LS104, a non-ATP-competitive small-molecule inhibitor of JAK2, is potently inducing apoptosis in *JAK2V617F*-positive cells

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Abstract

The activating *JAK2V617F* mutation has been described in the majority of patients with *BCR-ABL*-negative myeloproliferative disorders (MPD). In this report, we characterize the small-molecule LS104 as a novel non-ATP-competitive JAK2 inhibitor: Treatment of *JAK2V617F*-positive cells with LS104 resulted in dose-dependent induction of apoptosis and inhibition of JAK2 autophosphorylation and of downstream targets. Activation of these targets by JAK2 was confirmed in experiments using small interfering RNA. LS104 inhibited JAK2 kinase activity *in vitro*. This effect was not reversible using elevated ATP concentrations, whereas variation of the kinase substrate peptide led to modulation of the IC_{50} value for LS104. In line with these data, combination treatment using LS104 plus an ATP-competitive JAK2 inhibitor (JAK inhibitor I) led to synergistically increased apoptosis in *JAK2V617F*-positive cells. Furthermore, LS104 strongly inhibited cytokine-independent growth of endogenous erythroid colonies isolated from patients with *JAK2V617F*-positive MPD *in vitro*, whereas there was no significant effect on growth of myeloid colonies obtained from normal controls. Based on these data, we have recently started a phase I clinical trial of LS104 for patients with *JAK2V617F*-positive MPDs. To the best of our knowledge, this is the first report on a non-ATP-competitive kinase inhibitor being tested in a clinical trial. [Mol Cancer Ther 2008;7(5):1176–84]

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Introduction

The *JAK2V617F* mutation has recently been discovered as a common molecular marker in Philadelphia chromosome–negative myeloproliferative disorders (MPD) (1–5). The incidence of the *JAK2V617F* mutation is ~50% in both essential thrombocythemia and primary myelofibrosis and >90% in polycythemia vera (6, 7).

In vitro, the *JAK2V617F* mutation confers constitutive activation of JAK2 and the JAK-STAT pathway (2). Ba/F3 cells coexpressing a cytokine type I receptor like erythropoietin receptor (EpoR) or thrombopoietin receptor, together with mutant JAK2, consistently grow cytokine independently on growth factor withdrawal (4, 8). These features closely resemble observations made in primary patient material: cytokine hypersensitivity, constitutive activation of the JAK-STAT pathway, and cytokine-independent growth of the so-called endogenous erythroid colonies (EEC) in the majority of patients with MPDs (9). Nevertheless, many questions remain unanswered, such as how a single mutation leads to phenotypically distinct diseases like polycythemia vera, essential thrombocythemia, and primary myelofibrosis. Recent reports on murine bone marrow transplant models suggest that the *JAK2V617F* mutation alone is sufficient to induce a polycythemia vera–like phenotype in mice (10–12).

Identification of specific JAK2 inhibitors appears as an important step toward development of targeted therapy for MPDs.

LS104 [(*E*,*E*)-2-(benzylaminocarbonyl)-3-(3,4-dihydroxystyryl)acrylonitrite] is a novel kinase inhibitor that inhibits growth of leukemia cell lines and primary cells from leukemia patients *in vitro*. By *in vitro* kinase assays, LS104 has been shown to inhibit oncogenic kinases, such as Bcr-Abl and JAK2, whereas widely expressed Src family kinases appeared to be unaffected by LS104 (for details, see ref. 13). In this report, we characterize LS104 as a kinase inhibitor effectively targeting the JAK2V617F mutant. Furthermore, we provide data that LS104 has a distinct mode of action, which is ATP independent and substrate competitive. To our knowledge, this is the first report on a non-ATP-competitive JAK2 kinase inhibitor.

Materials and Methods

Cell Lines

Murine hematopoietic Ba/F3 cells expressing transgenic murine EpoR alone (Ba/F3-EpoR) or transgenic murine EpoR plus either transgenic murine JAK2V617F (Ba/F3-EpoR-VF) or transgenic murine wild-type JAK2

(Ba/F3-EpoR-WT) were maintained as described earlier (8). Human hematopoietic HEL and K562 cells were obtained from the German Collection of Microorganisms and Cell Cultures and maintained in RPMI 1640 containing 10% heat-inactivated FCS.

Inhibitors

LS104 was provided by LymphoSign (East Tower Markham) as a nanoparticle formulation. Stock and working solutions were prepared in distilled water and stored at 4°C. JAK inhibitor I was obtained from Calbiochem (Merck Biosciences). Stock solutions were prepared in DMSO and stored at 4°C. Working solutions have been prepared freshly for each experiment.

DNA Content Analysis

The percentage of cells featuring a sub-G_1 DNA peak corresponding to the apoptotic cell fraction was analyzed as described previously (14). Briefly, cells were incubated for 48 h in the presence of increasing doses of LS104 with or without interleukin-3 (IL-3) as indicated. Cells were washed with PBS and then resuspended in 300 μL HFS buffer, incubated at 4°C in the dark for 30 min, and then subjected to fluorescence-activated cell sorting analysis.

Annexin V Assay

Ba/F3-EpoR-VF cells were incubated for 48 h in the presence of increasing doses of LS104 in medium without IL-3. Cells were washed twice with cold PBS and then resuspended in binding buffer. FITC-labeled Annexin V and 7-AAD were added and incubated for 15 min at room temperature in the dark and subsequently subjected to fluorescence-activated cell sorting analysis.

Cell Treatment, Protein Extract Preparation, Immunoprecipitation, and Immunoblotting

Cells (2×10^6) were incubated in the presence of different concentrations of LS104 or JAK inhibitor I at 37°C in RPMI 1640. Preparation of cellular lysates, SDS-PAGE, and blotting onto nitrocellulose membranes (Amersham) were done as described previously (15). The following antibodies were used: anti-phospho-JAK2 (p-Tyr^{1007}/Tyr^{1008}), anti-JAK2, anti-phospho-STAT5 (p-Tyr^{694}/Tyr^{699}), anti-phospho-STAT3 (p-Tyr^{705}), anti-STAT3, anti-phospho-AKT (p-Ser^{473}), anti-AKT, anti-phospho–extracellular signal-regulated kinase (ERK; p-Thr^{202}/Tyr^{204}), and anti-ERK (all from Cell Signaling Technology); anti-STAT5α (Santa Cruz Biotechnology); anti-β-actin (ICN); and anti–glyceraldehyde-3-phosphate dehydrogenase (Biodesign International). For immunoprecipitation of JAK2, 5×10^6 cells were incubated and processed as described above. Protein extracts were then incubated with JAK2 antibody overnight at 4°C. Antibody complexes were precipitated by incubation with A/G agarose beads for 1 h at 4°C. Pellets were washed three times with 1:10 lysis buffer, subsequently resuspended in Laemmli buffer, and subjected to SDS-PAGE.

JAK2 Small Interfering RNA Experiments

Small interfering RNA (siRNA) specific for murine *JAK2* was designed using the Invitrogen online tool.[4] AllStars Negative Control siRNA labeled with Alexa Fluor 488 (Qiagen) was used as negative control. siRNA transfection was done according to a standard electroporation protocol. Transfection efficiency was 60% to 70% using Alexa Fluor 488–labeled control siRNA (data not shown).

Isolation of Mononuclear Cells and Granulocytes

Samples of peripheral blood or bone marrow blood were obtained from six patients with *JAK2V617F*-positive MPD and four normal controls after written informed consent has been given. Mononuclear cells were isolated immediately by means of Ficoll-Hypaque (Seromed) density gradient centrifugation. For peripheral blood samples, the red cell pellet at the bottom was treated with NH_4Cl_2 for 10 min. Granulocytes were then pelleted by centrifugation for 5 min at 1,800 rpm and subsequently used for DNA isolation.

JAK2 Mutation Analysis

Presence or absence of *JAK2V617F* mutation was tested using the ARMS assay published by Baxter et al. (1). Briefly, DNA from purified granulocytes was isolated using the Qiagen Blood DNA Purification kit (Qiagen). For allele-specific PCR, we used 50 ng granulocyte DNA in a 40-cycle reaction at an annealing temperature of 57°C. Reverse primer (0.4 pmol; 5′-CTGAATAGTCCCTACAGTGTTTTCAGTTTCA-3′), mutant JAK2-specific forward primer (0.4 pmol; 5′-AGCATTTGGTTTTAAATTATGGAGTATATT-3′), and control forward primer (0.4 pmol; 5′-ATCTATAGTCATGCTGAAAGTAGGAGAAAG-3′). This reaction setup gave a 364-bp product from all tested DNA samples as an internal control, whereas in the presence of a *JAK2V617F* mutation a second 203-bp product was visible. DNA extracts from HEL and K562 served as positive and negative controls, respectively.

Colony Assays

The EEC assay using freshly isolated peripheral blood mononuclear cells was done in duplicate in methylcellulose medium with and without erythropoietin (Methocult H4330 and H4230; Stem Cell Technologies) as described by others (16). LS104 was added in increasing doses to the medium without erythropoietin.

Freshly isolated bone marrow mononuclear cells from normal controls were plated in duplicate at a fixed density (2×10^4 cells/mL) in methylcellulose medium containing cytokines (Methocult H4534) and increasing doses of LS104. Colonies derived from granulocyte-macrophage progenitors (CFU-GM, CFU-G, and CFU-M) were counted on day 14.

JAK2 Kinase Assay

For determination of *in vitro* JAK2 kinase activity, we used HTScan JAK2 Kinase Assay Kit (Cell Signaling Technology), which contains recombinant wild-type JAK2. Structure of the JAK2 kinase domain has been described by Lucet et al. (17). Analysis was done according the manufacturer's protocol. Briefly, kinase reaction was set up in a total volume as follows: 60 mmol/L HEPES (pH 7.5), 5 mmol/L $MgCl_2$, 5 mmol/L $MnCl_2$, 3 μmol/L Na_3VO_4, 1.25 mmol/L DTT, 20 μmol/L ATP (200 μmol/L ATP for competition experiments), 1.5 μmol/L substrate peptide (0.3 and 0.15 μmol/L for substrate competition

[4] https://rnaidesigner.invitrogen.com/sirna/

experiments), and 10 units kinase. Increasing doses of LS104 were added to the reaction and incubated at 25°C for 1 h. The reaction was stopped by adding 50 μL/well 50 mmol/L EDTA (pH 8). Each reaction (25 μL) was transferred in triplicate to a 96-well streptavidin-coated plate (Perkin-Elmer Life Sciences); 75 μL distilled water/well was added and incubated at room temperature for 1 h. After washing with PBS/T, monoclonal anti-phosphotyrosine antibody (p-Tyr^{100}; Cell Signaling Technology) was added (1:1,000 in PBS/T with 1% bovine serum albumin) and incubated at room temperature for 1 h. After repeated washing with PBS/T, anti-mouse IgG, horseradish peroxidase–linked antibody (Cell Signaling Technology) was added (1:500 in PBS/T with 1% bovine serum albumin) and incubated for 30 min at 37°C. After subsequent washing, TMB substrate was added, incubated for 5 min at room temperature, and stopped with equal volume of 1 mol/L HCl. Absorbance was measured using a standard ELISA reader at 450 nm.

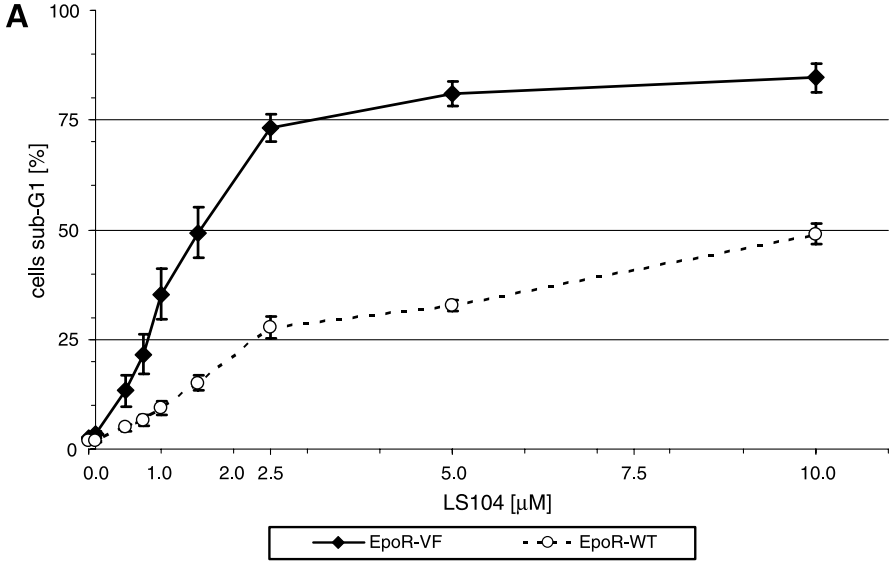
Statistical Calculations

All experiments were done in triplicate unless otherwise stated. For statistical evaluation of cell line responses on LS104 treatment, a paired Student's t test was applied. Statistical significance was assumed for $P < 0.05$. For analysis of drug effects in the combination treatment experiments, we used the CompuSyn software (18). Densitometric analyses were done using Gel-Pro Analyzer software (Media Cybernetics).

Results

LS104 Induces Apoptosis in *JAK2V617F*-Positive Cells

LS104 potently induced apoptosis in Ba/F3-EpoR-VF



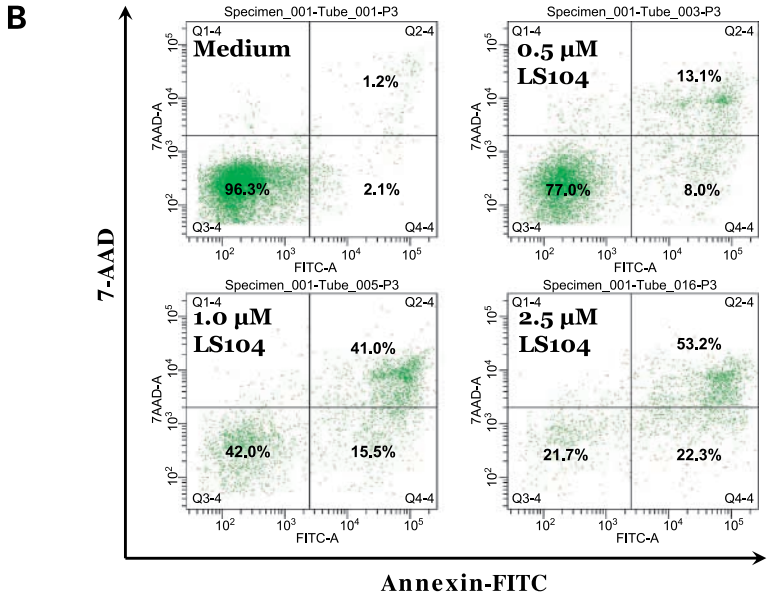


Figure 1. LS104 induces apoptosis in *JAK2V617F*-positive cells. **A,** induction of apoptosis in Ba/F3-EpoR-VF cells on LS104 treatment: for analysis of sub-G_1 DNA content, 8×10^3 cells per well (Ba/F3-EpoR-VF or Ba/F3-EpoR-WT) were seeded in a six-well plate. Ba/F3-EpoR-VF cells were grown without added growth factors; Ba/F3-EpoR-WT cells were grown in medium supplemented with 10% WEHI conditioned medium as a source of IL-3. After 48-h incubation with various concentrations of LS104, cells were stained with propidium iodide and fluorescence-activated cell sorting analysis was done. The estimated IC_{50} for Ba/F3-EpoR-VF cells in this assay was 1.5 μmol/L, whereas the IC_{50} for Ba/F3-EpoR-WT control cells was ~10 μmol/L. Mean ± SEM of seven independent experiments. **B,** Annexin V assay: using the same treatment as described in **A**, Ba/F3-EpoR-VF cells were harvested after 48-h incubation and costained with FITC-labeled Annexin V and 7-AAD. Representative scattergrams from one of four independent experiments.

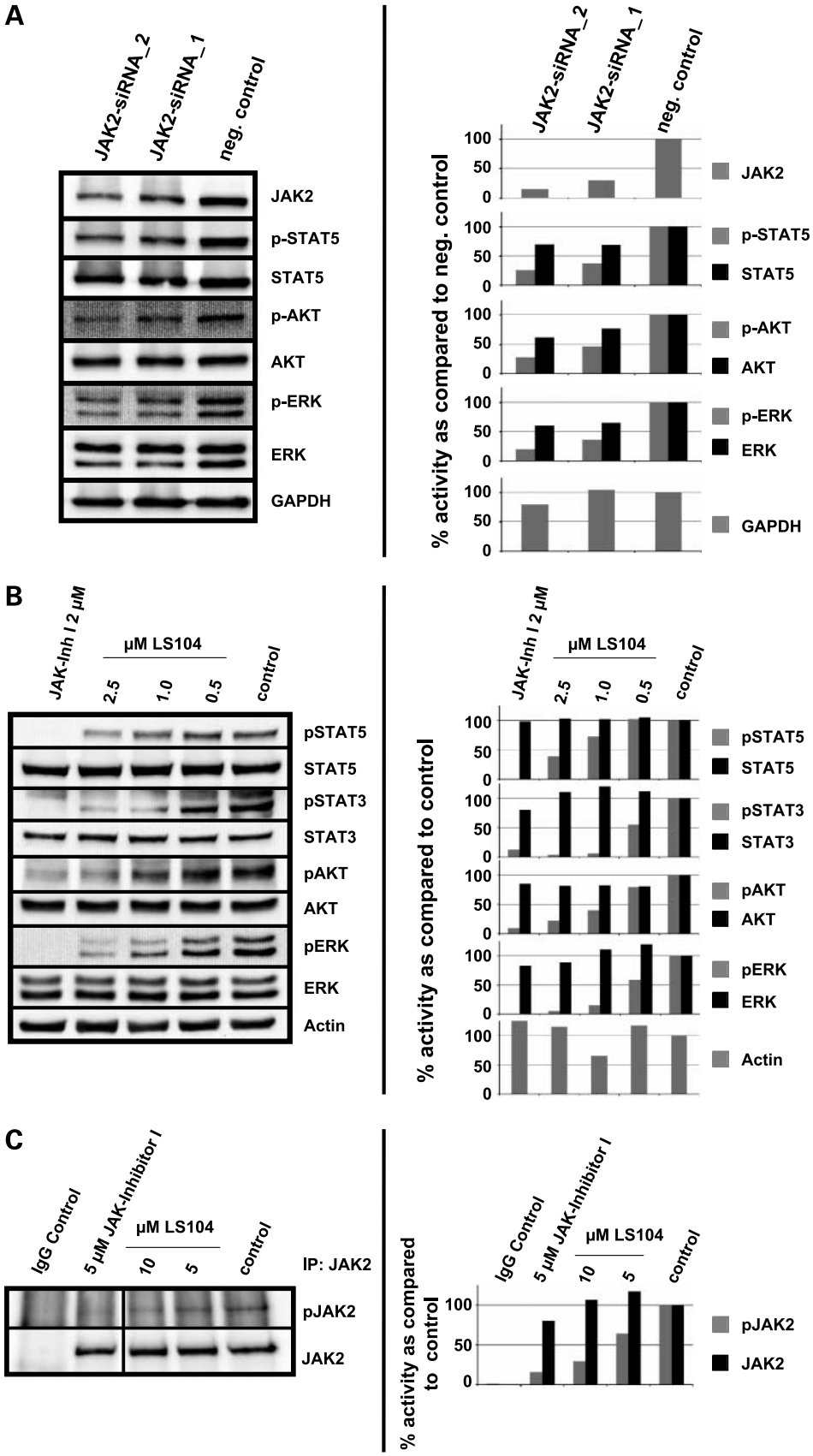
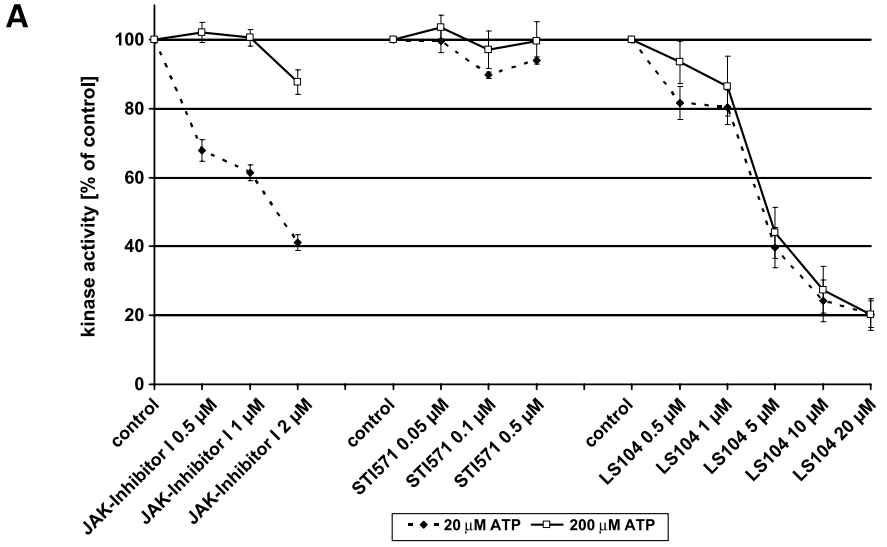
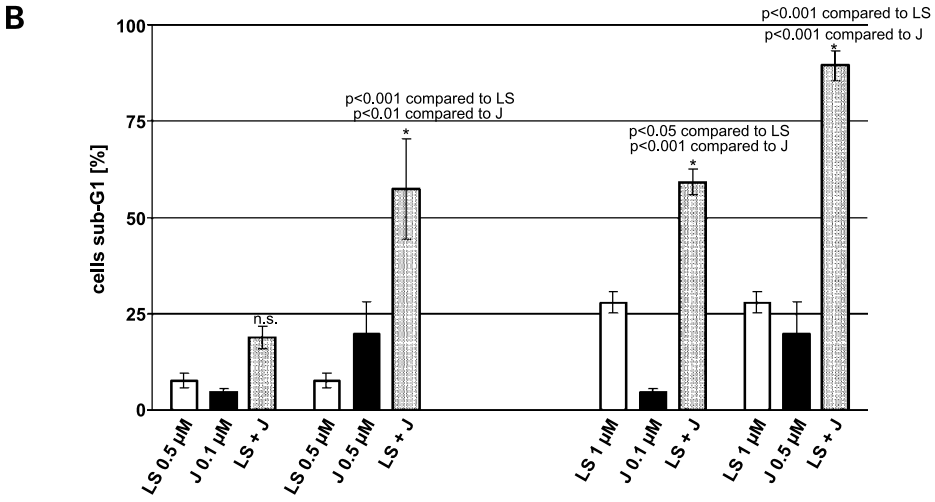


Figure 2. LS104 inhibits JAK2V617F autophosphorylation and its downstream signaling. **A,** JAK2 siRNA inhibits phosphorylation of JAK2 downstream targets: to verify which downstream targets depend on JAK2, we did siRNA mediated knockdown of JAK2 in Ba/F3-EpoR-VF cells. Immunoblotting of cellular lysates showed that phosphorylation of STAT5, AKT, and ERK is dependent on JAK2 activation. Densitometry of Western blots showed a 70% to 80% reduction of JAK2 expression on transfection using siRNA specific for JAK2. Consecutively, downstream target inhibition was 64% to 68% for phospho-STAT5, 56% to 66% for phospho-AKT, and 65% to 75% for phospho-ERK, respectively, when normalized for glyceraldehyde-3-phosphate dehydrogenase loading (*right*). **B** and **C,** LS104 inhibits JAK2 downstream signaling and JAK2 autophosphorylation in Ba/F3-EpoR-VF cells: Ba/F3-EpoR-VF cells treated for 6 h with either LS104 or JAK inhibitor I as indicated. Lysates were either immunoprecipitated with anti-JAK2 antibody (**C**) or referred directly to SDS-PAGE (**B**). Immunoblotting was done with antibodies as indicated. A dose-dependent decrease in autophosphorylation of JAK2 and JAK2 downstream targets is apparent on LS104 treatment. Densitometry of Western blots was done: downstream signaling was strongly inhibited at 2.5 μmol/L LS104: 62%, 96%, 73%, and 94% for phospho-STAT5, phospho-STAT3, phospho-AKT, and phospho-ERK, respectively (**B**, *right*). Inhibition of JAK2 autophosphorylation by LS104 was 45% at 5 μmol/L and 73% at 10 μmol/L. JAK inhibitor I inhibited JAK2 autophosphorylation by 81% at 5 μmol/L (**C**, *right*).





C

Dose LS104	Dose JAK-Inh I	Effect	CI	Synergism
0.50	0.10	0.19	0.84	++
0.50	0.50	0.57	0.48	+++
1.00	0.10	0.59	0.59	+++
1.00	0.50	0.90	0.28	++++

Scale: ++++ (strong synergism), +++ (synergisn), ++ (moderate synergism)

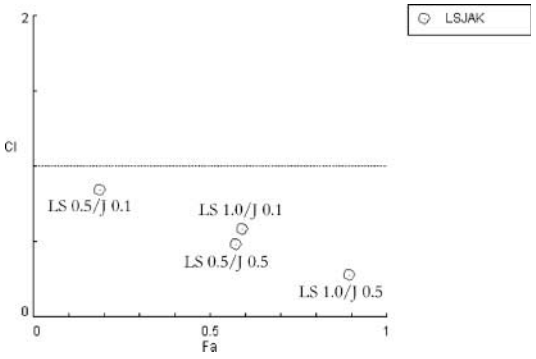


Figure 3. LS104 inhibits JAK2 kinase activity in an ATP-independent manner and has synergistic effects when combined with ATP-competitive kinase inhibitors. **A,** JAK2 kinase assay: specific inhibitory effects of LS104 and JAK inhibitor I on JAK2 kinase activity as measured by *in vitro* kinase assay. STI571 served as negative control. ATP dose escalation provided evidence that JAK inhibitor I but not LS104 acts via inhibition of ATP binding. Mean ± SEM of three independent experiments. **B,** induction of apoptosis by a combination of LS104 plus JAK inhibitor I in Ba/F3-EpoR-VF cells: combination treatment with LS104 (*LS*) plus JAK inhibitor I (*J*) showed strong additive effects that were statistically significant (*asterisks*) compared with each substance alone. Mean ± SEM of four independent experiments. **C,** combination of LS104 + JAK inhibitor I in Ba/F3-EpoR-VF cells is synergistic in a computer model: data from the experiments in **B** were used to calculate potential synergistic or antagonistic effects. A combination index <1 indicates synergism, combination index = 1 indicates additive effects, and combination index > 1 indicates antagonism at a given dose level. As depicted in the graph and the table insert, the calculated combination indices are <1 for all tested dose combinations. This indicates a moderate to strong synergistic effect of the combination of LS104 with JAK inhibitor I as shown in the table.

cells in a dose-dependent manner as evaluated by fluorescence-activated cell sorting analyses quantifying sub-G_1 DNA content (Fig. 1A). The estimated IC_{50} value for LS104 in this assay was 1.5 μmol/L. A control cell line (Ba/F3-EpoR-WT) expressing transgenic wild-type JAK2 was less sensitive to LS104 (Fig. 1A). However, some degree of sensitivity to LS104 in IL-3-treated control cells was observed and is most likely due to the presence of JAK2 within the IL-3 signaling cascade. A comparison of Ba/F3-EpoR-VF cells with Ba/F3-EpoR cells without overexpression of wild-type JAK2 exhibited similar results on treatment with LS104 (data not shown). Currently, there is no evidence that this is due to differential sensitivity of the JAK2V617F kinase to LS104 but rather reflects differential sensitivity of cells dependent on signals from a mutated kinase. This has been described previously as a typical feature of oncogene addiction (19). Induction of cell death by LS104 was also observed in HEL cells, harboring homozygous *JAK2V617F* mutation (data not shown). By using Annexin V-FITC/7-AAD costaining, we confirmed that JAKV217F-transformed cells undergo apoptosis on incubation with LS104 in a dose-dependent manner (Fig. 1B). The percentage of apoptotic cells (7-AAD and/or Annexin V-FITC positive) was 3.3% in controls and 21.1%, 56.5%, and 75.5% in cells treated with 0.5, 1.0, and 2.5 μmol/L LS104, respectively (Fig. 1B). These data show that induction of apoptosis is the mechanism of cell death induced by LS104 in JAK2V617F-dependent cells.

LS104 Inhibits JAK2 Autophosphorylation and Phosphorylation of JAK2V617F Signal Transduction

In Ba/F3-EpoR-VF cells, intracellular JAK2-responsive signaling pathways such as STAT5, AKT and ERK were found to be constitutively tyrosine phosphorylated (Fig. 2A). By introducing JAK2 mRNA-specific siRNA into Ba/F3-EpoR-VF cells, we successfully knocked down JAK2 expression by 70% to 80% and hereby confirmed that STAT5, AKT, and ERK activation in this cell model is indeed mediated by JAK2 activation (Fig. 2A).

Incubation of Ba/F3-EpoR-VF cells with LS104 resulted in dose-dependent inhibition of the JAK2-dependent signal transduction cascade (Fig. 2B) at dose levels similar to those observed to be active in the apoptosis experiments. Corresponding results were observed in HEL cells (data not shown). These data suggest that induction of apoptosis in *JAK2V617F*-positive cells by LS104 was mediated by inhibition of JAK2 and its downstream signals. We next investigated the effects of LS104 on JAK2 autophosphorylation: Immunoprecipitated JAK2 showed a dose-dependent decrease in autophosphorylation on LS104 treatment. However, surprisingly, for inhibition of constitutive JAK2V617F autophosphorylation, a LS104 dose of at least 5 μmol/L was necessary (data not shown; Fig. 2C). Similarly, others have observed a difference in dosages needed for inhibition of downstream target phosphorylation and for inhibition of kinase autophosphorylation, respectively, when using compounds acting as substrate binding inhibitors (20).

LS104 Is a JAK2 Substrate Inhibitor

Using constitutively activated recombinant JAK2 kinase and increasing doses of LS104 in an *in vitro* kinase assay, we confirmed the specific inhibitory effects of LS104 on JAK2 kinase activity (Fig. 3A). JAK inhibitor I and Imatinib were used as positive and negative controls, respectively. In this assay, the estimated IC_{50} value was ~5 μmol/L for LS104 and ~1 to 2 μmol/L for JAK inhibitor I.

To further examine the biochemical mechanism of action of LS104, we investigated the effects of a 10-fold higher ATP concentration on the inhibitory activity of the compound.

We reasoned that, under these conditions, kinase inhibitors acting via the ATP-binding pocket would be competed out, whereas the inhibitory effect of kinase inhibitors binding to other sites of the kinase, binding irreversibly, or acting allosterically would not be affected (21).

As expected, the ATP-competitive JAK inhibitor I showed dramatically decreased activity in this experimental setting, whereas JAK2 kinase inhibition by LS104 was virtually unaffected despite higher ATP concentrations (Fig. 3A). These results suggest that either LS104 has very high affinity to the ATP-binding pocket and inhibits ATP almost irreversibly or LS104 acts via a different biochemical mechanism (e.g., allosteric effects or substrate competition).

Therefore, next we examined the effects of increasing concentrations of substrate on the inhibitory activity of the compound in the presence of a constant amount of ATP. These studies revealed an increase in the IC_{50} values for inhibition of JAK2 activity using a higher substrate concentration (Table 1). Together, these data suggest that LS104 is not an ATP-competitive but a substrate-competitive tyrosine kinase inhibitor of JAK2.

Combination of LS104 with ATP-Competitive Kinase Inhibitors

To gain additional evidence that LS104 does not act via irreversible binding to the ATP pocket, an indirect approach using a combination of LS104 with the ATP-competitive JAK inhibitor I employed in an apoptosis assay was chosen. We reasoned that, if LS104 is irreversibly bound to the ATP pocket of JAK2V617F kinase, a combination with an ATP-competitive JAK2 inhibitor such as the JAK inhibitor I would not result in synergistic efficacy.

For this experiment, Ba/F3-EpoR-VF cells were incubated with either LS104 or JAK inhibitor I alone or in combination. Although each substance alone yielded not more than

Table 1. Influence of substrate concentration on IC_{50} values for LS104 induced inhibition of JAK2 kinase

Substrate peptide (μmol/L)	ATP (μmol/L)	IC_{50} (μmol/L LS104)
1.5	20	2.52
0.3	20	1.93
0.15	20	0.93

NOTE: *In vitro* JAK2 kinase assay was done using various concentrations of substrate peptide. IC_{50} values are calculated using CompuSyn software.

30% apoptosis, the combination resulted in dramatically enhanced efficacy with up to 89% apoptotic cells (Fig. 3B). We then applied a computational model to calculate the combination index. As depicted in Fig. 3C, this analysis showed that, at all doses employed, LS104 in combination with JAK inhibitor I has pronounced synergistic effects supporting our concept of a non-ATP-competitive mode of action for LS104.

LS104 Inhibits Growth of EECs in Patients with MPDs

The EEC assay tests for growth of EECs in the absence of added erythropoietin or any other cytokines and is specific for MPDs, whereas in healthy controls no colony growth is

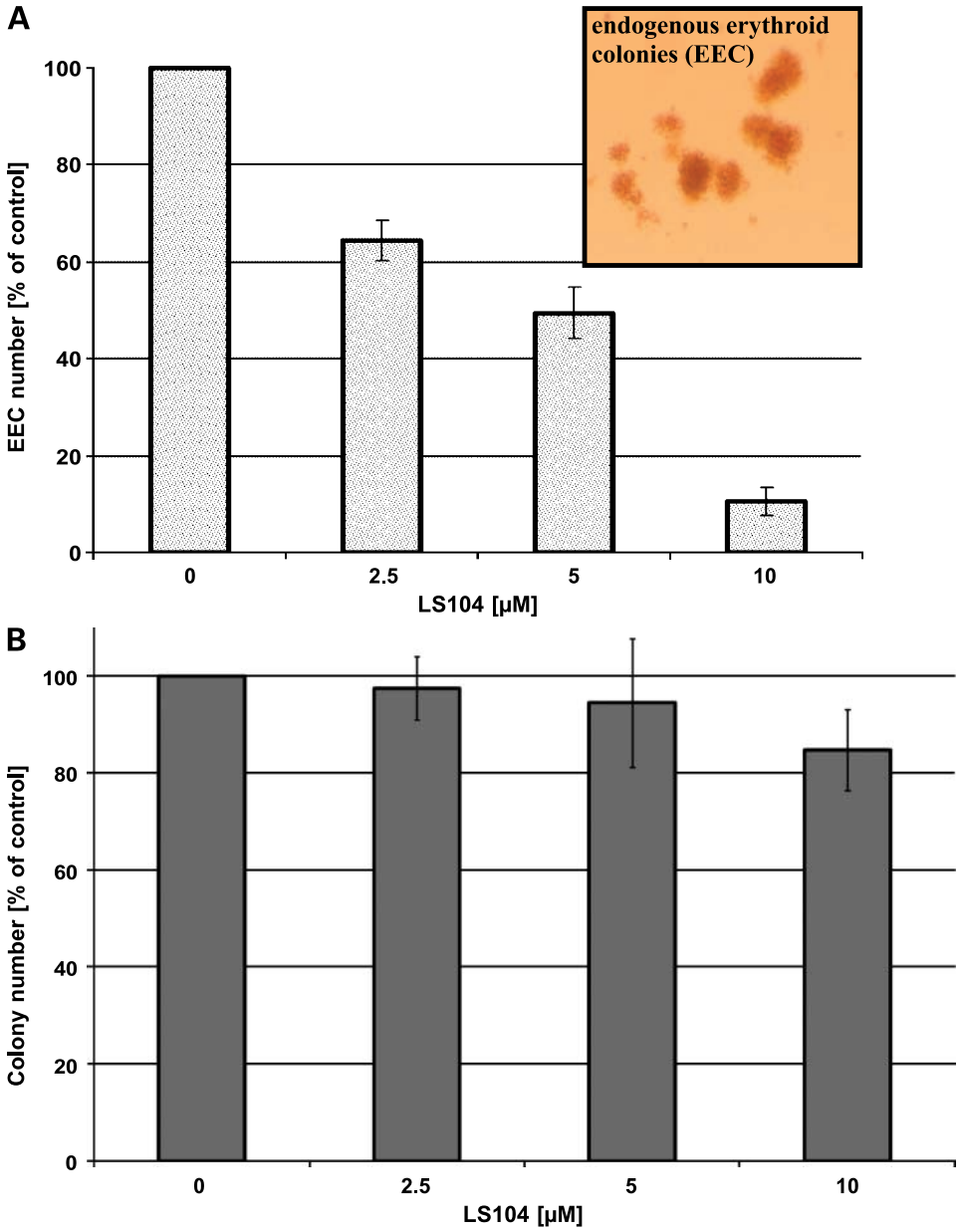


Figure 4. LS104 inhibits cytokine-independent colony growth from patients with *JAK2V617F*-positive MPDs, whereas it has no significant effects on cytokine-dependent colony growth from normal human bone marrow. **A,** effects of LS104 on number of EECs obtained from *JAK2V617F*-positive patients: peripheral blood samples from six patients with *JAK2V617F*-positive MPD were obtained after written informed consent has been given. Freshly isolated peripheral blood mononuclear cells were cultured in duplicate in methylcellulose culture medium in the absence of erythropoietin with and without LS104. The number of EECs was counted on day 14. Mean ± SEM. *Inset,* microscopic picture of typical EECs as observed with a ''Wilcovert'' microscope (original magnification, ×100). The picture was captured with a Canon EOS digital camera. **B,** effects of LS104 on cytokine-dependent colony growth from normal human bone marrow: bone marrow blood samples from four normal controls were obtained after written informed consent has been given. Freshly isolated bone marrow mononuclear cells were cultured in cytokine-containing methylcellulose culture medium in the presence and absence of LS104. The number of colonies derived from granulocyte-macrophage progenitors (CFU-GM, CFU-G, and CFU-M) was counted on day 14. Mean ± SEM.

observed under these conditions (16). We tested the effects of LS104 on EEC formation in six patients with *JAK2V617F*-positive MPD. LS104 significantly reduced the number of EECs in a dose-dependent manner. We observed 89% inhibition at 10 μmol/L LS104 (Fig. 4A). In a CFU assay using bone marrow mononuclear cells from normal controls, we did not observe a significant effect on the number of cytokine-dependent colonies derived from granulocyte-macrophage progenitors (Fig. 4B).

Discussion

The *JAK2V617F* mutation is an attractive and widespread target for new therapeutic approaches in MPDs. Several mouse models provide evidence that JAK2-mediated signaling is a key event in the pathogenesis of MPD and that inhibition of constitutively active JAK2 leads to reversal of the aberrant phenotype (10–12). The experimental data presented here characterize LS104 as a JAK2 kinase inhibitor that leads to apoptosis in JAK2V617F transformed cells by inhibiting JAK2 and its downstream signaling pathways. We also observed some effect of LS104 on the IL-3-dependent control cell line in our apoptosis assay. This may be explained by the fact that JAK2 is also involved in IL-3 signaling (22); thus, some degree of sensitivity to LS104 in IL-3-treated control cells may occur. From these data, we do not claim that there is differential inhibition of mutant and wild-type JAK2 by LS104. To our understanding, these data fit very well with the concept of oncogene addiction. This concept describes the phenomenon that cells dependent on oncogenic signals undergo apoptosis when they are taken away the oncogenic signal, for example, by tyrosine kinase inhibition (19). In line with this interpretation, colony assays of cytokine-dependent normal bone marrow cells yielded no evidence for a pronounced myelosuppressive potential of LS104 *in vitro*. Furthermore, LS104 inhibits cytokine-independent growth of primary cells obtained from patients with *JAK2V617F*-positive MPD in an *in vitro* EEC assay (23). We chose this EEC assay instead of a recently described fluorescence-activated cell sorting–based assay (9), because the EEC assay focuses on the terminal erythroid differentiation that is completely cytokine independent in MPD patients. Thereby, we could exclude interference with added cytokines, the signaling of which also depends on JAK2 (12). Western blotting experiments using Ba/F3-EpoR-VF cells show inhibition of JAK2 signaling pathways by LS104 treatment. In addition, we were able to show that JAK2 autophosphorylation is inhibited by LS104 treatment. The observation that inhibition of JAK2 autophosphorylation and inhibition of direct JAK2 downstream targets occurs at different dose levels was consistently detectable. A literature search on kinase inhibitors yielded several reports describing that non-ATP-competitive kinase inhibitors may not display identical IC_{50} values for inhibition of autophosphorylation and for inhibition of downstream signaling pathways (20, 24). These data led us to further investigate the mechanism of action of LS104. Modulation of ATP concentration in an *in vitro* kinase assay had no significant effect on the ability of LS104 to inhibit JAK2 kinase. This finding is in line with the hypothesis that LS104 is a non-ATP-competitive kinase inhibitor and leaves the following possible explanations: (*a*) LS104 binds irreversibly or with very high affinity to the ATP-binding pocket and thus cannot be competed out, (*b*) LS104 has allosteric effects and thus covalently modifies the structure of either enzyme or substrate, or (*c*) LS104 competes with substrate binding. Our kinase assays employing variation in substrate peptide concentrations suggest that LS104 is acting via the latter biochemical mechanism.

To further emphasize the alternative mechanism of action, we did combination experiments of LS104 and the ATP-competitive JAK Inhibitor I. These data showed a synergistic effect of both compounds, which argues against irreversible binding of LS104 to the ATP-binding pocket.

Taken together, our data show that LS104 selectively induces apoptosis in Ba/F3-EpoR-VF cells by suppression of JAK2 kinase activity. Furthermore, LS104 leads to a significant decrease in growth of EECs derived from patients with *JAK2V617F*-positive MPDs. We further provide data on a non-ATP-competitive but substrate-competitive mechanism of action of LS104 in an *in vitro* kinase assay. In addition, combination treatment of LS104 with an ATP-competitive kinase inhibitor resulted in synergistic effects as measured by induction of apoptosis. Based on these data, currently a phase I clinical trial of LS104 in patients with *JAK2V617F*-positive MPDs has been started. To our knowledge, this is the first report on a non-ATP-competitive kinase inhibitor being tested in a clinical trial.

Disclosure of Potential Conflicts of Interest

T. Fischer has received a research fund granted by LymphoSign. A patent describing LS104 as a JAK2V617F inhibitor has been recently submitted (T. Fischer and D.B. Lipka).

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