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## **PATHOGENETIC ROLE OF JAK3-STAT3 SIGNALING IN p210 BCR-ABL-POSITIVE CHRONIC MYELOGENOUS LEUKEMIA.**

This is an application for a second year extension of my Career Development Award from the Leukemia SPORE Grant. In summary, my preliminary data showed that STAT3 and JAK3 and their phosphorylated forms; pSTAT3<sup>Tyr705</sup> and pJAK3<sup>Tyr980</sup>, respectively, are constitutively expressed in the p210 BCR-ABL-positive cell lines K562 and KBM5. We have utilized 3 different pharmacologic inhibitors of JAK3: AG490, WPI-131, and WPI-154. In addition, we used an adenoviral vector carrying the dominant negative construct (AdSTAT3DN) to specifically block the effects of STAT3. These inhibitors induced concentration-dependent decrease in cell viability. Also, there were significant decrease in downstream targets of JAK3-STAT3 signaling that have direct impact on cell survival and apoptosis; specifically Mcl-1, Bcl-2, and Bcl-X<sub>L</sub>. The net result of these changes was apoptotic cell death. Of note, our preliminary findings showed that JAK3 and ABL coimmunoprecipitate, implicating that the two kinases are physically associated with possible significant biologic interaction. I have just obtained the fund for the first year. A postdoctoral fellow has been recruited and we started working on the experimental approaches proposed in my first year proposal (attached).

### ***Specific Aims for Second Year Extension:***

#### **Aim (1): To further investigate the role of JAK3 in p210 BCR-ABL-positive CML cell lines using RNA interference technique.**

I am currently working on developing JAK3 siRNA using standard techniques (Ambion). The next step is to transfer the most effective JAK3 siRNA into an adenoviral vector. This technique will allow to specifically evaluate the role of JAK3 in p210 BCR-ABL-positive CML, as it will eliminate the unknown, unpredictable, and non-specific effects of the pharmacologic agents. Biologic changes will be evaluated following treatment of the cells with JAK3 siRNA, including apoptosis, cell cycle changes, and soft agar colony formation. Also, alteration in downstream targets of JAK3 signaling will be examined. Importantly, changes in ABL kinase activity will be evaluated.

JAK3 siRNA will be also utilized in SCID mice implanted with CML cells, which will permit studying the effect of selective inhibition of JAK3 in CML *in vivo*.

#### **Aim (2): To identify target genes of JAK3 and STAT3 in p210 BCR-ABL-positive CML using cDNA microarray technique.**

The dominant negative STAT3 construct and JAK3 siRNA will be used to selectively block STAT3 and JAK3; respectively, and the changes in genomic profiling will be evaluated in p210 BCR-ABL-positive cell lines. Validation of the results will be confirmed by Western blot and RT-PCR.

**Aim (3): To identify the role of interleukins in activating JAK3-STAT3 signaling in p210 BCR-ABL-positive CML.**

Unlike other members of JAK family of kinases that can be activated by a large number of cytokines, JAK3 activation is limited to interleukins that possess the common gamma chain in their receptors; namely IL-2, IL-4, IL-7, IL-9, and IL-15. Previous studies have shown a role of IL-9 in the survival of myeloid neoplastic cells and progression of myeloid neoplasms. We are currently investigating the possible role of IL-9 in the constitutive activation of JAK3 in p210 BCR-ABL-positive CML cell lines using anti-IL-9 neutralizing antibody. Changes in pJAK3 and pSTAT3 levels will be examined. Changes in JAK3 and ABL kinase activity will be evaluated. In addition, occurrence of apoptotic cell death and cell cycle alterations will be investigated. We will also study the changes in down-stream targets of JAK3-STAT3 signaling in these cells after treatment with anti-IL-9 neutralizing antibody.

**The Mentors for my proposal:**

*Clinical Mentor:*

Jorge Cortes, MD  
Deputy Chairman and Associate Professor  
Department of Leukemia

*Basic Science Mentor:*

Ralph Arlinghaus, PhD  
Professor and Chairman  
Department of Molecular Pathology

**PATHOGENETIC ROLE OF JAK3-STAT3 SIGNALING IN p210 BCR-ABL-POSITIVE CHRONIC MYELOGENOUS LEUKEMIA.**

**Background:**

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor with oncogenic potential (1, 2). Selective inhibition of STAT3 signaling has recently been proposed to become a molecular target for cancer therapy (3). STAT3 normally resides in the cytoplasm. The phosphorylation of STAT3 at its Tyr<sup>705</sup> residue (pSTAT3<sup>Tyr705</sup>) leads to its activation, which is followed by dimerization and translocation to the nucleus. Thereafter, pSTAT3 binds to specific consensus DNA sequences located in the promoters of several genes, where it controls the transcription of several proteins known to directly affect cell survival and cell cycle progression. These genes include the *cyclin D* family, *cyclin E*, *Bcl-X<sub>L</sub>*, *Bcl-2*, *c-Jun*, *c-Kit*, *c-Myc*, *JunB*, *Bax*, *p21<sup>waf1/cip1</sup>*, *p27<sup>kip1</sup>*, *Mcl-1*, *Timp1*, *SOCS*, and *survivin*. Most likely, other still unidentified genes are under the influence of the transcriptional activity of pSTAT3. Three major systems are involved in the phosphorylation and activation of STAT3. The first is via interaction with surface receptors that possess innate tyrosine kinase activity, e.g., receptors of epidermal growth factor and platelet-derived growth factor. The second mechanism is mediated through the direct effect of tyrosine kinases primarily residing in the cytoplasm, e.g., Abl and Src. The third mechanism for STAT3 phosphorylation is via its interaction with members of receptor-associated Janus tyrosine kinases (JAK1, JAK2, JAK3, and TYK2) after docking to surface receptors that lack tyrosine kinase activity, such as interferons and interleukins receptors (4). Despite that the exact mechanism for JAK phosphorylation and activation is not completely understood, Abl has been proposed to play a significant role in JAK phosphorylation. Three naturally occurring major systems are known to negatively regulate JAK-STAT signaling. These systems include the suppressors of cytokine signaling (SOCS), proteins induced by activated STAT (PIAS), and SH-containing phosphatases (SHP).

Philadelphia-positive chronic myelogenous leukemia (CML) is the most frequently encountered chronic myeloproliferative disease and it accounts for 15-20% of all cases of leukemia. The molecular counterpart of the Philadelphia chromosome is the chimeric fusion gene *bcr-abl* that generates the p210 BCR-Abl protein, which possesses protein kinase activity and is responsible for the development of CML (5-7). BCR-Abl has been shown to induce its pathogenetic effects via interaction with several signaling pathways. Recently, the relationship between BCR-Abl and JAK-STAT signaling has been emphasized, as BCR-Abl appears to activate STATs, either directly or through the phosphorylation of JAKs (8, 9). Whereas the significance of STAT5 and STAT6, as well as JAK1 and JAK2 has been studied in some detail in CML, less studies have addressed the possible pathogenetic significance of STAT3 and JAK3 (10).

**Preliminary Results:**

We have utilized K562 and KBM5 cells that express the p210 BCR-Abl fusion protein characteristic of CML (11, 12). Using Western blotting we have shown that STAT3 and JAK3 and their activated forms (pSTAT3<sup>Tyr705</sup> and pJAK3<sup>Tyr980</sup>) are constitutively expressed in CML cell lines. Thereafter, we utilized 3 pharmacologic inhibitors of JAK3; AG490, WHI-P131, and WHI-P154. In addition, we used an adenoviral vector carrying the dominant negative construct (AdSTAT3DN) to specifically block the effects of STAT3. Inhibition of JAK3-STAT3 signaling induced down-regulation of pSTAT3 and pJAK3. The inhibitors caused

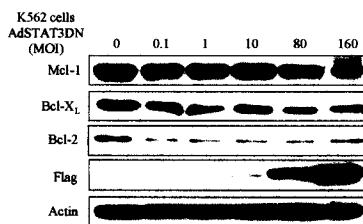
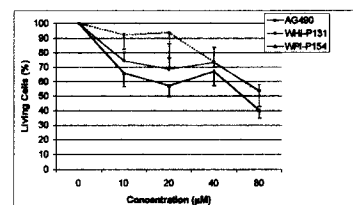


Figure (2)

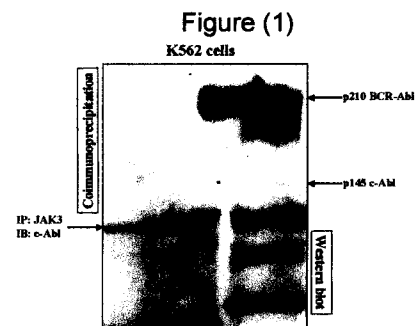


Figure (3)

concentration-dependent decrease in cell viability as demonstrated by staining the cells with trypan blue dye (Fig. 1 shows the effects of the pharmacologic inhibitors of JAK3 on the cell viability). K562 cells showed morphological changes consistent with apoptotic cell death, in the form of cell shrinkage and nuclear condensation, after treatment with JAK3 inhibitors or AdSTAT3DN. Western blotting showed significant changes in major antiapoptotic down-stream targets of JAK3-STAT3 signaling including Mcl-1, Bcl-X<sub>L</sub>, and Bcl-2, after treating the cells with JAK3-STAT3 signaling inhibitor (Fig. 2 shows the effects of AdSTAT3DN).  $\beta$ -actin is equal in all lanes and FLAG shows the gradual increase in intracellular AdSTAT3DN with increasing MOI (Fig. 2). Importantly, our coimmunoprecipitation study showed that JAK3 and p210 BCR-Abl are physically associated (Fig. 3), which implicates possible functional interaction between the two kinases.

**Specific Aims:**

**Aim (1): Analysis of the pathogenetic role of JAK3-STAT3 signaling in p210 BCR-Abl-positive CML cell lines and primary tumors:**

A) The frequency of expression of JAK3 and STAT3 and their activated forms, pSTAT3<sup>Tyr705</sup> and pJAK3<sup>Tyr980</sup>, will be evaluated in CML cell lines and primary tumors from patients in the different stages of the disease by using standard immunohistochemistry staining, Western blotting, and Northern blotting techniques. Significant correlation with survival or response to current therapeutic interventions will be statistically evaluated.

B) Functional relationship between JAK3 and p210 BCR-Abl will be explored in CML cell lines by analyzing the effects of specific inhibitors of Abl (STI571) and of JAK3 (e.g., WHI-P131 and WHI-P154) as well as siRNA to specifically block the effect of JAK3.

**Aim (2): Effect of inhibition of JAK3-STAT3 signaling in vivo using SCID mouse implanted with p210 BCR-Abl-positive CML cells:**

The effects of inhibition of JAK3-STAT3 signaling will be investigated *in vivo* using SCID mouse implanted with STI571-sensitive as well as STI571-resistant [KBM5-STI571(R1.0)] CML cell lines. Response to inhibition of JAK3-STAT3 signaling will be evaluated by morphologic examination and molecular studies of animal tissues including spleen, liver, and bone marrow.

**Aim (3): Expression and alterations of the naturally occurring inhibitors of JAK3-STAT3 signaling pathway in p210 BCR-Abl-positive CML cell lines and primary tumors:**

The frequency of expression of naturally occurring inhibitors of JAK3-STAT3 signaling, including SOCS, PIAS, and SHP, will be investigated in CML cell lines and primary tumors from patients. In addition, possible alterations by mutation and/or methylation of genes regulating these proteins will be evaluated.

**References:**

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