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Short Communication

The Btk inhibitor LFM-A13 is a potent inhibitor of Jak2 kinase activity

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Abstract

LFM-A13, or α-cyano-β-hydroxy-β-methyl-N-(2,5-dibromophenyl)propenamide, was shown to inhibit Bruton's tyrosine kinase (Btk). Here we show that LFM-A13 efficiently inhibits erythropoietin (Epo)-induced phosphorylation of the erythropoietin receptor, Janus kinase 2 (Jak2) and downstream signalling molecules. However, the tyrosine kinase activity of immunoprecipitated or *in vitro* translated Btk and Jak2 was equally inhibited by LFM-A13 in *in vitro* kinase assays. Finally, Epo-induced signal transduction was also inhibited in cells lacking Btk. Taken together, we conclude that LFM-A13 is a potent inhibitor of Jak2 and cannot be used as a specific tyrosine kinase inhibitor to study the role of Btk in Jak2-dependent cytokine signalling.

Keywords: cytokine signalling; erythropoiesis; tyrosine kinase.

Cytokine receptors are devoid of intrinsic kinase activity and require the association with cytoplasmic tyrosine kinases to transmit signals upon ligand binding. Janus tyrosine kinase 2 (Jak2) activity is crucial for most cytokine receptors including the erythropoietin receptor (EpoR; Parganas et al., 1998). Epo binding to the EpoR induces a conformational change which juxtaposes the associated Jak2 kinases resulting in cross-phosphorylation, activation and subsequent phosphorylation of the EpoR and induction of multiple signalling intermediates (for review see Wojchowski et al., 1999). These include signal transducer and activator of transciption 5 (Stat5), protein kinase B (PKB), the mitogen-activated protein kinase (Erk1/2) pathways but also tyrosine kinases like Lyn, a member of the Src-family, and Bruton's tyrosine kinase (Btk), a member of the Tec-family (Chin et al., 1998; Schmidt et al., 2004).

Tec-family members harbour an N-terminal pleckstrin homology (PH) domain, a proline-rich (PR) domain, a SRC homology 3 (SH3) domain, a Src homology 2 (SH2) domain and a kinase domain (SH1). Upon membrane recruitment via its PH domain, activation of Tec-family members occurs through phosphorylation of tyrosine residues in the kinase domain by Src kinases (amino acid 551 in Btk; Rawlings et al., 1996). This phosphorylation event is believed to release the intramolecular interaction between the PR and the SH3 domain, thereby releasing the kinase domain from structural restraints (Andreotti et al., 1997; Brazin et al., 2000). Trans-phosphorylation results in activation of the kinase and subsequent autophosphorylation of a tyrosine residue within the SH3 domain (amino acid 223 in Btk; Rawlings et al., 1996). Both membrane recruitment and kinase activation are of paramount importance for Btk function (Kurosaki and Kurosaki, 1997; Nisitani et al., 1999; Saito et al., 2001). Btk has been shown to play a role in phospholipaseC-γ1/2 activation (Takata and Kurosaki, 1996; Fluckiger et al., 1998; Rawlings, 1999) and cytoskeleton organisation via WASP (Guinamard et al., 1998). Furthermore, it has been shown that Tec and Jak1 are able to cross-phosphorylate each other, indicating that Tec family kinases may have a modulatory effect on Jak kinases or *vice versa* (Takahashi-Tezuka et al., 1997; Yamashita et al., 1998).

To analyse the role of Btk in EpoR signalling, we used the chemical compound α-cyano-β-hydroxy-β-methyl-N-(2,5-dibromophenyl)propenamide or in short LFM-A13. This inhibitor was reported to bind specifically to the catalytic pocket of the Btk kinase domain and not of other related tyrosine kinases like Jak1, Jak3 and Src kinases (Mahajan et al., 1999). Erythroid progenitors (cell line R10) were factor deprived in the presence and absence of 100 μM LFM-A13, the recommended concentration for this inhibitor (Mahajan et al., 1999), and subsequently stimulated with Epo or left unstimulated. Cells treated with LFM-A13 during starvation did not show any abnormalities with respect to cell shape and viability compared to untreated cells (data not shown). While Epo efficiently induced phosphorylation of the EpoR, Jak2, Btk, Stat5 and Erk1/2, this was significantly reduced in the presence of 100 μM LFM-A13 (Figure 1A, B). The co-immunoprecipitating bands in the EpoR immuno-precipitate representing Stat5 and Shc (Figure 1A; identified by specific antibodies) were absent when cells were pre-treated with LFM-A13. Thus LFM-A13 severely impairs EpoR phosphorylation and the recruitment of proteins. Since

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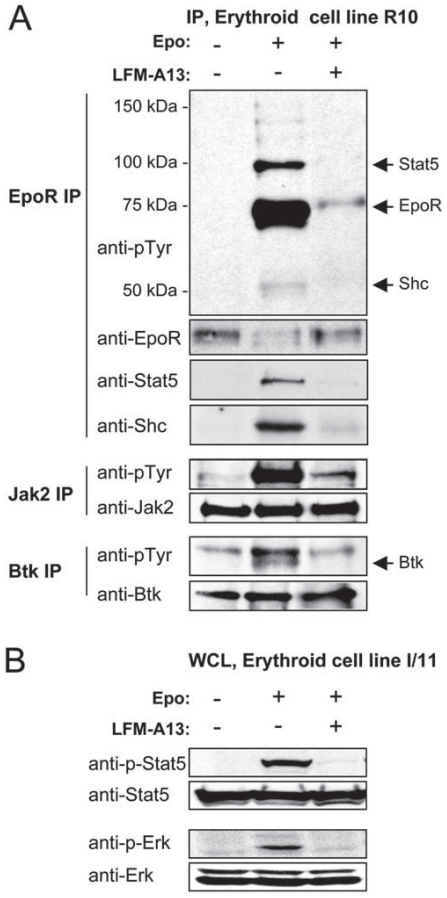

Figure 1 Epo-induced phosphorylation of EpoR, Jak2, Btk, Stat5 and Erk1/2 is inhibited by pre-treatment with 100 μM LFM-A13.

R10 cells were factor deprived in plain Iscoves medium (Invitrogen, Breda, The Netherlands) for 4 h in the presence or absence of LFM-A13 (100 μM) and subsequently stimulated with Epo (5 U/ml for 10 min; 40–80×10⁶ cells/ml) at 37°C. Ten volumes of ice-cold PBS were added to stop the reaction. Preparation of cell extracts, immunoprecipitations, SDS-polyacrylamide gel electrophoresis and Western blots were performed as described by van Dijk et al. (2000).

(A) The EpoR, Jak2 and Btk were immunoprecipitated to analyse Epo-induced phosphorylation on Western blots. The upper panels represent anti-phosphotyrosine stained blots; the lower panels represent the same blots re-probed with the antibody used in the immunoprecipitation (anti-EpoR, anti-Jak2 and anti-Btk) as indicated. In the EpoR immunoprecipitate the position of size markers is indicated and arrows indicate the EpoR and known proteins co-immunoprecipitating. Btk (lower band) is indicated with an arrow in the Btk immunoprecipitate. (B) In the upper panels, Stat5 and Erk1/2 phosphorylation was detected with phospho-specific antibodies in whole cell lysates, lower panels represent the total amount of Stat5 and Erk1/2 detected with anti-Stat5 and anti-Erk1/2, respectively. Antibodies against the murine EpoR (#SC-697), Erk1/2 (#SC-94), Stat5 (#SC-836) and phosphotyrosine (PY99, #SC-7020) were purchased from Santa Cruz (tebu-bio, Heerhugowaard, The Netherlands), phospho-specific Erk1/2 (#9106L) from Cell signalling (Westburg, Leusden, The Netherlands), antibodies against mouse Jak2 (#06–255), phospho-specific Stat5 (#05–495) from Upstate Biotechnology (Campro-Scientific, Veenendaal, The Netherlands). Recombinant human Epo was a kind gift from Ortho Biotech (Tilburg, The Netherlands). LFM-A13 was manufactured by Boehringer Ingelheim (Germany) as described by Mahajan et al. (1999).

we found that Btk is required for efficient signal transduction by the ligand-activated EpoR (Schmidt et al., 2004), we expected LFM-A13 to inhibit Epo-induced signal transduction.

However, the strong effect of LFM-A13 on Epo-induced signalling could also be due to direct inhibition of Jak2 by LFM-A13. To investigate whether LFM-A13 could act directly on Jak2, Btk and Jak2 were immunoprecipitated from lysates of factor-deprived cells and subjected to *in vitro* kinase assays in the presence of increasing amounts of LFM-A13. Surprisingly, the kinase activity of Btk and Jak2 (auto-phosphorylation) appeared to be inhibited with similar dose-response curves. Both kinases were already inhibited at 10 μM LFM-A13, while complete inhibition occurred at 200 μM LFM-A13 (Figure 2A). The same results were obtained when Btk and Jak2 were immunoprecipitated from Epo-stimulated cells (Figure 2B). Epo stimulation leads to Btk and Jak2 phosphorylation, resulting in a lower pool of non-phosphorylated Btk or Jak2, hence explaining the lower auto-phosphorylation in the Epo-stimulated lanes. These data suggest that LFM-A13 directly inhibits Jak2 kinase activity.

However, it could still be possible that the effect of LFM-A13 on Jak2 is mediated by Btk. If both proteins interact with each other, the presence of Btk in the Jak2 immunoprecipitate could affect Jak2 auto-phosphorylation. To rule out Btk interference in the *in vitro* kinase assay, we used *in vitro* transcription/translation (ITT) to synthesise recombinant Jak2 that was subsequently subjected to an *in vitro* kinase assay in presence or absence of LFM-A13. Expression of Jak2 was detected by immunoblotting using anti-Jak2 antibodies. Jak2 was

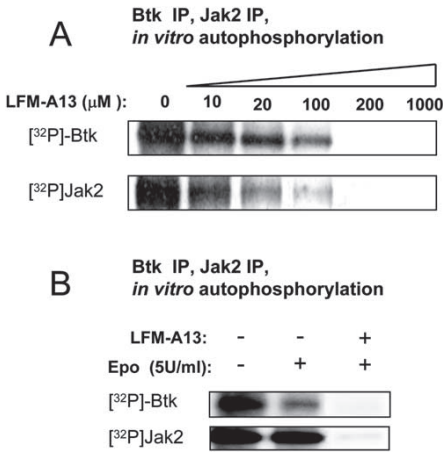

Figure 2 LFM-A13 inhibits *in vitro* auto-phosphorylation of Btk and Jak2.

(A) Jak2 and Btk were immunoprecipitated from factor-depleted erythroid progenitors (R10) and tested for auto-phosphorylation in an *in vitro* kinase assay. Sepharose G beads (Sigma, Zwÿndrecht, The Netherlands) with immunoprecipitated Jak2 and Btk complexes were split into 6 equal portions and subsequently subjected to an *in vitro* kinase assays as described by von Lindern et al. (2000) in the presence of increasing concentrations LFM-A13 as indicated on the top. (B) Jak2 and Btk were immunoprecipitated from cells non-stimulated (−) or Epo-stimulated (+; 5 U/ml) and similarly subjected to an *in vitro* kinase assay. 100 μM LFM-A13 was added to precipitates from stimulated cells.

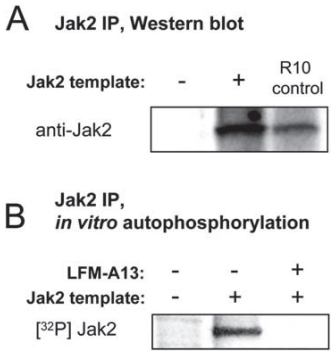

Figure 3 LFM-A13 inhibits auto-phosphorylation of recombinant Jak2 produced by *in vitro* transcription/translation (ITT).
The *in vitro* transcription/translation was carried out using the TnT® T_7 coupled reticulocyte lysate system (#L4611; Promega, Leiden, The Netherlands) according to the manufacturer's protocol.
(A) Jak2 was immunoprecipitated from ITT reactions in the absence (lane1) or presence (lane2) of Jak2 template or from the erythroid progenitor cell line R10 and analysed for their presence on Western blots using an anti-Jak2 antibody. (B) To test the effect of LFM-A13 on recombinant Jak2, immunoprecipitated ITT Jak2 was subjected to an *in vitro* kinase assay in the absence and presence of 100 μM LFM-A13 (lanes 2 and 3). Lane 1 represents Jak2 immunoprecipitate on ITT-lysate with no Jak2 template present.

only present when the template was added to the ITT and the mobility of recombinant Jak2 was the same as immunoprecipitated Jak2 when compared to the erythroid progenitor cell line R10 (Figure 3A). Auto-phosphorylation of *in vitro* synthesised Jak2 in an *in vitro* kinase assay was fully inhibited by 100 μM LFM-A13 (Figure 3B). This proves that LFM-A13 directly inhibits Jak2 kinase activity.

Finally, we tested the effect of LFM-A13 in cells devoid of Btk. Immortalised cultures of erythroid progenitors established from Btk⁻ or Btk*wt*, p53-deficient bone marrow cells, were factor deprived in the presence or absence of 100 μM LFM-A13 and stimulated with Epo or left unstimulated. Epo-induced phosphorylation of the EpoR is impaired in Btk-deficient cells (Schmidt et al., 2004; Figure 4A). However, the remaining Epo-induced phosphorylation of the EpoR is still blocked by LFM-A13. Importantly, Epo-induced phosphorylation of Jak2, Stat5 and Erk1/2, was clearly inhibited by 100 μM LFM-A13 in Btk-deficient cells (Figure 4A, B), providing further evidence that LFM-A13 directly affects Jak2, independently of Btk. Finally, we tested whether LFM-A13 still showed some of its alleged specificity. COS cells were transfected in duplo with the kinases Jak2, Tec, Btk and Lyn. Half of the cells were treated with LFM-A13, the other half left untreated. Cell lysates were harvested and examined for auto-phosphorylated kinase by Western blotting. LFM-A13 inhibited auto-phosphorylation of Jak2, Tec and Btk, but it did not affect Lyn kinase auto-phosphorylation (Figure 5). Thus, LFM-A13 does not inhibit the activity of all tyrosine kinases.

Both Jak2 and Tec-family kinases, including Btk, are prominent cytoplasmic tyrosine kinases in hematopoietic cells which co-operate in signalling pathways activated by e.g. cytokine receptors, the B cell receptor and the

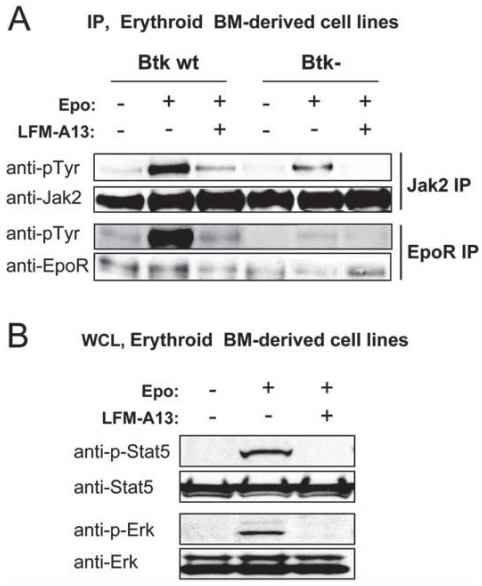

Figure 4 LFM-A13 inhibits Jak2, Stat5 and Erk1/2 phosphorylation in Btk-deficient erythroid progenitor cells.
The murine erythroid cell lines 2B6 (Btk*wt*) and 3G4 (Btk⁻) were established from p53-deficient mouse bone marrow of *wt* and Btk-deficient mice, respectively, as previously described (von Lindern et al., 2001; Schmidt et al., 2004). These Btk-deficient cells were factor-depleted in the presence and absence of 100 μM LFM-A13 as indicated and stimulated with Epo (5 U/ml; 10 min) where indicated.
(A) The EpoR and Jak2 proteins were immunoprecipitated and assayed for tyrosine phosphorylation [anti-p-Tyr (PY99); upper panels] and for the presence of the EpoR and Jak2 by specific antibodies. (B) Lysates of the Btk⁻ cells (3G4) were assayed on Western blots using phospho-specific antibodies for Stat5 or Erk1/2. The lower panels indicate the same blots re-probed with anti-Stat5 or anti-Erk1/2 antibodies to check for equal loading.

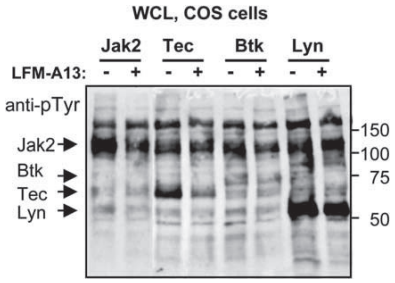

Figure 5 LFM-A13 inihibits Jak2, Tec and Btk, but not Lyn auto-phosphorylation in COS cells.
COS cells were transfected with pSG5-based expression plasmids encoding Jak2, Tec, Btk or Lyn, using calcium-phosphate precipitates as previously described (Van Dijk et al., 2000). Two dishes were used for each construct and LFM-A13 (100 μM) was added 24 h after transfection to one of the dishes. Forty-eight h after transfection the cells were lysed and total cell lysates were examined for phosphorylated proteins on a Western blot using the anti-phosphotyrosine antibody PY99 (anti-pTyr). The kinases expressed in COS cells are indicated on top of the lanes, the position of the kinases is indicated by arrows. The position and of size markers is indicated at the right hand side (molecular mass in kDa).

Fc_ε receptor (Qiu et al., 2000). Moreover, Jak1 and Tec can associate and cross-phosphorylate each other (Takahashi-Tezuka et al., 1997) and we recently demonstrated that Btk similarly associates with Jak2 (Schmidt et al., 2004). Since lack of Btk impairs EpoR signalling, the effect of LFM-A13 on Epo-induced signal transduction was initially expected. However, since LFM-A13 inhibited intrinsic auto-phosphorylation of cellular or recombinant Jak2 in *in vitro* kinase assays and significantly decreased Epo-induced Jak2 phosphorylation in cells lacking Btk, we conclude that LFM-A13 directly inhibits Jak2 phosphorylation independent of its effect on Btk.

It was previously reported that the kinase activity of Jak1 and Jak3, close homologues of Jak2, was not inhibited by high concentrations (139 to 278 μM) of LFM-A13 in *in vitro* kinase assays (Mahajan et al., 1999). Importantly, the sequences of the kinase domains of Jak kinases are highly conserved. LFM-A13 incorporation into the catalytic site of Btk requires hydrogen bonds between the side chain of LFM-A13 and Arg^{525} and Asp^{539} of Btk and a hydrophobic pocket for the aromatic ring. The kinase domains of Btk and Jak kinases both contain arginine and aspartic acid residues at comparable positions, but there are several differences regarding the hydrophobic pocket trapping the aromatic ring of LFM-A13. Mahajan et al. (1999) discussed the consequences of these differences with respect to LFM-A13 binding affinity, but complete understanding of these affinities is still elusive. Jak1 and Jak3 kinase activity was shown to be unaffected by LFM-A13 (Mahajan et al., 1999) but no data was shown for Jak2, leaving open the possibility that Jak2 is the exception in the Jak kinase family with respect to LFM-A13 sensitivity.

In conclusion, we have shown that LFM-A13 is not specific for Btk but also inhibits Jak2 kinase activity *in vitro* and *in vivo*. LFM-A13 is proposed to be a useful anti-leukaemic and anti-thrombotic agent (Uckun, 2002, 2003). Although the successful use of STI571 to treat chronic myelogenous leukaemia proves that an inhibitor does not necessarily need to be specific in order to be effective (Mauro, 2002), it is important that its specificity is known. Using LFM-A13 to elucidate the role of Btk in signal transduction of Jak2-dependent cytokine receptors could result in wrong conclusions due to simultaneous inhibition of Jak2 and Btk by LFM-A13.

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