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Article

Focal adhesion kinase inhibition decreases cell viability and induces apoptosis of JAK2 V617F positive cells

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Focal Adhesion Kinase (FAK) protein participates in proliferation, migration, cell survival, and apoptosis process. It has been described as overexpressed in several neoplasms being a promising target for therapy. BCR-ABL negative chronic Myeloproliferative Neoplasms (MPN) are clonal disorders characterized by the excess of proliferation and apoptosis resistance. The identification of the acquired JAK2 V617F mutation in MPN patients allowed a better understanding of pathogenesis. However, there is still no pharmacological treatment that leads all patients to molecular remission, justifying new studies. The present study aimed to evaluate FAK involvement in the viability and apoptosis of HEL and SET-2 cells, both JAK2 V617F positive cell lines. The FAK inhibitor PF 562,271 was used. Cell viability was determined using MTT assay and apoptosis verified by cleaved PARP, cleaved Caspase 3 and Annexin-V/PI staining detection. FAK inhibition significantly reduced HEL and SET-2 cells viability and induced apoptosis. Considering the role of JAK/STAT pathway in MPN, further investigation of FAK participation in the MPN cells proliferation and apoptosis resistance, as well as possible crosstalk between JAK and FAK and downstream pathways may contribute to the knowledge of MPN pathophysiology, the discovery of new molecular targets, and JAK inhibitors resistance mechanisms.

Keywords: FAK. Apoptosis. Myeloproliferative Neoplasms.

INTRODUCTION

Focal Adhesion Kinase (FAK) is a non-receptor tyrosine kinase encoded by the protein tyrosine kinase 2 (*PTK2*) gene, which expression is regulated by molecules such as NF-kB and P53 (Sulzmaier, Jean, Schlaepfer, 2014). FAK can be activated by auto-phosphorylation of Tyr397 residue in response to stimulus as transcriptional factors, neuropeptides, and cytokines then interacting with other proteins as integrins, SRC kinase, PI3K, and GRB2 and triggering signaling pathways that regulate cell survival, apoptosis, proliferation, migration, invasion and angiogenesis (Sulzmaier, Jean, Schlaepfer, 2014). Several studies have demonstrated that FAK is deregulated in many cancers such as breast cancer, colon, prostate, and pancreas (Sulzmaier, Jean, Schlaepfer, 2014; Kessler *et al*., 2016). Deregulation of FAK was also described in hematological neoplasms as

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Acute Myeloid Leukemia (AML). Carter *et al.* (2017) found that 40% of AML patients included in their study presented FAK overexpression and the expression was associated with higher blasts proliferation, higher bone marrow cellularity, and worse prognosis. The authors also showed that FAK inhibitor VS-4718 has anti-leukemic effects in AML (Carter *et al*., 2017) and that VS-4718 increases BCL-2 inhibitor ABT-199 effect in AML cell apoptosis (Wang *et al*., 2020). El-Sisi *et al.* (2021) analyzed the serum levels of FAK in AML patients and found higher levels compared to controls. Furthermore, they found a negative correlation between FAK serum levels and overall survival. Some studies also found a link between BCR-ABL kinase in Chronic Myeloid Leukemia (CML) and FAK. It was demonstrated that BCR-ABL interacts with FAK, altering cell adhesion of hematopoietic cells (Maia *et al*., 2013). It is worth to emphasizing that FAK has already been described as involved in hematopoietic cells differentiation, including erythroid, lymphoid, and myeloid progenitors (Lu *et al*., 2012).

BCR-ABL negative Chronic Myeloproliferative Neoplasms (MPN) are clonal disorders characterized by the accumulation of mature myeloid cells because of the excess proliferation and apoptosis impairment (Vainchenker, Kralovics, 2017). This group of diseases includes Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF). JAK2 V617F mutation is found in a near 95% of PV patients and 50% of ET and PMF. JAK2 V617F negative patients harbor other mutations of *JAK2*, *MPL,* and *CALR* genes or also may be triple negative, being genotype linked to clinical phenotype (Vainchenker, Kralovics, 2017). JAK2 is a tyrosine kinase activated by cytokines receptors, which triggers pathways such as PI3K/AKT, MAPK, and others, increasing cell proliferation and reducing apoptosis (Hu *et al*., 2021). Nowadays, MPN treatment is still mainly for control constitution symptoms and complications (Tavares *et al*., 2019). Ruxolitinib, a JAK2 inhibitor, is approved for intermediate or high-risk PMF patients and those PV patients with inadequate response or intolerance to hydroxyurea, leading to an improvement of constitutional symptoms and splenomegaly but not inducing a complete molecular response or bone marrow fibrosis reduction in MF (Tavares *et al*., 2019; Tefferi, 2021). Furthermore, many patients present intolerance or develop resistance to ruxolitinib, mainly by loss of response. The only potential curative option for these patients is the hematopoietic stem cell transplantation, but it is related to high mortality rates and morbidity (Tavares *et al*., 2019; Tefferi, 2021).

Considering this scenario, we aimed to investigate whether FAK inhibition affects JAK2 V617F positive cell lines. Therefore, we evaluated the cell viability and apoptosis of HEL 92.1.7 cells and SET-2 cells, both study models for myeloproliferative neoplasms.

MATERIAL AND METHODS

HEL 92.1.7, a erythroleukemia cell line homozygous for JAK2 V617F mutation and SET-2 cells, a megakaryocyte cell line heterozygous for *JAK2* V617F mutation, were cultured in RPMI 1640 (LONZA, 12-702F) supplemented with 1% Penicillin/Streptomycin (LONZA, 21F095302) at 37ºC and 5% CO2. For SET-2 cells, the medium was also supplemented with 20% heat-inactivated fetal bovine serum (LGC Biotecnologia, 10-BIO500). For HEL cells, the medium was also supplemented with 10% heat-inactivated fetal bovine serum and 1mM Sodium Pyruvate. The cell lines were gently provided by Prof. Fabíola Attié de Castro (School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil). FAK inhibitor PF-562,271 was purchased from Sigma Aldrich (#PZ0387). For cell viability assay and IC50 determination, HEL cells were plated at 2 x 10^4 cells/well and SET-2 cells were plated at 4 x 10^4 cells/well and then treated with PF-562,271 at different concentrations according to the experiment, for 48 h and 72 h, respectively. After that, 20 µL of a 5mg/mL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Sigma Aldrich, M5655) solution was added to the wells and incubated at 37ºC for 4 hours. Then, the plate was centrifuged at 2000 rpm for 3 minutes at room temperature, the supernatant was removed and DMSO was added to the wells. The plate was incubated at 37ºC for 30 minutes and the

absorbance was measured at 570nM (ThermoFisher Multiskan FC 51119000. Doxorubicin was used as a positive control for cell death. DMSO, the vehicle for PF 562,271 was used as negative control (CTRL). For protein detection by western blot, PF 562,271 treated cells were washed three times with PBS buffer and the cell lysate was obtained using RIPA buffer (ThermoFisher, 89900) plus phosphatase inhibitors (2.5mM Sodium Pyrophosphate; 1mM Sodium Orthovanadate and 1mM β-glycerolphosphate) and 1% Proteases Inhibitor Cocktail (Sigma Aldrich, P8340). After flushing and centrifugation at 6000 rpm for 20 minutes, protein quantification was performed using BCA Protein Assay Kit (Pierce™, 23227), according to the kit instructions. Laemmli buffer (1M Tris, 10% SDS, 20% glycerol, bromophenol blue, and 10% beta-mercaptoethanol) was added to cell lysate, which was boiled at 95ºC for 5 minutes and stored at -20ºC. Equal amounts of protein were separated by SDS-PAGE and transferred to a PVDF membrane (Amersham Hybond P0.45). The membrane was blocked with albumin and incubated overnight with the following antibodies: anti-SRC (CST#2108), anti-P-SRC (CST#2101S), anti-cPARP (CST#9541S), anti-cCASP3 (#CST9664) and anti-Actin (BIORAD VMA00078). Signals were detected using horseradish peroxidase (HRP) conjugated secondary antibodies (anti-mouse IgG from GE Amersham NA934V and anti-rabbit from GE Amersham NA931V) and Amersham ECL Prime kit (GE Health Care RPN2232). Band densitometry was performed using Image J (Schneider, Rasband, Eliceiri, 2012). For Annexin-V/PI evaluation, HEL cells were plated in a 24-well plate at 0.5 x 10^6 cells/mL and treated with PF 562,271. After treatment, the cells were recovered and washed with cold PBS buffer, and *Dead Cell Apoptosis Kit with Annexin V Alexa Fluor™ 488 & Propidium Iodide (PI)* kit (Thermo, V13241) was used. The acquisition was performed in the FACSVerse cytometer (BD Bioscience) and the analysis in the FCS Express 3.0 software. Regarding statistical analysis, IC50 values were calculated using a nonlinear regression analysis on R studio and "drc package" (Ritz *et al.*, 2015). For comparison of cell viability and Annexin-V/PI positivity, ANOVA followed by Tukey post-test was performed using JAMOVI software (The Jamovi Project, 2023).

RESULTS AND DISCUSSION

PF 562,271 significantly reduced HEL cell viability at 5 μM (52.2%) and 10 μM (12.9%) in comparison to CTRL ($P<0.0001$), as well for Doxorubicin at 1 μM (6.2%) ($P<0.0001$) (Figure 1A). In SET-2 cells, the inhibitor PF-562,271 at 5μM and 10 μM reduced the cell viability by 71.5% and 85.5%, respectively, compared to CTRL ($P< 0.0001$) (Figure 1B). The IC50 of PF-562,271 was 3.9020 μM for HEL cells and 3.1183 μM for SET-2 cells (Figure 1C and 1D).

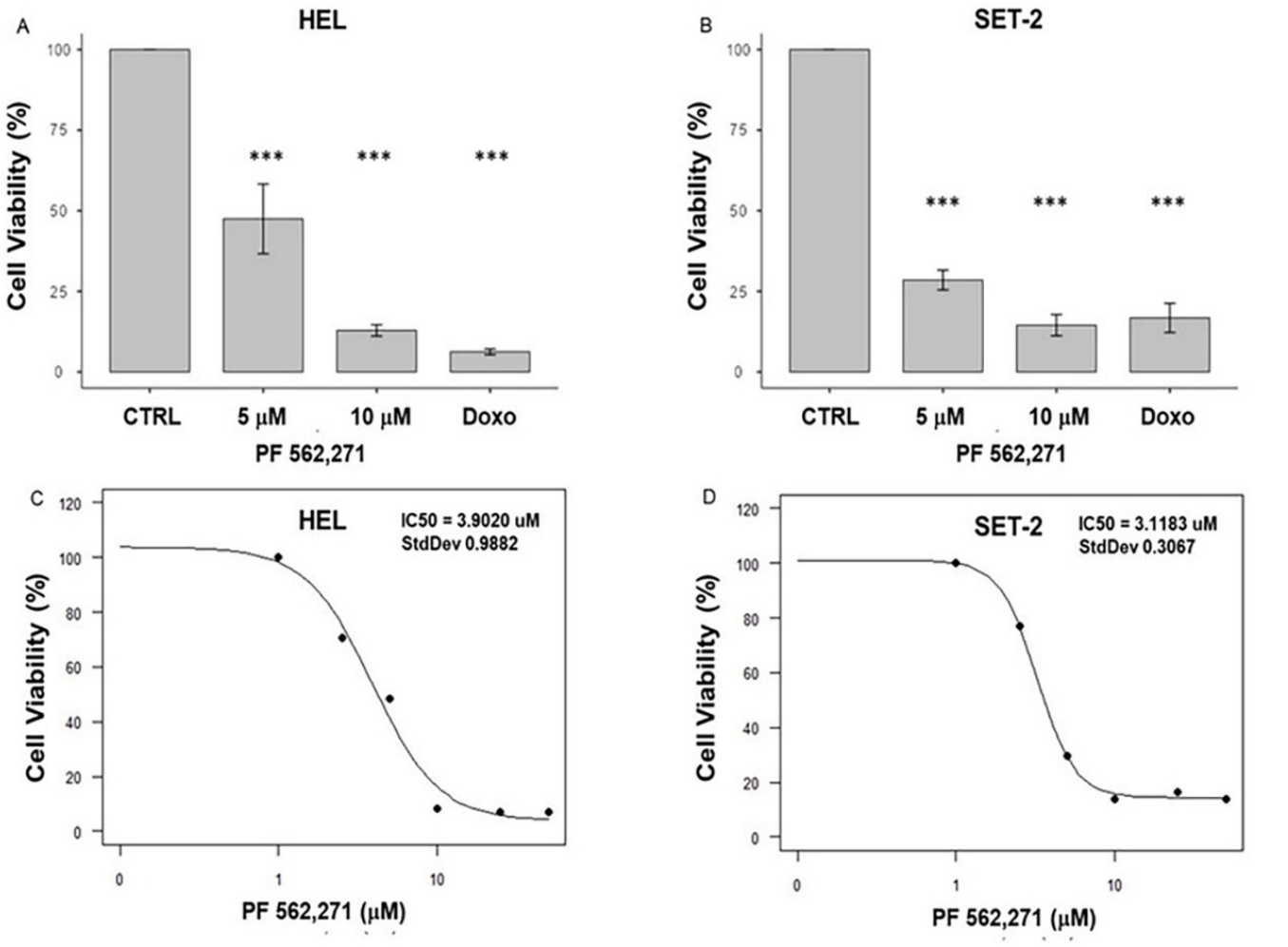

FIGURE 1 - Cell viability evaluated by MTT after FAK inhibition with PF 562,271. Cell viability results after treatment with PF 562,271 at 5 µM and 10 µM are shown as percentages in (A) HEL cells and (B) SET-2 cells. (C) Dose-Response Curve of PF-526,271 (1 µM, 2.5 µM, 5 µM, 10 µM, 25 µM, and 50 µM) in HEL cells and (D) SET-2 cells for IC50 determination. *** P<0.001. IC50: Inhibitory Concentration for 50% of viability. StdDev: Standard Deviation. CTRL: DMSO treated cells. Doxo: Doxorubicin.

FAK inhibition also induced apoptosis in both cells. SET-2 cells treated with PF 562,271 at 5 µM and 10 µM for 72h showed a clear increase in cPARP and cCasp3 (Figure 2A). It was possible to confirm that P-SRC/SRC ratio decreased in cells treated in both concentrations using densitometry. P-SRC/SRC ratio in 5 µM and 10 µM was 0.334 and 0.339 times P-SRC/SRC ratio in control (Figure 2A). cPARP was also detected in HEL cells treated with PF 562,271 at 10 µM for 48h and P-SRC inhibition was evident at 10 µM (Figure 2B). It was not possible to detect cCasp3 in HEL cells. So, the apoptosis induction in HEL cells was also evaluated by Annexin-V/PI staining as illustrated in Figure 2C. HEL cells treated with PF 562,271 at 1 µM, 5 µM and 10 µM for 16h showed an increase of apoptosis (Annexin-V positivity) compared to control (P<0.0001, P<0.05, and P<0.0001, respectively) (Figure 2D).

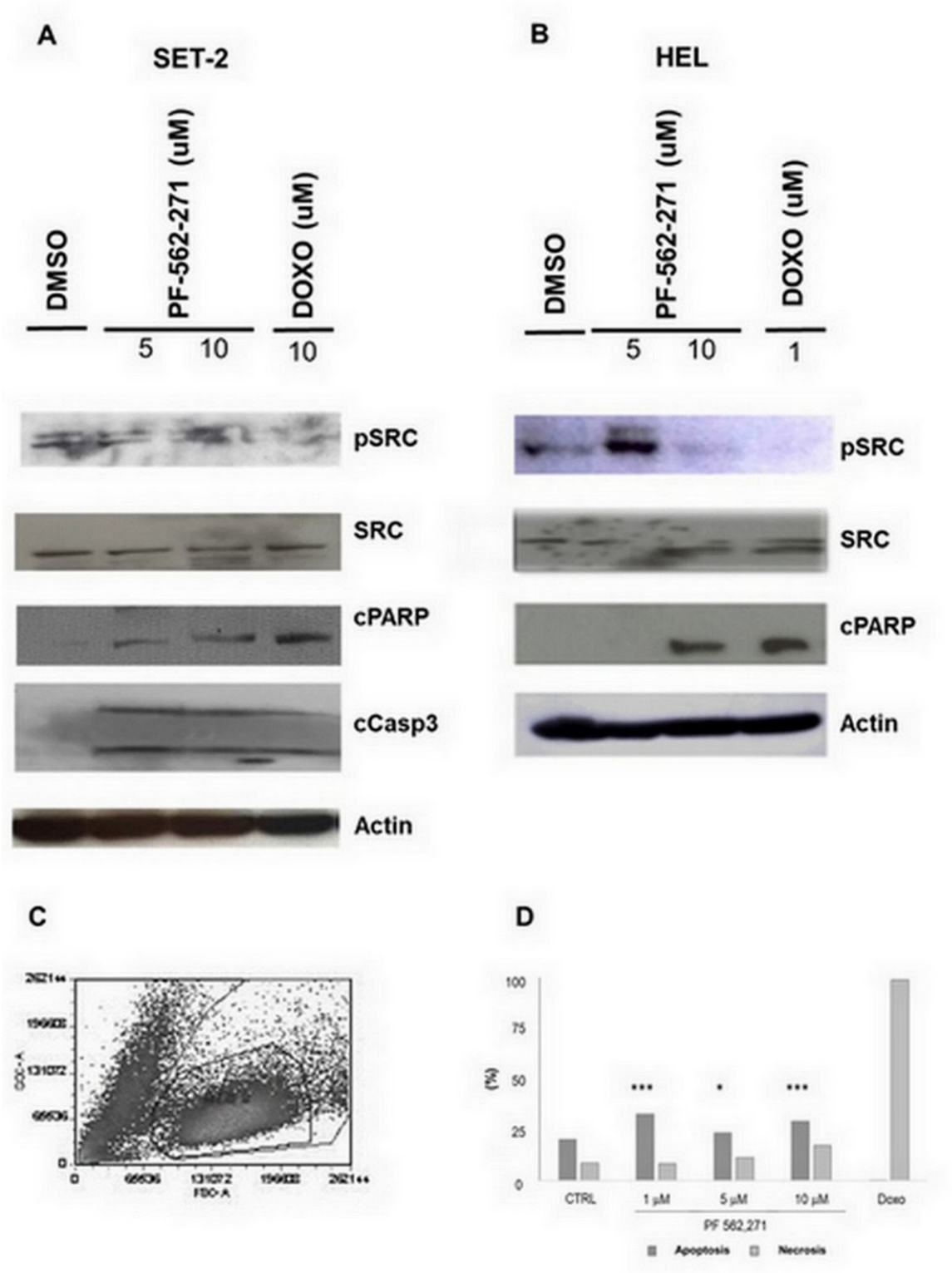

FIGURE 2 - FAK inhibition induces apoptosis in HEL and SET-2 cells. (A) cPARP and cCASP3 expression increased after FAK inhibition in SET-2 cells as well (B) cPARP increased in HEL cells. (C) Representation of the gate used for Annexin-V-AlexaFluor/PI evaluation in HEL cells by flow cytometry. (D) Apoptosis and necrosis percentage for HEL cells treated with PF 562,271 at 1 µM, 5 µM and 10 µM for 48 hours. *** P<0.001 and *P<0.05, comparing apoptosis' percentage among all groups using ANOVA followed by Tukey post-test. CTRL: cells treated with DMSO. Doxo: Doxorubicin.

The involvement of FAK and SRC proteins in cancer progression was already demonstrated. FAK and SRC interact and participate in adhesion, migration, survival, apoptosis and cell cycle progression, being correlated with tumor metastasis and angiogenesis (Sulzmaier, Jean, Schlaepfer, 2014). For example, FAK inhibition in ovarian carcinoma and cervix cancer cells inhibited cell proliferation (Ward *et al*., 2013). PF 562,271 diminished thyroid cancer and other solid tumors in animal models (Slack-Davis *et al*., 2009). FAK participation in cell survival, proliferation and apoptosis is due to the activation of pathways such as PI3K-AKT-mTOR (Chuang *et al*., 2022). It was demonstrated in HL60 cells, a promyelocytic leukemia cell line, a resistance to oxidative stress and etoposide-induced apoptosis when FAK is overexpressed and the resistance was due to activation of PI3K-AKT, NF-κB and an increase in antiapoptotic molecules (Chuang *et al*., 2022, Sonoda *et al*., 2000). Another important role of FAK in cell survival is mediated by its interaction with p53 and MDM2. FAK interacts, inhibits the p53 activity and promotes p53 degradation by an MDM2-dependent mechanism (Lim *et al*., 2008). Interestingly, JAK2 seems to promote direct activation of FAK and FAK can active STAT3, leading to apoptosis resistance when induced by drugs (Bousoik, Montazeri Aliabadi, 2018; Cho *et al*., 2018).

In conclusion, considering the role of the JAK/STAT pathway in MPN and the findings of the present study, further investigations regarding FAK participation in the MPN cells proliferation and apoptosis resistance may contribute to the knowledge of MPN pathophysiology, to the discovery of new molecular targets and to better understanding of JAK inhibitors' resistance mechanisms.

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AUTHORS CONTRIBUTION

ACMMV performed experiments, analyzed data, and wrote the manuscript. GHLF and ACRB performed experiments. CCSA performed flow cytometry experiments. MBMP and RTR conceived the project, designed the experiments, performed experiments, and wrote the manuscript. All authors critically revised the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Bousoik E, Montazeri Aliabadi H. "Do We Know Jack" About JAK? A Closer Look at JAK/STAT Signaling Pathway. Front Oncol. 2018;8:287. doi: 10.3389/fonc.2018.00287.

Carter BZ, Mak PY, Wang X, Yang H, Garcia-Manero G, Mak DH, et al. Focal Adhesion Kinase as a Potential Target in AML and MDS. Mol Cancer Ther. 2017;16(6):1133-44. doi: 10.1158/1535-7163.MCT-16-0719.

Cho HJ, Park JH, Nam JH, Chang YC, Park B, Hoe HS. Ascochlorin Suppresses MMP-2-Mediated Migration and Invasion by Targeting FAK and JAK-STAT Signaling Cascades. J Cell Biochem. 2018;119(1):300-13. doi: 10.1002/jcb.26179.

Chuang HH, Zhen YY, Tsai YC, Chuang CH, Hsiao M, Huang MS, et al. FAK in Cancer: From Mechanisms to Therapeutic Strategies. Int J Mol Sci. 2022;23(3). doi: 10.3390/ijms23031726.

El-Sisi MG, Radwan SM, Saeed AM, El-Mesallamy HO. Serum levels of FAK and some of its effectors in adult AML: correlation with prognostic factors and survival. Mol Cell Biochem. 2021;476(5):1949-63. doi: 10.1007/s11010-020-04030-z.

Hu X, Li J, Fu M, Zhao X, Wang W. The JAK/STAT signaling pathway: from bench to clinic. Signal Transduct Target Ther. 2021;6(1):402. Epub 20211126. doi: 10.1038/s41392-021-00791-1.

Kessler BE, Sharma V, Zhou Q, Jing X, Pike LA, Kerege AA, et al. FAK Expression, Not Kinase Activity, Is a Key Mediator of Thyroid Tumorigenesis and Protumorigenic Processes. Mol Cancer Res. 2016;14(9):869-82. doi: 10.1158/1541-7786.MCR-16-0007.

Lim ST, Chen XL, Lim Y, Hanson DA, Vo TT, Howerton K, et al. Nuclear FAK promotes cell proliferation and survival through FERM-enhanced p53 degradation. Mol Cell. 2008;29(1):9-22. doi: 10.1016/j.molcel.2007.11.031.

Lu J, Sun Y, Nombela-Arrieta C, Du KP, Park SY, Chai L, et al. Fak depletion in both hematopoietic and nonhematopoietic niche cells leads to hematopoietic stem cell expansion. Exp Hematol. 2012;40(4):307-17.e3. doi: 10.1016/j.exphem.2011.11.010.

Maia V, Ortiz-Rivero S, Sanz M, Gutierrez-Berzal J, Alvarez-Fernández I, Gutierrez-Herrero S, et al. C3G forms complexes with Bcr-Abl and p38α MAPK at the focal adhesions in chronic myeloid leukemia cells: implication in the regulation of leukemic cell adhesion. Cell Commun Signal. 2013;11(1):9. doi: 10.1186/1478-811X-11-9.

Ritz C, Baty F, Streibig JC, Gerhard D. Dose-Response Analysis Using R. PLoS One. 2015;10(12):e0146021. doi: 10.1371/journal.pone.0146021.

Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012;9(7):671-5. doi: 10.1038/nmeth.2089.

Slack-Davis JK, Hershey ED, Theodorescu D, Frierson HF, Parsons JT. Differential requirement for focal adhesion kinase signaling in cancer progression in the transgenic adenocarcinoma of mouse prostate model. Mol Cancer Ther. 2009;8(8):2470-7. doi: 10.1158/1535-7163.MCT-09-0262.

Sonoda Y, Matsumoto Y, Funakoshi M, Yamamoto D, Hanks SK, Kasahara T. Anti-apoptotic role of focal adhesion kinase (FAK). Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. J Biol Chem. 2000;275(21):16309-15. doi: 10.1074/jbc.275.21.16309.

Sulzmaier FJ, Jean C, Schlaepfer DD. FAK in cancer: mechanistic findings and clinical applications. Nat Rev Cancer. 2014;14(9):598-610. doi: 10.1038/nrc3792.

Tavares RS, Nonino A, Pagnano KBB, Nascimento ACKVD, Conchon M, Fogliatto LM et al. Guideline on myeloproliferative neoplasms: Associacão Brasileira de Hematologia, Hemoterapia e Terapia Cellular: Project guidelines: Associação Médica Brasileira - 2019. Hematology, transfusion and cell therapy. 2019;41 Suppl 1(Suppl 1). doi: 10.1016/j.htct.2019.03.001.

Tefferi A. Primary myelofibrosis: 2021 update on diagnosis, risk-stratification and management. Am J Hematol. 2021;96(1):145-62. doi: 10.1002/ajh.26050.

The Jamovi Project (2023). Jamovi (Version 2.3) [Computer Software]. Retrieved from https://www.jamovi.org.

Vainchenker W, Kralovics R. Genetic basis and molecular pathophysiology of classical myeloproliferative neoplasms. Blood. 2017;129(6). doi: 10.1182/blood-2016-10-695940.

Wang X, Mak PY, Mu H, Tao W, Rao A, Visweswaran R, et al. Combinatorial Inhibition of Focal Adhesion Kinase and BCL-2 Enhances Antileukemia Activity of Venetoclax in Acute Myeloid Leukemia. Mol Cancer Ther. 2020;19(8):1636-48. doi: 10.1158/1535-7163.MCT-19-0841.

Ward KK, Tancioni I, Lawson C, Miller NL, Jean C, Chen XL, et al. Inhibition of focal adhesion kinase (FAK) activity prevents anchorage-independent ovarian carcinoma cell growth and tumor progression. Clin Exp Metastasis. 2013;30(5):579-94. doi: 10.1007/s10585-012-9562-5.

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