib (RX,

Jakavi®, INCB18424) was the first drug approved for the therapy of patients with myelofibrosis in Europe [3–5]. The former preclinical experiments revealed that proliferation of Ba/F3-EpoR-JAK2V617F cells and JAK2 mutant HEL cells is

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inhibited by INCB18424 in a mechanism of increased apoptosis [6]. In contrast, such effect was not observed in relation to cell lines, characterized by c-KIT mutations or transformed with the Bcr-Abl oncoprotein [6]. Although, up-regulation of the pro-apoptotic protein Bim and down-regulation of the anti-apoptotic Bcl-xL was observed, the exact mechanism of apoptosis related to JAK2 inhibitors remain to be elucidated [7-9].

Gradually, we are starting to realize that the pathogenesis of MPNs is very complex. For example, beside the abnormal JAK/STAT pathway, constitutive activation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway was discovered [10, 11]. Recently, it has been shown in preclinical studies that a combination of everolimus (rapamycin analogue) and RX demonstrated strong synergism in inhibiting different MPNs cells [12, 13]. Moreover, everolimus was efficient (phase I/II trial) in reducing splenomegaly and systemic symptoms in patients with myelofibrosis. The results of these trials indicate that the PI3K/Akt/mTOR pathway might be another interesting target for MPNs therapy [14].

The aim of this study was to determine the cytotoxicity, if present, of RX when used as a single agent or in combination with other cytotoxic drugs such as hydroxyurea (HU), busulphan (BS), rapamycin (RAPA) and LY294002 (PI3K inhibitor) against JAK2-positive human cell lines (SET-2, HEL). We also studied the mechanisms of apoptosis regulation caused by RX.

Material and Methods

Cell Lines

The following cell lines were tested: erythroleukemia-derived HEL cells and essential thrombocythemia-derived SET-2 cells (both lines carrying the *JAK2 V617F* mutation) (DSMZ, Braunschweig, Germany). The cells were treated with RX (Novartis Pharma AG, Switzerland) alone and in combination with other anticancer drugs. After the tests assessing increasing RX concentrations (1 nM – 500 nM), the lowest drug concentration inducing significant cytotoxicity (5 nM/mL) compared to the untreated samples was chosen for further experiments. Combinations of RX with HU (Sigma-Aldrich Chemie GmbH, Germany), RAPA (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany), LY-294002 (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) or BS (Sigma-Aldrich Chemie GmbH, Germany) were also checked for cytotoxicity. Naive parallel cultures

were used as a control. The optimal concentrations of compounds used in this study were as follows: 10 μ M of HU, 81.2 μ N of BS, 50 nM of RAPA, and 10 μ M of LY-294002.

Assessment of Drug Cytotoxicity

Cytotoxicity was assessed based on propidium iodide (PI; Sigma Aldrich, Germany) staining. The difference between the percentage of dead cells after incubation with drugs and that seen in the control culture (cytotoxic index, CI) was calculated.

Assessment of Apoptosis Its Mechanisms and Cell Cycle Analysis

The rate of apoptosis was determined by annexin-V (Ann-V) assay after 48 h incubation with the studied drugs. Subsequently, the apoptotic index (AI) was assessed as a percentage of Ann-V positive cells. The mechanisms of apoptosis were judged by a drop of mitochondrial transmembrane potential ($\Delta\psi_m$), activation of caspases (3, 8, 9) and expression of proteins involved in apoptosis regulation (Bax, Bad, Bid, Bcl-2, Mcl-1, XIAP, survivin, p53). The apoptotic, hypodiploid sub-G₁ fraction was evaluated on the basis of the DNA histogram. In addition, the cell cycle phase specificity of the apoptosis caused by the study compounds was assessed by the same settings. The percentage of cells in sub-G₁, G₀/G₁ and S/G₂M phases of the cell cycles was determined in relation to a DNA histogram analysis.

A detailed methodology of $\Delta\psi_m$ assessment, detection of active caspase-3, -8 and -9, expression of apoptosis-regulating proteins and cell cycle analysis was described in our previous publication [15].

Fluorescence Measurements

All tests were done on flow cytometry (FAC-Scan; Becton Dickinson, San Jose, CA, USA) and 10,000 cells per sample were measured in all tests.

Statistics

Statistical software (STATISTICA version 10.0, Tulsa, OK, USA) was used to determine the range, mean, median and standard deviation (SD) of the measured variables. The differences between values were evaluated with the Student's *T*-test. *P* values less than 0.05 were considered statistically significant.

Results

Cytotoxic Effects of RX on SET-2 Cell Line

After 48 h of treatment with 5 nM of RX, the mean CI was 18.7% (vs control $p = 0.002$). Mean cytotoxicity induced by BS was 18.0% (vs control $p = 0.004$). HU and LY294002 induced the following mean CIs: 22.3% ($p < 0.001$), and 13.95% (vs control $p = 0.011$), respectively. RAPA had the lowest impact on the CI of SET cells (ns. vs control) (Fig. 1).

A statistically significant increase in cytotoxic effect was observed in combinations of RX with HU, BS, RAPA and LY294002 (RX + HU – mean CI 19.7%; vs control $p = 0.03$, RX + BS 21.3%; vs control $p < 0.001$, RX + RAPA 19.7%; vs control $p = 0.002$ and RX + LY294002 54.1%; vs control $p < 0.001$). The combinations RX + BS, and RX

+ LY294002 exerted a statistically significant increase in cytotoxicity compared to the single drugs (for both combinations $p < 0.001$). RX + HU and RX + RAPA did not significantly increase CIs compared to the drugs used alone (Fig. 1).

Cytotoxic Effects of RX on HEL Cell Line

After 48 h of treatment with 5 nM/mL of RX the mean CI was 12.2% (vs control $p = 0.010$). Mean cytotoxicity induced by BS was 14.7%; vs control $p = 0.009$. HU and LY294002 induced the following mean CIs: 15.3% ($p < 0.007$), and 12.6% (vs control $p = 0.011$), respectively. RAPA had no significant impact on the CI of HEL cells (vs control ns.) (Fig. 2).

A statistically significant increase in cytotoxic effect was observed in combinations of RX with HU, BS, RAPA and LY294002 (RX + HU – mean CI 14.1%; vs. control $p = 0.008$, RX + BS 28.3%;

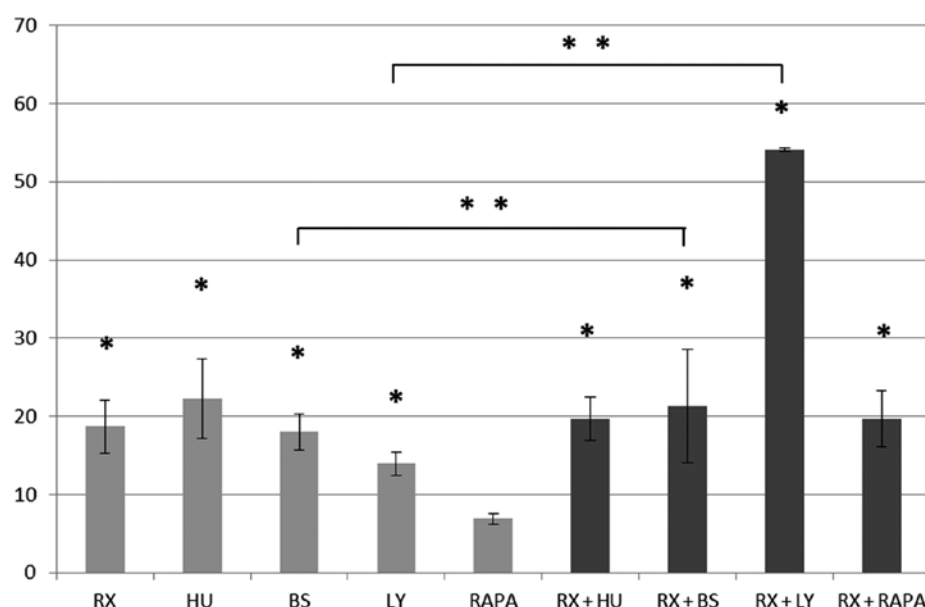


Fig. 1. Cytotoxicity of ruxolitinib (RX) alone and in combination with hydroxyurea (HU), busulphan (BS), rapamycin (RAPA) and LY294002 as measured by propidium iodide (PI) assay against SET-2 cells, after 48 h of incubation. Means and standard deviations are shown

* statistically significant differences vs control

** statistically significant differences between combinations of drugs and drugs used as a single agent

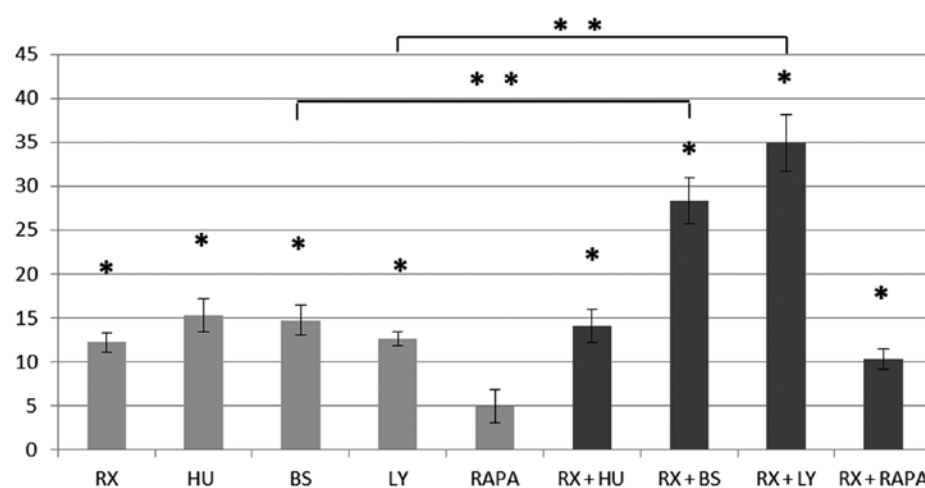


Fig. 2. Cytotoxicity of ruxolitinib (RX) alone and in combination with hydroxyurea (HU), busulphan (BS), rapamycin (RAPA) and LY294002 as measured by propidium iodide (PI) assay against HEL cells, after 48 h of incubation. Means and standard deviations are shown

* statistically significant differences vs control

** statistically significant differences between combinations of drugs and drugs used as a single agent

vs control $p < 0.001$, and RX + LY294002 34.9%; vs control $p < 0.001$). Also, RX + RAPA exerted moderately significant cytotoxicity in comparison to untreated controls (10.3%; vs control $p = 0.032$). The combinations RX + BS, and RX + LY294002 exerted a statistically significant increase in cytotoxicity compared to single drugs ($p < 0.021$ and $p < 0.010$, respectively). According to RX + HU and RX + RAPA, those combinations did not statistically increase CIs compared to the drugs used alone (Fig. 2).

Proapoptotic Effects of RX on SET-2 and HEL Cell Lines

Ann-V/PI Assay

Ann-V/PI staining showed a pro-apoptotic activity of RX against both SET-2 and HEL cells. After 48 h of treatment, the mean AIs were 19.5% for SET-2 and 14.3% for HEL cell lines (vs control $p = 0.012$ and $p = 0.021$, respectively) (Fig. 3).

Drop of Mitochondrial Potential ($\Delta\psi_m$)

The drop of $\Delta\psi_m$ after 48 h of incubation with RX was distinctly higher than in untreated cultures. The mean percentage of both SET-2 and HEL cells with a drop of $\Delta\psi_m$ was 20.2% and 12.3%, respectively (vs control, $p = 0.003$ and $p = 0.015$, respectively) (Fig. 3).

Drug-Induced Caspase Activation

Forty-eight hours of incubation with RX significantly elevated caspase-3, -8 and -9 activation in comparison with untreated control samples. Namely, the

mean percentages of SET-2 and HEL cells showing caspase-3 activation were 16.3% and 12.5%, respectively (vs control, $p = 0.03$ and $p = 0.031$, respectively), caspase-8 activation 13.9% and 8.4%, respectively (vs control $p = 0.041$ and ns., respectively) and caspase-9 activation 18.5% and 13.2%, respectively (vs control, $p = 0.002$ and $p = 0.008$, respectively) (Fig. 3).

Expression of Apoptosis-Regulating Proteins

The expression of apoptosis-regulating protein inducing programmed cell death (Bax, Bak, Bid, Bad, p53), as well as chosen apoptosis inhibitors (Bcl-2, Mcl-1, XIAP, survivin), were tested in response to RX exposure of SET-2 and HEL cell lines. In SET-2 cells, RX was associated with a significant influence on increase in Bax, Bad and Bad pro-apoptotic proteins, and decrease in the Bcl-2 anti-apoptotic protein. Regarding the HEL cell line, RX stimulated a statistically significant increase in Bax expression and down-regulation of Bcl-2 and Mcl-2 anti-apoptotic proteins (Table 1).

Cell Cycle Analysis

After 48 h of incubation with RX, a significant pro-apoptotic effect was observed as measured by the rate of sub- G_1 fractions, as well as distinct changes in other particular fractions of the cell cycle. Interestingly, after 48 h of incubation of the SET-2 cell line with RX, significant inhibition was also observed of the G_0/G_1 fraction cells (Fig. 4).

Discussion

Since the *JAK2V617F* mutation probably does not represent the driver mutation in MPNs, it seems unlikely that eradication of the pathogenic

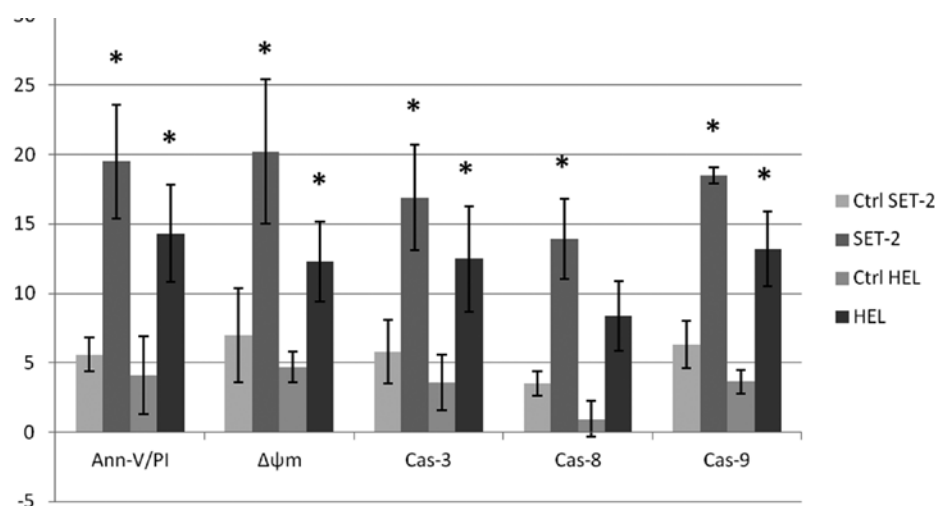


Fig. 3. The proapoptotic effect of ruxolitinib (RX) as measured by annexin-V/propidium iodide (Ann-V/PI) assay, the drop of mitochondrial potential ($\Delta\psi_m$), activation of caspases-3, -8 and -9 in either SET-2 or HEL cells, after 48 h of incubation with the drug. Means and standard deviations are shown

* statistically significant differences vs control.

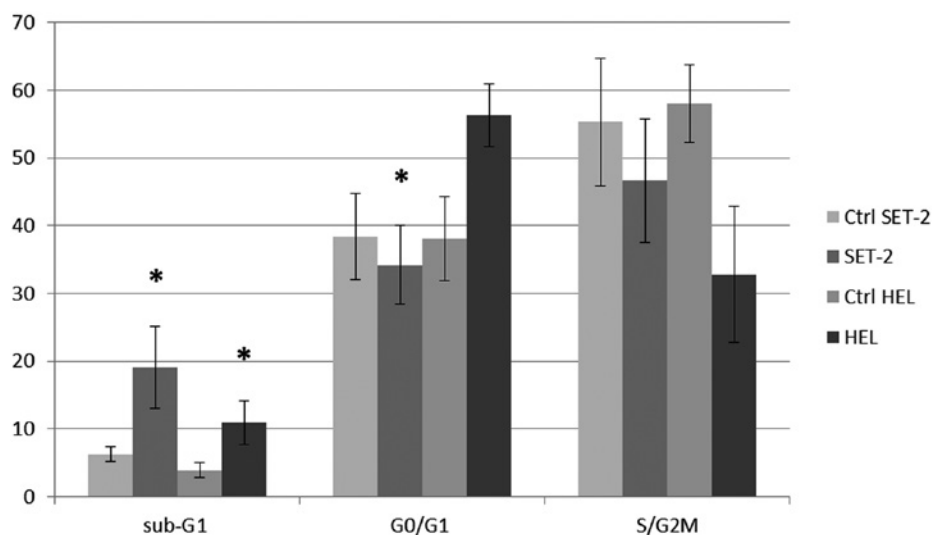


Fig. 4. The effect of ruxolitinib (RX) on the cell cycle of SET-2 and HEL cells after 48 h of incubation with the drug. Bars represent the means (\pm standard deviations) estimated by the rate of cells in the following cell cycle fractions: sub-G1, G0/G1, and S/G2M
* statistically significant differences vs control

Table 1. Expression of apoptosis-regulating proteins measured as mean fluorescence intensity (MFI) by flow cytometry after 48 h of incubation of SET-2 and HEL cells with ruxolitinib (RX)

Protein	SET-2 cell line		HEL cell line	
	N-fold increase/decrease* in median MFI**	statistics	N-fold increase/decrease* in median MFI**	statistics
Bax	1.73	p = 0.012	1.46	p = 0.023
Bak	1.53	p = 0.042	1.05	ns.
Bid	1.1	ns.	1.16	ns.
Bad	2.16	p = 0.007	1.21	ns.
p53	1.34	ns.	1.12	ns.
Bcl-2	0.36*	p = 0.017	0.37*	p = 0.003
Mcl-1	0.69	ns.	0.73*	p = 0.035
XIAP	1.0	ns.	1.13	ns.
Survivin	0.73	ns.	0.81	ns.

** N-fold – proportion of protein expression (MFI) in examined sample vs untreated control, ns. – not significant.

clone can be 100% achieved with the currently available JAK2 inhibitor RX [16]. Hence, other compounds like panobinostat (histone deacetylase inhibitor), 5-azacytidine (methyltransferase inhibitor), lenalidomide, pomalidomide (thalidomide analogs), everolimus and BMK120 (PI3K/mTOR inhibitors) are being studied in combination with RX in phase 1/2 trials in patients with myelofibrosis [17].

This study is the first to address the cytotoxicity of RX in combination with HU, BS and PI3K/Akt/mTOR pathway inhibitor (LY294002) on cell lines carrying the *JAK2V617F* mutation. Until now, there has been strong evidence supporting the involvement of the PI3K/Akt/mTOR pathway inhibitor in MPNs pathogenesis. Constitutive activation of Akt and mTOR kinases has been observed

in *JAK2V617F* positive cell lines and megakaryocytes of MPNs patients [1, 18]. These findings led to the development of different Akt, PI3K or dual mTORC1/mTORC2 complex inhibitors.

The results of the study indicate that mutated cell lines are sensitive to the studied drugs, except RAPA. Thus, our data showed that LY294002 is a more potent inducer of apoptosis of SET2 and HEL cells than RAPA, which may be related to differences in the inhibition target (Akt1 vs mTORC1), as similar differences were observed on *JAK2V617F* mutated human and murine leukemia cell lines between the ATP mimetic inhibitor PP242 (inhibitor of both mTORC complexes) and the allosteric inhibitor RAD001 (everolimus, inhibitor of mTORC1) [12, 19]. The authors conclude that inhibition of *JAK2V617F* positive cells

by RAD001 was more likely due to a cytostatic than an apoptotic effect and suggested that inhibition of both TORC1 and TORC2 complexes is essential for induction of apoptosis.

Moreover, our findings show that, in contrast to a RX + RAPA combination, co-treatment of RX with LY294002 results in significant anti-proliferative activity against the SET2 and HEL cell lines. This is in contrast to results by Bogani et al. [12] which show that, like JAK2 inhibitors, RAD001 exerts an anti-proliferative effect on *JAK2V617F* positive cells. Overall, these results justify the development of clinical trials with RX in combination with PI3K or catalytic mTORC1/mTORC2 inhibitors. As a matter of fact, RAD001, has been already used in a phase 1/2 clinical trial in patients with myelofibrosis, resulting in a significant reduction of spleen volume in 43% of patients and constitutional symptoms in 80% [14]. The path of improvement was different than that taken by RX mechanisms since it was not connected to the normalization in concentrations of different inflammatory cytokines.

The results of this study confirm previous reports suggesting that RX has a cytotoxic effect on cells carrying the *JAK2V617F* mutation. Quintas-Cardama et al. [6] showed that treatment with INCB018424 (the previous name of RX) markedly increased apoptosis of Ba/F3-EpoR-*JAK2V617F* cells, as assessed by Ann-V staining, and by a drop of $\Delta\psi_m$, suggesting the involvement of the intrinsic apoptotic pathway. A similar effect was observed in the HEL cell line [6].

The present study also demonstrated a down-regulation of Bcl-2 protein in both cell lines consistent with the previous reports [20–22]. The Bcl-2 family proteins contain proapoptotic Bax and Bak-like proteins, the antiapoptotic Bcl-2 proteins and the BH3-only proteins. Bim, as important member of the BH3-only protein group, initiates apoptosis by binding and neutralizing prosurvival Bcl-2 proteins [23]. Will et al. [7] suggest that Bim is a key effector protein in JAK2 inhibition-related apoptosis since knockdown of Bim was found to dramatically decrease the apoptosis of both HEL and SET-2 cells. It was also demonstrated that the addition of ABT-737 (BH3 mimetic) enhanced the apoptosis induced by JAK2 inhibition in HEL and SET-2 cells. Interestingly, Bim can be also induced through the PI3K-Akt pathway [24].

The synergistic effect of RX + LY294002 on the apoptosis of HEL and SET-2 cells observed in our study may be connected with this mechanism. Also, Rubert et al. [8] suggest that Bim activation plays a key role in regulating the survival of SET-2 cells triggered by JAK2 inhibitor. They found that Bim activation following JAK2 inhibition led to enhanced sequestration of Bcl-xL and Mcl-1 anti-apoptotic proteins. Although we did not assess Bim expression, we observed Mcl-1 down-regulation in HEL cells after RX treatment. We also found up-regulation of proapoptotic Bax protein in both HEL and SET-2 cells. In our previous study, we found that patients with essential thrombocythemia positive for the *JAK2V617F* mutation had markedly higher Bax expression than *JAK2V617F* negative cases [25]. As reported before, Mcl-1 protein has a crucial role in regulating the survival of hematopoietic stem cells [26].

In another study, Gozgit et al. [20] assessed the effect of AZ960 (a JAK2 inhibitor) on Pim/Bad/Bcl-xL survival signaling in the SET-2 cell line. The authors conclude that JAK2 inhibition induces apoptosis by direct and indirect regulation of the antiapoptotic protein Bcl-xL. They observed a decrease in Pim1 protein levels and inhibition of Bad phosphorylation at serine 112, which promotes the apoptotic process [27–29].

To the best of our knowledge, till now there were no reports on the influence of RX on the cell cycle phase of *V617F* mutation-positive cells. In our study, we observed not only a significant proapoptotic effect, as measured by the rate of sub-G₁ fractions, but also distinct changes in other particular fractions of the cell cycle. Interestingly, after 48 h of incubation of the SET-2 cell line with RX, we observed the significant inhibition in G₀/G₁ fraction cells.

In conclusion, this study shows that a combination of RX with PI3K kinase inhibitor LY294002 provokes a proapoptotic effect in *JAK2V617F* mutated cell lines. This may justify commencement of trials exploiting the combination of RX with compounds containing PI3K or mTOR. Our findings demonstrate that the apoptosis of SET-2 and HEL cells induced by RX therapy is executed mainly by caspase-9, which implies that apoptosis triggered by RX involves mainly the intrinsic pathway of caspase activation.

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