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Role of Bruton's Tyrosine Kinase inhibitors in HIV-1 infected cells

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Abstract

Many cellular cofactors have been documented to be critical for various stages of viral replication. Using high throughput proteomic assays, we have previously identified Bruton's tyrosine kinase (BTK) as a host protein that was uniquely up-regulated in the plasma membrane of HIV-1 infected T-cells. Here, we have further characterized the BTK expression in HIV-1 infection and show that this cellular factor is specifically expressed in infected myeloid cells. Significant up-regulation of the phosphorylated form of BTK was observed in infected cells. Using size exclusion chromatography, we found BTK to be virtually absent in the uninfected U937 cells, however new BTK protein complexes were identified and distributed in both high molecular weight (~600 kDa) and a small molecular weight complex (~60–120 kDa) in the infected U1 cells. BTK levels were highest in cells either chronically expressing virus or induced/infected myeloid cells and that BTK translocated to the membrane following induction of the infected cells. BTK knockdown in HIV-1 infected cells using siRNA resulted in selective death of infected, but not uninfected, cells. Using BTK specific antibody and small molecule inhibitors including LFM-A13 and a FDA approved compound, Ibrutinib (PCI – 32765), we have found that HIV-1 infected cells are sensitive to apoptotic cell death and result in a decrease in virus production. Overall, our data suggests that HIV-1 infected cells are sensitive to treatments targeting BTK expressed in infected cells.

Keywords

HIV-1 replication; BTK; host cell protein; inhibitor; cell death

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Conflict of interest

The authors declare that they have no conflict of interest.

Introduction

The human immunodeficiency virus (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS) that infected more than 60 million people worldwide (UNAIDS Report 2012). Despite the success of combined antiretroviral therapy (cART) in treating HIV-1 infection and preventing progression to AIDS (Bagasra, 2006; Chun *et al*, 1999; Cohen, 2005; Jordan *et al*, 2003), limitations include the generation of resistant viral strains. Moreover, even though the cART is efficient in eradicating the circulating virus in plasma by inhibiting productive infection, the cART is not able to completely inhibit virus production from the infected cells (Richman *et al*, 2009). HIV-1 is capable of maintaining latent infection in reservoirs such as resting CD4⁺ memory and naive T cells, myeloid cells and CD34⁺ multipotent hematopoietic stem cells (Carter *et al*, 2010; Cobos-Jimenez *et al*, 2011; Evans *et al*, 2013; Gougeon *et al*, 2012; Wightman *et al*, 2010). Thus, the identification of new markers of non-productive HIV-1 infection as well as determination of the new potential targets for therapeutic interventions is the critical tasks of the current HIV/AIDS research.

Using comparative proteomics analysis, we have previously identified the Bruton's tyrosine kinase (BTK) as one of the 17 candidate proteins unique to latent HIV-1 infected T-cells, where BTK was localized to the cytoplasm and translocated to the plasma membrane upon viral activation (Berro *et al*, 2007). BTK is a member of the Tec family of non-receptor protein tyrosine kinases, and is encompassed within the Src superfamily, an important regulator of lymphocyte signaling (Berg *et al*, 2005; Mano, 1999). BTK is structurally characterized to contain the Src homology-1 (SH1) tyrosine kinase domain and additional protein-protein interacting domains SH2 and SH3 (Lindvall *et al*, 2005). Moreover, it harbors a pleckstrin homology (PH) domain located at the N-terminus which permits PH domain-mediated BTK translocation to the plasma membrane (Mohamed *et al*, 1999; Salim *et al*, 1996; Varnai *et al*, 1999). BTK characterization studies have been mostly performed in B cells, where it has been shown to influence multiple signaling pathways including calcium influx, cytoskeletal remodeling, apoptosis, survival, proliferation, differentiation, and gene expression (Contreras *et al*, 2007; Fluckiger *et al*, 1998; Liu *et al*, 2011; Matsuda *et al*, 2009; Satterthwaite *et al*, 1998; Uckun, 1998). Of interest, BTK has been shown to play a dual role in cell survival and apoptosis in a cell-type specific manner, conferring an anti-apoptotic effect in B cells while promoting apoptosis in HeLa cells (Islam and Smith, 2000; Uckun, 1998). BTK is primarily a cytoplasmic enzyme but can be recruited to the plasma membrane as a result of phosphoinositide 3-kinase (PI3K) activation in a PI3K-dependent manner (Mohamed *et al*, 1999; Mohamed *et al*, 2000; Salim *et al*, 1996; Scharenberg and Kinet, 1998; Varnai *et al*, 1999). PH-mediated membrane recruitment occurs through binding to phosphatidylinositol-3,4,5-trisphosphate (PIP₃) after PI3K activation. BTK is required for proper tyrosine phosphorylation and activation of phospholipase C- γ (PLC- γ) which catalyzes the formation of inositol-3-4-5 triphosphate (IP₃) and diacylglycerol, which in turn mediate calcium mobilization and activation of protein kinase C (PKC), respectively (Scharenberg and Kinet, 1998). The IP₃ and PKC pathways lead to activation of transcription factors such as NF- κ B, BAP-135 and TFII-I, that regulate gene expression required for B cell survival and cell cycle progression (Bajpai *et al*, 2000; Novina *et al*,

1999; Petro *et al*, 2000; Yang and Desiderio, 1997). In addition to a cytoplasmic and membrane location, BTK was also found in the nucleus (Mohamed *et al*, 2000; Saito *et al*, 2003; Webb *et al*, 2000b). Regulation of the nuclear translocation of this protein remains to be explored. However, a recent study showed that BTK nucleocytoplasmic shuttling requires its interaction with Lyn-interacting ankyrin repeat protein (Liar), utilizing a Crm1-mediated mechanism of nuclear export (Gustafsson *et al*, 2012).

In the present study we have characterized the expression profiles of BTK in different chronically HIV-1 infected cells including monocytes/macrophages. We demonstrate that BTK is upregulated during HIV-1 replication. Relevant to the BTK membrane translocation and nuclear shuttling activities observed earlier in B cells, we show that treatment with the PI3K inhibitors LY294002 and wortmannin in the presence of activated HIV-1 lead to decreased BTK translocation to the plasma membrane (Berro *et al*, 2007). We also show that selective depletion or inhibition of BTK kinase activity results in apoptosis of infected, but not uninfected cells. Taken together, our results suggest that viral regulation of receptor signaling can be mediated by redistribution of cytoplasmic and probably nuclear BTK to the cell membrane. Since BTK inactivation leads to selective apoptosis of HIV-1 infected cells, targeting the host BTK pathway could potentially serve as an attractive host-based therapeutic strategy for the elimination of latent HIV-1 infection.

Results

BTK expression is induced in HIV-1 infected cells

Using membrane proteomics analysis of HIV-1 infected T-cells, we have previously identified that amongst other proteins, BTK is upregulated in infected cells (Berro *et al*, 2007). In order to further explore the BTK expression profile during infection, western blot was performed in various cell types including uninfected (CEM and Jurkat) and HIV-1 infected T cells (positive control; ACH2 and J1.1), matched uninfected and infected monocytic cells (U937 and U1), and PMA-activated U937 and U1 cells that are considered as a model of monocyte derived macrophages (MDM model: U937 mΦ/U1 mΦ). The B cell line (BJAB) was used as a positive control for BTK expression. Results shown in Figure 1A indicate that BTK levels are much higher in the HIV-1 infected cells when compared to the uninfected counterparts for T cells, monocytes and MDM model cells. These results further show selective upregulation of BTK across a panel of cell lines that are chronically infected with HIV-1. To further examine BTK upregulation during *de novo* infection, we transfected the infectious HIV-1 clone pNL4.3 into Jurkat T cells (control) and monocytic U937 cells, and assessed the BTK distribution status by western blot. Results in Figure 1B indicate that there is significant upregulation of BTK expression at 48 hours post-infection (lane 2) when compared to mock-treated cells (lane 1). Even though BTK expression was detectable in the both uninfected T and monocytic cells, we observed significant upregulation of the phosphorylated form of BTK in all infected cells including both chronically (Fig. 1A) and newly infected (Fig. 1B) cell lines. Previously, we have shown in a latency model that increased BTK phosphorylation may be linked to its HIV-1-specific translocation and enrichment at the plasma membrane, where it becomes further phosphorylated (Berro *et al*, 2007). Finally, we were interested in exploring whether BTK localization was differently

affected in HIV-1 infected cells. The western blot of cytoplasmic vs nuclear extracts, shown in Figure 1C revealed that BTK levels were increased in infection both at the cytoplasmic and nuclear fractions (lanes 2 and 4) when compared to uninfected fractions (lanes 1 and 3). Interestingly, BTK found in the cytoplasm of infected cells was mainly phosphorylated, whereas both phosphorylated and unphosphorylated forms of BTK were present in the nucleus. In contrast, BTK is found mainly in the nucleus of uninfected cells in an unphosphorylated form, with little to no BTK detectable in the cytoplasm. Thus, increased phosphorylation in *de novo* infection may be indicative of increased BTK activation and possible translocation to the plasma membrane where it partakes in the BTK signalosome, modulating signal transduction in a pro- or anti-apoptotic pathway (Islam and Smith, 2000).

We finally asked whether BTK associated complexes could be modified between infected and uninfected cells. Our rationale came from our previous work where complexes such as Cdk9/T1 complex, GSK, and IKK were present in both large and small complexes after infection (Guendel *et al.*, 2013; Kehn-Hall *et al.*, 2011; Narayanan *et al.*, 2012). Importantly, these novel small complexes were highly kinase active and were formed only after infection. Here, we used total cell lysates of both uninfected U937 and infected U1 cells and loaded onto a sizing column in the presence of high salt to minimize non-specific binding (500 mM NaCl). FPLC fractions were then precipitated and used for western blot analysis. Results of such an experiment are shown in Figure 1D, where BTK was virtually absent in the uninfected U937 cells, however the BTK protein complexes were distributed in both high molecular weight (~600 kDa) and a small molecular weight complex (~60–120 kDa, possibly the phosphorylated form) in the infected U1 cells. We also observed more actin distribution in the U1 infected cells compared to uninfected cells. The initial loading of actin onto the column was similar between infected and uninfected cells (lane 1). These data on distribution of BTK between large and small complexes further indicate that infection may cause a distribution between both active and inactive forms of BTK in cell.

Presence of BTK on cell surface membrane

We next examined the presence of BTK protein on both chronically infected and latent cells. We used J1-1 cells (Jurkat T-cell background) where wild type virus is secreted into the media from these cells and compared to U1 cells, which contain 2 copies of silent viruses (one wild type and one mutant). In both cases cells were grown to mid-log phase of growth and stained with BTK antibody, without any fixation. We also used U937 as control cells for infected U1 cells either with or without PMA treatment. Results in Figure 2A show that J1-1 cells (which actively secrete virus) have a ~5 fold induction and presence of BTK on the membrane. Interestingly, U1 myeloid cells, that don't secrete virus, had minimal BTK on the surface, but upon activation BTK levels increased by about ~5 fold (Panel C) as compared to just a 70% increase for uninfected U937 cells. It is interesting to note that even though U1 cells contained higher levels of BTK protein, as determined by sizing column and western blots (Figure 1A and D), most of the protein becomes membrane associated after viral induction. Collectively, these results indicate that the BTK levels were highest in cells either chronically expressing virus or induced/infected myeloid cells and that BTK translocates to the membrane following induction of the infected cells.

Selective BTK depletion induces apoptosis

Due to dual role of BTK regarding apoptosis, we were interested to determine if inhibiting BTK expression through siRNA knockdown would have an effect on cell survival. The HLM-1 cells (Sadaie *et al*, 1990) were transfected with two siRNAs against BTK (BTK #1 and BTK #2) and cultured for 24, 48, and 72 hrs post-transfection. In the absence of induction, HLM-1 cell line is negative for virus particle production but displays basal transcription (i.e. latent phenotype). Small interfering RNA against luciferase was used as a negative control. Results in Figure 3A indicate that BTK #1 siRNA transiently but dramatically decreased the presence of phosphorylated BTK at 24 and 48 hrs post-transfection, respectively (lanes 4 and 5), while BTK #2 siRNA decreased level of this BTK form only at 24 hours (lane 7). The non-phosphorylated BTK was detected as a minor form of this enzyme in these cells. To evaluate if loss of BTK resulted in apoptosis in these cells, we performed western blot analysis against caspase 3 and PARP, canonical markers of the apoptotic cascade activation. The activation of caspase 3 leads to downstream cleavage events of cytoplasmic and nuclear substrates including PARP, attributing morphological features of apoptotic cell death (Huppertz *et al*, 1999). Results in Figure 3B indicate that upon BTK selective depletion, cleavage of both procaspase-3 and PARP was observed (lanes 2–3 and 5–6) in contrast to their respective luciferase control samples (lanes 1 and 4) or when the RNA-mediated knockdown had resolved at 72 hours post-transfection (lanes 8–9). As a further confirmation, we then treated HLM-1 cells with either siRNA and performed a cell viability assay. Results in Figure 3C indicate that either siRNAs were capable of inhibiting or apoptosing cell growth after 72 hrs of treatment. Taken together, these data indicate that selective down-regulation of BTK, and especially the phosphorylated form of this enzyme, triggers programmed cell death pathways suggesting that BTK may play an anti-apoptotic role in HIV-1 infected cells.

Treatment with BTK antibody results in selective death of HIV-1 infected cells

Monoclonal antibodies against plasma membrane proteins that are overexpressed in cancer cells, such as VEGF and EGFR, have been successfully used as targeted therapeutics (Ludwig *et al*, 2003). In the case of HIV-1 infection, there are currently two humanized monoclonal antibodies for CCR5, Pro-140 and HGS Ab004, which inhibit viral entry (Castagna *et al*, 2005; Lederman *et al*, 2006) that are in clinical trial. Since BTK localizes specifically to the plasma membrane of HIV-1 infected cells, we tested whether antibody treatment against BTK could induce cell death in HIV-1 infected cell lines. A variety of uninfected and infected cell lines were incubated with a titration of monoclonal BTK antibody or IgG as a non-specific target control. Twenty-four hours post-treatment, trypan blue staining and CellTiter-Glo assays were used to assess cellular viability or growth. Results in Figure 4A depict a panel of cells treated with α -BTK antibodies and control IgG for 24 hours. Infected T cells (control; ACH2 and J1.1) and monocytes (U1) displayed >35% loss of cell viability upon treatment with BTK antibody, whereas corresponding uninfected cells were largely unaffected by the treatment. Similarly, reproduction of these results in uninfected Jurkat and infected J1.1 cells indicated a preferential decrease in viability of the HIV-1 infected cells upon exposure to α -BTK antibody (Figure 4B). Moreover, to confirm these observations in an inducible system, the activation of viral

replication in HLM-1 cells was stimulated with the HDAC inhibitor sodium butyrate (NaB) for 72 hours prior to treatment with BTK antibody. Results in Figure 4C indicate that at 48 hours post-treatment, cells which had undergone viral induction were more susceptible to selective BTK antibody cell killing. We have previously shown that viral induction in HLM-1 cells results in virus-specific translocation of BTK to the plasma membrane (Berro *et al*, 2007). These data suggest a feasible correlation between BTK localization in the presence of active HIV-1 and susceptibility to therapeutic targeting of BTK.

We have previously identified the anti-apoptotic protein X-linked inhibitor of apoptosis (XIAP) as one of the proteins enriched in the membrane fractions of latently infected cells (Berro *et al*, 2007). To further test the effect of BTK antibody on infected cells, we subjected U937 and U1 MDM cell models to treatment with antibodies against BTK and XIAP. Antibody treatment against CD45 was used as a positive control for macrophage-specific induced cell death (Steff *et al*, 2003). Six days post-treatment, we tested the effect of antibodies on cell viability by using CellTiter-Glo assay. Results in Figure 4D indicate that treatment with anti-CD45 antibody effectively induced cell death in both uninfected (92% cell death) and infected cells (96% cell death). Furthermore, BTK antibody treatment resulted in decreased viability only in the infected U1 MDM cell model (87% cell death). Interestingly, XIAP antibody treatment did not induce cell death in either U937 or U1 MDMs, suggesting that the regulatory mechanism of cell apoptosis exerted by HIV-1 via XIAP is different from that of BTK. Collectively, these results indicate that HIV-1 infected cells are more sensitive to BTK antibody treatment possibly due to the specific upregulation of BTK in infected cells.

BTK antibody treatment results in decreased HIV-1 viral replication

Therapeutic approaches that result in the killing of infected cells may affect release of immature viral particles into the extracellular space or maturation of the virions. Therefore, we next asked whether the treatment of infected cells with α -BTK antibody could modulate viral release after triggering cell death. Our rationale for these experiments came from previous findings that certain treatments that stimulate apoptosis may both decrease or increase viral replication. For instance, our earlier study showed that irradiation of chronically infected cells increases apoptosis, but also simultaneously activates cells and induces viral replication and release (Clark *et al*, 2000). We treated both HLM-1 cells that have undergone viral activation and the chronically infected J1.1 T cells that produce HIV-1 without additional activation with either α -BTK antibody or control IgG. Antibody treatment was performed once and supernatants were collected two days post-treatment for RT activity. Results in Figure 5A indicate that BTK antibody treatment inhibited viral replication in HLM-1 cells. Likewise, α -BTK also decreased RT activity in the supernatant from chronically infected J1.1 cells (panel B). Finally, we asked whether genomic RNA levels also decreased following antibody treatment. Experiments in panel A and B used reverse transcriptase protein as a read out of the functional viral particles in the supernatant, however unspliced RNA molecules were not assayed following antibody treatment. Experiments in panel C and D using both HLM-1 and J1.1 cells showed reduced levels of genomic RNA following BTK antibody treatment. Interestingly IgG treatment showed an increase of viral RNA levels but not RT activity in the supernatants, indicating that IgG

alone could increase RNA levels, but not functional virus. Taken together, these results suggest that BTK antibody treatment selectively induces apoptosis in infected cells and also decreases viral shedding from these cells.

BTK antibody treatment induces cell death in HIV-1 infected PBMCs

To determine whether BTK antibody treatment had an effect on primary cells, we infected PHA/IL-2 activated PBMCs with the dual tropic HIV-1 89.6 virus and subsequently treated once with either BTK antibody or control IgG. Uninfected PBMCs were also treated in parallel as controls. On day 10, cells were stained with propidium iodide and analyzed by flow cytometry to determine whether antibody treatment resulted in alteration of the cell cycle or induction of apoptosis. Results in Figure 6A show that the treatment with BTK antibody induced substantial apoptosis (73.78%) in infected PBMCs (upper panel), while the uninfected PBMCs remained unaffected (3.97% apoptosis, lower panel). Interestingly, antibody-mediated suppression of membrane-localized BTK resulted in complete death of the cells in G₀/G₁ and G₂/M stages of the cell cycle, whereas the cells with replicating DNA in the S phase of the cell cycle remained viable (Fig. 6B), suggesting either reduced exposure of BTK on the plasma membrane of these cells or that the BTK activity is not essential for viability of the cells in the S phase. Earlier studies of cancer cells treated with the BTK inhibitor LFM-A13 have been reported to exhibit a G₂/M-arrest during cell cycle progression and lower level of cell death (Uckun, 2007), indicating that stronger pro-apoptotic effect in our experiment could be associated with HIV-1 activation. Collectively, the present data indicate that targeting BTK may result in death of HIV-1 infected primary cells. In addition, these data further suggest that BTK antibody treatment does not exhibit apparent toxicity to uninfected primary cells.

The BTK inhibitor LFM-A13 and Ibrutinib induce cell death in HIV-1 infected, but not in uninfected cells

In addition to therapeutic effects observed by monoclonal α -BTK antibody treatment on HIV-1 infected cells, we were interested in exploring treatment effects with commercially available small molecule inhibitors such as alpha-cyano-beta-hydroxy-beta-methyl-N-(2,5-dibromophenyl)propenamide or LFM-A13. LFM-A13 is a leflunomide metabolite analog designed to target the TEC family protein tyrosine kinase BTK (Mahajan *et al*, 1999; Uckun *et al*, 2003). To determine the effect of the specific pharmacological BTK inhibitor LFM-A13 on cell viability, uninfected CEM and Jurkat and HIV-1 chronically infected T cells, alongside the uninfected monocytic U937 and infected U1 cells were subjected to LFM-A13 or vehicle for 72 hours. Results in Figure 7A show that LFM-A13 decreased cell viability in a dose-dependent manner in HIV-1 infected cells when compared to DMSO-treated cells. BTK was originally identified in B cells and it has been found more recently in myeloid cells, including monocytes, macrophages, neutrophils, and mast cells (Mohamed *et al*, 2009). Thus, the BJAB B cell line was used as a positive control for compound toxicity in cells that constitutively expresses the target protein. The compound did not show cell toxicity in B cells or towards the other uninfected cells, that confirm our earlier conclusions about the importance of BTK activity for the viability of HIV-1 infected, but not uninfected cells. Interestingly, we observed the strongest effect of BTK inhibition on the cell viability in the ACH2 and U1 HIV-1 infected cells, which produce minimal amount of HIV-1

particles (Clark *et al.*, 2000), whereas the effect of LFM-A13 was minimal on the J1.1 cells that persistently produces higher amount of HIV-1 virions without activation.

To complement the effects of LFM-A13 observed on the infected cells, we explored its effect on the viral replication. LFM-A13 treatment was performed once on infected cells, and supernatants were collected at day 3 for RT analysis. Results in Figure 7B indicate that LFM-A13 triggers a dose-dependent decrease of viral replication in infected cells. These results are consistent with anti-BTK antibody treatment (Fig. 4 and 5) where BTK inhibition also induced death of HIV-1 infected cells and simultaneous decrease of viral production. We next utilized another BTK inhibitor, Ibrutinib (PCI – 32765), that was in a Phase III clinical trial for treatment of chronic lymphocytic leukemia (CLL) and recently approved by the FDA. We treated infected and uninfected cells with three concentrations of Ibrutinib for 24 and 48 hours. Results in Figure 7C indicate that HIV-1 infected cells were very sensitive to Ibrutinib as evident by the increased apoptosis. Similar to LFM-A13, Ibrutinib showed a decrease in the intracellular p24 expression (using western blot) by more than 90% in infected cells (data not shown). Finally we compared both LFM-A13 and Ibrutinib side by side in cells and looked for presence of HIV-1 genomic RNA. Results in panel D indicate that both LFM-A13 and Ibrutinib inhibited genomic RNA present in the supernatants of infected cells in a dose-dependent manner. Collectively, these data point to the use of various BTK inhibitor drugs as an alternative means of cell killing in infected cells.

Target of BTK inhibitor in infected cells

To characterize the putative mechanisms by which LFM-A13 could exert its effects on HIV-1 infected cells, we analyzed uninfected and chronically infected cells by reverse phase protein microarray (RPMA) technology. Cells were treated with LFM-A13 or DMSO for 24 hrs prior to preparation of the cell lysate. For phosphoproteomic analysis, cell lysates were immobilized by printing onto nitrocellulose membrane slides and probed with antibodies specific for phosphorylated or total forms of signaling proteins. The antibodies were selected to screen molecular networks involved in host responses believed to be affected by viral infection, specifically immune response, cell survival and apoptosis, inflammation and cell growth, and differentiation. RPMA has been extensively validated in previous studies regarding antibody specificity, sensitivity, and accuracy of phosphoprotein detection in cell lysates (Wulfschlegel *et al.*, 2006). Moreover, RPMA has been recently used to characterize the phosphoproteome and signaling networks induced by Rift Valley Fever Virus infection (Popova *et al.*, 2010). Table 1 shows a summary of significant changes in signal transduction upon LFM-A13 treatment of infected cells. Changes in protein expression were normalized to DMSO treated infected and LFM-A13 treated uninfected cells. To map these signaling pathways and discover important signaling pathway nodes mediated by LFM-A13, we annotated a narrow protein-protein interaction network (Fig. 8). Although PI3K was not identified as a LFM-A13 responsive node, it is functionally linked to the BTK signalosome and subsequent effector proteins. As shown in the signaling pathways, tyrosine kinases PI3K/AKT cascade, effector kinases serine/threonine GSK-3 β and Aurora, p90RSK, substrates BAD, MDM2, c-Myc, mTOR, and microtubule dynamics-associated protein LC3B, were involved in the LFM-A13-mediated phosphorylation signaling pathways. Taken together, these data demonstrate that BTK inhibitor LFM-A13 induces cell death in HIV-1

infected cells suggesting that the cell death is induced largely through modulation of the PI3K/AKT signaling network.

Discussion

Current cART uses selective inhibitors that target only viral proteins. Novel targeted host-based therapies serve as an attractive approach for the development of new treatment alternatives as well as to advance the understanding of disease mechanisms. In this study we have further characterized BTK as a cellular factor important for HIV-1 replication and viability of infected cells. The demonstrated role of BTK in HIV-1 infection along with the undetectable effect on uninfected cells supports this host protein as a potential new target for anti-HIV interventions.

BTK was originally identified as the mutated gene product in X-linked agammaglobulinemia (Tsukada *et al*, 1993; Vetrie *et al*, 1993). BTK is a member of the Tec family of non-receptor protein tyrosine kinases (PTKs), which is encompassed within the Src superfamily, an important regulator of lymphocyte signaling (Berg *et al*, 2005; Mano, 1999). The enzyme has been widely characterized in B cells as an important regulator of development, differentiation, cell survival, and growth as part of a membrane associated signalosome (Kurosaki, 1997; Kurosaki, 2000; Rawlings and Witte, 1994; Uckun, 1998). Moreover, BTK was the first tyrosine kinase identified as a dual-function regulator of apoptosis in what may seem a cell-type specific function (Uckun, 1998). We have previously identified BTK as one of 17 proteins that is uniquely upregulated in the plasma membrane of HIV-1 latently infected T-cells (Berro *et al*, 2007). After viral activation in infected cells, there is a virus-specific redistribution of nuclear and cytoplasmic BTK to punctuated foci at the plasma membrane that does not occur in the absence of viral particle production. Of particular interest, Smith *et al*. (Smith *et al*, 1994a) originally established Tec kinase expression in hematopoietic cells with the exception of plasma and T cells, where mRNA levels of BTK were below detection levels by the Northern blot. Generally, it is widely accepted that there is a minimal BTK expression in T-lymphocytes (de Weers *et al*, 1993; Smith *et al*, 1994a; Smith *et al*, 1994b; Yu *et al*, 2008), whereas higher expression is seen in myeloid cells, including monocytes, macrophages, neutrophils, and mast cells (Mohamed *et al*, 2009). Thus, the observed BTK upregulation in HIV-1 infected cells sheds a new light on the involvement of Tec kinase members in infection.

To further evaluate the status of BTK expression in HIV-1 infection, we explored expression of this protein in a variety of uninfected and infected HIV-1 target cells. We have shown up-regulation of BTK expression and phosphorylation in HIV-1 chronically infected cells, as well as in *de novo* infected cells and monocytic cell models. Transient RNAi selective depletion of the enzyme in latently infected cells that have undergone viral reactivation indicated that down-regulation of BTK resulted in increased detection of apoptotic markers, namely activated caspase 3 and cleaved PARP and that infected cells were more susceptible to anti-BTK antibody. Treatment of a panel of uninfected and infected cells and monocytes with LFM-A13 or Ibrutinib, reflected a similar apoptotic phenotype of BTK antibody-treated cells, with infected cells generally being more sensitive to treatment. Importantly, LFM-A13 successfully inhibited viral replication on a post-transcriptional stage of the viral

replication *in vivo* in a humanized animal model (data not shown). Collectively, our findings indicate that BTK is specifically up-regulated in both HIV-1 infected T cells and macrophages and is associated with the plasma membrane of infected cells. We speculate that BTK is normally intracellular but may be associated with membrane (phosphorylated form) in infected cells, hence becoming accessible to antibody treatment.

The leflunomide metabolite analog LFM-A13 is a rationally-designed specific inhibitor of BTK (Mahajan *et al*, 1999; Robak and Robak, 2012). Recently, a number of tyrosine kinase inhibitors became available for both preclinical and clinical trials including several BTK inhibitors such as LFM-A13, GDC-0834, AVL-101, AVL-292, and PCI-32765 (Robak and Robak, 2012). Avila Therapeutics (AVL-292) and Pharmacyclics (PCI-32765) both have developed covalent BTK inhibitors that are currently in the clinical trial pipeline for treatment of various malignancies associated with BTK dysregulation. PCI-32765 or Ibrutinib, a selective and irreversible inhibitor, has gained significant ground in clinical development and was being investigated in patients with B cell malignancies, and recently FDA approved, that includes chronic lymphocytic leukemia/small lymphocytic lymphoma, mantle cell lymphoma, diffuse large B cell lymphoma, and multiple myeloma (Advani *et al*, 2013; Carmi *et al*, 2012; Harrison, 2012; Robak and Robak, 2012; Winer *et al*, 2012). Moreover, the advancement of these compounds in Phase I and II clinical trials could be useful towards cART complementation once their effects on HIV-1 infection have been further explored.

The exact mechanism by which BTK monoclonal antibody or small molecule inhibitor induce apoptosis in HIV-1 infected cells is not yet clear. Previously, we have identified BTK enrichment in the membrane proteome of latently infected cells, and also observed the accumulation of BTK in distinct foci of the cell membrane upon viral reactivation (Berro *et al*, 2007). As previously described, BTK contains a PH domain that targets it to the plasma membrane allowing it to play a pivotal role in B cell signaling (Kurosaki, 2000). Interestingly, a recent study identified a lipid raft BCR signaling tuning mechanism regulated by differential posttranslational modification (Schmidt *et al*, 2009), hinting towards the complex regulatory loops governing the BTK signalosome. This signalosome is believed to occur predominantly in the cholesterol and sphingolipid-rich lipid raft or caveolae environments, where additional signal transducing molecules also assemble (Fruman *et al*, 2000; Guo *et al*, 2000; Vargas *et al*, 2002). Once commenced, BTK signaling at the plasma membrane conveys the integration of cytoplasmic and nuclear events. For example, BTK and PLC- γ 2 have been shown to play a critical role in the activation of NF- κ B after lipopolysaccharide stimulation of the Toll-like receptor (TLR) or B cell receptor (BCR) engagement (Bajpai *et al*, 2000; Horwood *et al*, 2003; Petro *et al*, 2000), and NF- κ B signaling is compromised in BTK deficiency (Yu *et al*, 2008). Also, it has been shown that BTK-mediated B-cell-activating factor receptor (BAFF-R)-dependent activation of NF- κ B promotes B cell survival (Shinnars *et al*, 2007). NF- κ B is a canonical transcription factor that plays an essential role in innate and adaptive immunity, and given its involvement in the HIV-1 viral life cycle, it is possible that HIV-1 is exerting BTK-mediated NF- κ B regulation to fine tune cell survival outcomes of the infected cell as well as viral transcription. Moreover, two functional NF- κ B binding sites located at the BTK promoter have been

recently described (Yu *et al*, 2008). These have been shown to function in a positive proteasome-dependent autoregulatory feedback mechanism that BTK uses to stimulate transcription from its own promoter. Therefore, increased BTK expression in infection may be directly linked to NF- κ B availability in the presence of viral infection and T cell activation. Additionally, BTK has been shown to directly target other transcription factors including BAP-135, TFII-I, TFB, and BAM11; indicative of BTK's role in gene expression and regulation (Hirano *et al*, 2004; Kikuchi *et al*, 2000; Novina *et al*, 1999; Webb *et al*, 2000a; Webb *et al*, 2000b; Yang and Desiderio, 1997).

In this study, we have demonstrated using antibody, siRNA or other inhibitor molecule that BTK positively regulates productive HIV-1 infection either by activating the infected cells or enhancing the release of virus from the infected cells. Nevertheless, other members of this family have been reported to also have a direct effect on HIV-1 replication (Prins *et al*, 2012; Readinger *et al*, 2008; Schiralli Lester *et al*, 2013). For example, ITK (inducible T cell kinase), another member of the Tec family tyrosine kinase, induces HIV-1 replication by regulating multiple steps of viral life cycle such as T cell activation, actin polymerization/polarization, virion assembly and release. Specifically, ITK has been shown to co-localize with HIV-1 Gag on plasma membrane during virus release (Schiralli Lester *et al*, 2013). ITK has also been shown to be involved in inducing activation signals during the formation of virological synapse that enhances cell to cell spread of HIV-1 (Prins *et al*, 2012). Future studies will focus on elucidating the mechanistic details regarding the importance of BTK and other Tec family members for HIV-1 infection and how targeting the overexpressed Tec tyrosine kinases could eliminate HIV-1 infection especially in the latently infected cells.

Materials and methods

Cell cultures and reagents

HLM-1 cells obtained from the AIDS Research and Reference Reagents Program (NIH, Bethesda, MD, USA) were grown in DMEM supplemented with 10% heat-inactivated FBS, 1% L-glutamine, and 1% streptomycin/penicillin (Gibco/BRL, Gaithersburg, MD, USA). HLM-1 cells are HeLa-T4+ cells containing one integrated copy of HIV-1 genome with a Tat-defective mutation at the first AUG of Tat gene. In the absence of stimulation, HLM-1 is completely negative for virus particle production, and viral transcripts (unspliced) are completely absent (Berro *et al*, 2006; Guendel *et al*, 2013). HLM-1 cells were stimulated with 2 mM sodium butyrate for viral induction. The HIV-1 infected human cells J1.1, ACH2 and their uninfected counterparts Jurkat and CEM cells, as well as the infected monocytic U1 cells and the corresponding uninfected U937 cells, were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 1% L-glutamine, and 1% streptomycin/penicillin (Guendel *et al*, 2013). The uninfected B-cells (BJAB) were also grown in the same media. The J1.1 cell line is a Jurkat E6.1 derivative chronically infected with HIV-1 (LAI strain) (Perez *et al*, 1991). ACH2 T cells are also infected with HIV-1 LAI strain. ACH2 and J1.1 cells contain a single integrated copy of HIV-1 genome (Clouse *et al*, 1989), whereas U1 cells harbor two copies (one wild type and one mutant) of the viral genome (LAV-1 strain) (Folks *et al*, 1987; Guendel *et al*, 2013). Monocytic U937 and U1 cells were induced to differentiate into monocytes derived macrophages (MDM) with 250 nM of phorbol-12-

myristate-13-acetate (PMA) for 96 hrs. All cells were incubated at 37°C in the presence of 5% CO₂.

Therapeutic treatments

Antibodies against BTK (611116) and XIAP (610762) were purchased from BD Transduction Laboratories (San Diego, CA, USA) and anti-CD45 (H-130) antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Mouse monoclonal anti-BTK IgG antibody (clone 53/BTK from BD Biosciences) recognizes human BTK, but does not cross react with ITK (Tec kinase family member). This antibody is also specific for human BTK as neither wildtype nor BTK deficient chicken B cells show any reactivity with this antibody (Douhan Iii *et al*, 2007). Inhibitions studies were always performed with fresh antibodies. Fifty thousand cells were plated per well in a 96-well plate and treated the next day with the appropriate antibody dilution to a final concentration of 1, 5 or 10 µg/ml. Antibodies against β-actin (C-11), caspase 3 (H-277), and PARP-1/2 (H-250) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The commercially available BTK inhibitor, LFM-A13 or α-Cyano-β-hydroxy-β-methyl-N-(2,5-dibromophenyl)propanamide, was obtained from Sigma Saint Louis, MO, USA) and prepared as a 10 mM stock solution dissolved in DMSO. Ibrutinib was purchased from Selleckchem, Catalog #S2680.

Cell viability assay

Viability was assessed by the trypan blue exclusion method using a Neubauer counting chamber. Fifty thousand cells were plated per well in a 96-well plate and treated the next day with 1 and 5 µg of antibody against BTK. After 48 hrs, CellTiter-Glo (Promega, Madison, WI, USA) was used to measure viability following the manufacturer's recommendations. CellTiter-Glo is a luminescent assay used to measure cell viability by analyzing the ATP levels. The reagent was added to the wells (1:1 reagent:media) and incubated at room temperature for 10 min protected from light. The luminescence signal was recorded using the GloMax-Multi Detection System (Promega)(Guendel *et al*, 2013). The viable cell number was normalized with control group and the results were expressed as relative cell viability

Protein extracts and immunoblotting

Cells were washed with PBS and lysed in a buffer containing Tris-HCl pH 7.5, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM Na₃VO₄, 1 mM DTT and one tablet of complete protease inhibitor cocktail per 50 ml. Cell lysis was performed on ice for 30 min and before centrifugation (at 14,000 rpm) for 5 minutes at 4°C (Van Duyne *et al*, 2011). For separating cytoplasmic and nuclear proteins a differential salt extraction method was employed. First, cells were collected and washed twice with PBS w/out Ca²⁺ and Mg²⁺. Cytosolic extracts were then prepared by resuspending cells in 80 µl Buffer A (10 mM KCl, 10 mM MgCl₂, 10 mM HEPES, 1 mM EDTA, 1 mM DTT, 0.5% NP-40, 0.1% PMSF and one EDTA-free complete protease inhibitor tablet per 50 mL) and incubating for 10 min on ice. Samples were spun at 5,000 g for 5 min at 4°C and the supernatants saved as cytosolic extract (CE). The pelleted nuclei were washed with 200 µl of Buffer A, and spun at 5,000 g

for 5 min at 4°C. The supernatants were removed and nuclei resuspended in 80 µl of Buffer B (450 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, 0.5 mM EDTA, 1 mM DTT, 0.5% NP-40, 0.1% PMSF and one EDTA-free complete protease inhibitor tablet per 50 mL) and incubated for 10 min on ice (Popova *et al.*, 2010). Samples were spun at 20,000 g for 10 min at 4°C and supernatants saved as nuclear extract (NE). The protein concentration in the supernatant was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). For separating cytoplasmic versus nuclear proteins a differential salt extraction method was employed. Cell extracts were resolved by SDS PAGE on a 4–20% tris-glycine gel (Invitrogen, Carlsbad, CA, USA) and analyzed by WB using specific antibodies.

Reverse transcriptase activity assay

For RT assays, supernatants from infected cells (10 µl) were incubated in a 96-well plate with RT reaction mixture containing 1× RT buffer (50 mM Tris-HCl, 1 mM DTT, 5 mM MgCl₂, 20 mM KCl), 0.1% Triton, poly(A) (10–2 U), poly(dT) (10–2 U) and [3H]TTP. The mixture was incubated overnight at 37°C and 5 µl of the reaction mix was spotted on a DEAE Filter mat paper (PerkinElmer, Shelton, CT, USA) washed four times with 5% Na₂HPO₄, three times with water, and then dried completely (Van Duyne *et al.*, 2011). RT activity was measured in a Betaplate counter (Wallac, Gaithersburg, MD).

RNA extraction and RT-PCR

Humanized mice were treated with LFM-A13 post- and pre-infection with dual-tropic HIV-1 89.6 virus. Total RNA was isolated from the infected mouse PMBCs using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Five hundred ng of total RNA was used to generate cDNA with the iScript cDNA Synthesis kit (Bio-Rad) using oligo-dT reverse primers (Guendel *et al.*, 2013). The HIV-1 LTR and env genes were amplified by RT-PCR using primers specific for LTR and env as previously described (Narayanan *et al.*, 2013; Zhou *et al.*, 2004).

RT reaction and Quantitative PCR

For quantitative analysis of HIV-1 RNA, total RNA was isolated from culture supernatants of HIV-1 infected cells at different time points. RNA was purified using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. A total of 0.5 µg of RNA from the RNA fraction was treated with 0.25 mg/ml DNase I RNase-free (Roche, Mannheim, Germany) for 60 minutes in the presence of 5 mM MgCl₂, followed by heat inactivation at 65° C for 15 minutes. A 200–500 ng aliquot of total RNA was used to generate cDNA with the GoScript Reverse Transcription System (Promega, Madison, WI) using oligo-dT reverse primer. Subsequent quantitative real-time PCR analysis was performed with 2 µl of undiluted and 10⁻¹, and 10⁻² diluted aliquots of RT reaction mixes. The iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) was used with the primers specific for HIV-1 gag gene: Gag1483-F (5'-AAGGGGAAGTGACATAGCAG-3') and Gag1625-R (5'-GCTGGTAGGGCTATACATTCTTAC-3') amplified 143 nt. fragment of HIV-1 gag gene. Serial dilutions of DNA from 8E5 cells (CEM cell line containing a single copy of HIV-1 LAV provirus per cell) were used as the quantitative standards. Real-time PCR

reactions were carried out at least in triplicate using the CFX96 Real-Time PCR System (BioRad) and SFX Manager Software ver. 2.0.

Transfection assays

For siRNA-mediated knockdown of BTK, HLM-1 cells were transfected with 10 nM of double-stranded duplex HsBTK2 (BTK #1) or HsBTK3 (BTK #2) FlexiTube siRNA (SI00001029, SI00001036) HP Validated siRNA (Qiagen, Valencia, CA, USA) or luciferase (Dharmacon, Lafayette, CO, USA) using HiPerfect reagent according to the manufacturer's recommendations (Qiagen) in 24-well plates. HIV-1 proviral DNA construct pNL4.3 (5 µg) was also transfected in U937 and Jurkat cells using Effectene (Qiagen) according to the manufacturer's instructions (Jaworski *et al*, 2014).

Size-exclusion chromatography

Both uninfected U937 and infected U1 monocytic cells were washed with PBS without Mg^{2+} and Ca^{2+} and resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM Na_3VO_4 , 1 mM DTT, and one complete protease cocktail tablet per 50 ml) and incubated on ice for 20 minutes, with gentle vortexing. Lysates were then centrifuged at 4°C at 10,000 rpm for 10 min, and protein concentration in the supernatants was determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). For each cell line, 2.5 mg of protein was brought up to 1 ml total volume using chromatography running buffer (0.2 M Tris-HCl pH 7.5, 0.5 M NaCl, and 5% glycerol). The lysates were run on a Superose 6 10/300 size-exclusion chromatography column (GE Healthcare Bio-Sciences, Uppsala, Sweden) using the ÄKTA Purifier system (GE Healthcare Bio-Sciences, Piscataway, NJ, USA)(Guendel *et al*, 2013). A quarter inch gap was introduced to the top of the Superose 6 column to better separate small molecular weight complexes from fractions eluting off the far right side of the chromatogram. After sample injection (using 1 ml loop), running buffer was set at a flow rate of 0.3 ml/minute, and 0.5 ml fractions of the flow-through were collected at 4°C for a total of approximately 60 fractions. Every 5th fraction was acetone precipitated (~250 µl) using 4 volumes of ice-cold 100% acetone, incubating for 15 minutes on ice. Precipitates were centrifuged at 4°C for 10 min at 14,000 rpm, supernatants were removed, and the pellets were dried for about 10 min at 95°C (Guendel *et al*, 2013). The pellets were resuspended in Laemmli buffer and analyzed by immunoblotting with BTK (BD Biosciences) and actin antibodies (Santa Cruz).

Cell cycle analysis and apoptosis

Cells were washed with PBS and fixed with 70% ethanol. Following rehydration in PBS, cells were stained in PBS containing 25 µg/ml propidium iodide (Sigma, Saint Louis, MO, USA), 10 µg/ml RNase A (Sigma) and 0.1% NP-40. Cells were analyzed on a BD FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). Acquisition was done with CELLQuest software, and cell cycle analysis was performed with ModFit LT software (Verity Software House, Topsham, ME, USA). Aggregates and debris were excluded by gating on the FL2W and FL2A parameters. The gates were at channel 200 for FL2 width versus FL2 area, with a doublet discriminator. Apoptosis was considered to be the population of cells localized in sub-G1 area.

Surface Staining of BTK and FACS Analysis

For each cell line analyzed, 5 mL of culture (5×10^5 cells/mL starting density) were incubated in T25 flasks at 37°C in 5% CO₂. For the U937 and U1 cell lines, two cultures were maintained either with or without 50 nM PMA stimulation. After 48 hours of incubation, cells were counted and then 5×10^5 cells from each cell line were pelleted. Next, cells were resuspended in 100 µL of PBS + 0.1% BSA plus α-BTK mouse monoclonal antibodies (equal parts BD Transduction (Cat# 611116) and Santa Cruz Biotechnology (Cat# sc-28387)) and incubated on ice for 30 minutes. Two cell pellets, one Jurkat and one U937, were incubated with PBS + 0.1% BSA alone to serve as negative controls for non-specific secondary antibody binding. Cells were then washed with 2 mL of cold PBS + 0.1% BSA and resuspended in 100 µL of PBS + 0.1% BSA plus FITC-labeled α-mouse secondary antibody and incubated on ice for 30 minutes in the dark. Cells were washed again, and resuspended in 100 µL of PBS + 0.1% BSA plus 100 µL of 2% paraformaldehyde. FACS analysis was then carried out using a FACSCalibur (BD Biosciences) and data analyzed using CellQuest software (BD Biosciences).

Reverse Phase Microarray

Approximately 30 nL of each sample (equivalent to 40 lysed cells) were arrayed onto nitrocellulose slides (Whatman, MA) by direct contact printing using a high-resolution 2470 arrayer (Aushon Biosystems, Billerica, MA). Samples were printed as duplicates of an 8.2-fold dilution series (Popova *et al*, 2010). The linear range of each dilution curve was then used for quantitative comparisons between samples. Slides were stored at -20°C prior to detection. To estimate the total protein amount in each spot, several randomly selected slides were stained with Sypro Ruby Protein Blot Stain (Molecular Probes, Eugene, OR) and visualized on a Fluorchem imaging system (Alpha Innotech, San Leandro, CA). Slides were stained on a Dako Autostainer with specific antibodies using a biotin-linked peroxidase-catalyzed signal amplification (Dako, CSA kit). Specifically, the arrayed slides were placed into Re-Blot solution (Chemicon, Temecula, CA) for 15 min, washed twice in PBS 5 min each, placed into I-Block solution (Applied Biosystems, Foster City, CA) in PBS with 0.1% Tween-20 for at least 2 h, and then immunostained using an automatic slide stainer (Autostainer, Dako Cytomation) and the manufacturer-supplied reagents (Popova *et al*, 2010). Briefly, the slides were incubated for 5 min with hydrogen peroxide, rinsed with high-salt Tris-buffered saline (CSA Buffer, Dako) supplemented with 0.1% Tween-20, blocked with avidin block solution for 10 min, rinsed with CSA buffer, and then incubated with biotin block solution for 10 min. After another CSA buffer rinse, 5 min incubation with protein block solution was followed by air drying (Popova *et al*, 2010). The slides were incubated with either a specific primary antibody diluted in Dako antibody diluent or with only diluent as a control, for 30 min. Every antibody underwent extensive validation ensuring a specific detection of the antigenic protein as a single band on the Western blot of cell lysate. The slides were washed with CSA buffer and incubated with a secondary biotinylated goat anti-rabbit IgG antibody (1:7500) (Vector Labs, Burlingame, CA) for 15 min. Slides were incubated with diaminobenzidine chromogen diluted in Dako diaminobenzidine diluent for 5 min, washed in deionized water and imaged using a Umax PowerLook III scanner (Umax, Dallas, TX) at 600 dpi. The images were analyzed with

software AlphaAse FC (Alpha Innotech)(Popova *et al*, 2010). For each antibody, the average pixel intensity value for negative control (staining with second antibody only) was subtracted from the average pixel intensity value for specific antibody and then normalized by the corresponding average value of the total protein intensity. Each sample dilution curve in each experiment was fitted with a nonlinear approximation equation and statistically evaluated using GraphPad Prism ver5 software (Graphpad Software, CA)(Popova *et al*, 2010). All measurements were performed in triplicate. Data are represented as mean values with standard deviation (two-tail t-test, $\alpha = 0.05$, $n = 3$).

Data analysis and bioinformatics

Quantitative data were analyzed by two-way ANOVA (OriginPro v. 8.0) and Student's *t* test (Microsoft Excel). Standard deviation was calculated in all quantitative experiments for at least three independent preparations. The difference was considered to be statistically significant when $P < 0.05$ (Iordanskaia and Nawshad, 2011). The function of the RPMA identified proteins was elucidated by SWISS-PROT database <http://www.expasy.org> and the interaction between the differentially expressed/modified proteins. A protein-protein interaction network was drawn by STRING 9.0 <http://string-db.org/> using proteins identified in this work and data from SWISSPROT function annotation as input (Popova *et al*, 2010).

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Fig. 1. BTK expression is induced in HIV-1 infected cells

(A) To assess BTK expression in a series of HIV-1 matched cell targets, 50 μ g of whole cell extracts from B cells (BJAB), uninfected T cells (CEM and Jurkat), uninfected monocytic cells (U937 and U937-derived macrophage model cells, m Φ), HIV-1 infected T cells (ACH2 and J1.1), and infected monocytic cells (U1 and U1 m Φ) were run on a 4–20% SDS-PAGE and immunoblotted against BTK and β -actin. (B) Jurkat and U937 cells were transfected with the HIV-1 clone pNL4-3 using Effectene transfection reagent, harvested at 48 hr and lysed in Laemmli sample buffer. Twenty microgram of whole cell extract was assayed by

western blot using α -BTK and α - β -actin antibodies. Phosphorylated BTK (p-BTK) is suggestive of BTK activation. **(C)** Cytosolic and nuclear extracts were prepared from Jurkat and J1.1 cells as described in Materials and Methods. The extracts were analyzed by western blot using anti-BTK antibody. Lamin A was used as a nuclear control. **(D)** Total cell lysates of both uninfected U937 and infected U1 cells were loaded onto a size-exclusion chromatography column and proteins separated in the presence of high salt buffer (500 mM NaCl) to minimize non-specific binding. No detergent was used during fractionation. A sampling (250 μ l) of every 5th fraction from 10 – 55 was acetone precipitated and resuspended in 30 μ l of Laemmli buffer, and then 15 μ l were run on a gel for western blotting using BTK and β -actin antibody. More soluble actin was present in the U1 fractions as compared to uninfected U937 cells.

Fig. 2. Extracellular staining of both infected and uninfected cells with BTK antibody

(A) To assess BTK expression, chronically infected J1.1 cells (positive control) were used in these assays. Cells were not treated with either detergent or a fixative prior to staining, thereby preserving the natural state of the BTK localization on the membrane. These cells are chronically expressing virus and show a ~5-fold increase in surface BTK staining as compared to the uninfected parental Jurkat T-cell line. (B) Uninfected U937 cells (negative control) of myeloid lineage show baseline expression of surface BTK levels prior to differentiation with PMA treatment and an increase of 70% following PMA treatment. (C) Latently infected U1 cells (experimental) were used for staining with BTK antibodies. These cells do not normally express virus, however after the addition of PMA, they are differentiated to macrophages and express virus. These cells show low basal BTK surface staining and ~ 5-fold increase of BTK upon PMA stimulation.

Fig. 3. siRNA mediated BTK knockdown induces apoptosis in HIV-1 infected cells

(A) Latently infected HLM-1 cells were used for selective depletion with siLuc (control) or two different siRNAs targeting BTK (siBTK #1 and siBTK #2). Log phase growing cells (5×10^5 /ml) were transfected with either siLuc or siBTK (10 nM) using Effectene. Cells were harvested at 24, 48 and 72 h post-transfection, and 20 μ g of whole cell lysates were resolved by SDS-PAGE and immunoblotted against BTK to assay for effective siRNA-mediated BTK downregulation. (B) Similar to panel A, where cell lysates were further probed with α -caspase 3 and α -PARP antibodies as markers of program cell death. Western blots against β -actin were performed as a loading control. (C) Similar to panel A, where HLM-1 cells were transfected with siLuc, siBTK #1 and siBTK #2 (50 nM). Viability was assessed using CellTiter-Glo assay 72 hours post-treatment. Results are shown as mean of three independent experiments $\pm$ SD.

Fig. 4. BTK antibody treatment induces cell death in HIV-1 infected, but not uninfected cells

(A) Uninfected cells (CEM and U937) and HIV-1 infected cells (ACH2, J1.1 and U1) were treated with 5 µg/ml of IgG as a control or two concentrations of anti-BTK antibody (1 µg/ml and 5 µg/ml). Cell viability was determined by trypan blue dye exclusion cell quantitation (100 cells per sample) at 24 hours post-treatment. BTK antibody treatment decreased viability when compared to the respective uninfected controls by 36% in ACH2 cells, 59% in J1.1 cells, and by 58% in U1 cells at 5 µg/ml. (B) Jurkat (uninfected) and J1.1 chronically HIV-1 infected cells were treated with 1 µg/ml of IgG or antibody against BTK.

Viability was assessed using CellTiter-Glo assay 48 hours post-treatment. Error bars show standard deviation of three independent measurements. **(C)** Latently HIV-1 infected HLM-1 cells were activated with 2 mM sodium butyrate (NaB) for 72 hours followed by treatment with 5 µg/ml of BTK antibody. Cells were subjected to CellTiter-Glo viability assay at 48 hours post-treatment. Results are mean $\pm$ SD of three independent preparations. **(D)** Uninfected monocytic control cell line U937 and latently HIV-1 infected U1 cells were subjected to differentiation with 250 nM PMA for 96 hours. The monocyte derived macrophages (U937 mΦ and U1 mΦ) were treated with 10 µg/ml of α-BTK and α-XIAP antibodies. The α-CD45 antibody was used as a positive control for receptor-mediated macrophage cell death. Cells were subjected to CellTiter-Glo viability assays at 48 hr post-treatment. Treatments were performed in triplicate and the mean plus-minus standard deviation is displayed. The double asterisks indicate $p < 0.01$. Viability is expressed as percent of IgG control.

Fig. 5. BTK antibody treatment results in decreased HIV-1 replication

(A) Latently infected HLM-1 cells were treated with 5 µg/ml of α-BTK antibody or control IgG. Antibody treatment was performed once and supernatants were collected at 48 hr post-treatment for RT activity analysis. (B) HIV-1 infected J1.1 T cells were treated with 5 µg/ml of IgG or α-BTK antibody. Antibody treatment was performed once and supernatants were collected at 48 hr post-treatment for RT activity analysis. RT activities are expressed as percent of IgG control. Results are expressed as mean ±SD of three independent measurements. (C, D) Similar to panels A and B, except that the supernatants were assayed for presence of viral particle, using qRT/PCR for unspliced HIV-1 RNA. HLM-1 cells were

treated with sodium butyrate for 72 hr to activate the latent HIV-1 provirus. Results are mean $\pm$ SD of three independent preparations. Single asterisk indicates $p < 0.01$; double asterisks - $p < 0.05$.

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Fig. 6. BTK antibody treatment induces cell death in HIV-1 infected primary cells
(A) Phytohemagglutinin (PHA) and IL-2 activated PBMCs from healthy donors were maintained in culture for 48 hr prior to infection. Approximately 2.5×10^7 PBMCs were infected with HIV-1 89.6 virus (35,520 RT units). After 24hrs of infection, cells were subjected to one treatment with anti-BTK antibody or IgG control at 10 $\mu\text{g/ml}$. At day 10 post-infection, cells were stained with propidium iodide and analyzed for stages of cell cycles using flow cytometry. The data were acquired on a Becton Dickinson FACScalibur. On the histogram plot the dead cells are marked with blue color, the cells in G₁ phase are

red. **(B)** Samples from panel A are plotted for the presence of cells at various stages of cell cycle including G₀/G₁, S, and G₂/M. “Sub-G1” represents cells that are in the process of cell death.

Fig. 7. BTK inhibitors induce cell death in HIV-1 infected cells

(A) A panel of matched infected (ACH2, J1.1 and U1) and uninfected (CEM, Jurkat and U937) cells was treated with 10, 50 or 100 μ M of LFM-A13 for 72 hours. BJAB served as positive control. Viability was assessed by CellTiter-Glo assay as described by the manufacturer. Treatments were performed in triplicate and the mean $\pm$ SD is presented. The asterisks indicate $p < 0.05$. Viability is expressed as percent of DMSO control. (B) Latently HIV-1 infected ACH2 cells were similarly treated with the above concentrations of LFM-A13 for 72 hours. Supernatants were collected at day 3 for RT activity analysis. Treatments

were performed in triplicate and the mean $\pm$ SD of three representative experiments is provided. RT activity is expressed as percent of DMSO control. **(C)** Uninfected B-cells (BJAB, lanes 1–4), uninfected CEM T cells (lanes 5–8), and infected cells (ACH2, lanes 9–12), were treated with either DMSO or Ibrutinib (10, 50 or 100 nM) for 72 hr. Cell deaths following drug treatments were monitored by comparing the levels of luminescence with DMSO treatment. Results are mean $\pm$ SD of three independent measurements. Single asterisk indicates $p < 0.05$. **(D)** Infected cells (ACH2), were treated with either DMSO, LFM-A13 (10, 50 or 100 μ M), or Ibrutinib (10, 50 or 100 nM) for 48 hr. The supernatants were assayed for presence of viral particle, using qRT/PCR for unspliced HIV-1 RNA. Results are mean $\pm$ SD of three independent preparations. The asterisks indicate $p < 0.01$.

Fig. 8. Functional categories of LFM-A13-regulated phosphoproteins and signaling networks
 Uninfected CEM and HIV-1 infected ACH2 cells were treated with DMSO or LFM-A13 for 24 hr, cell lysates were prepared and analyzed with RPMA as described in Materials and Methods. Significantly altered proteins are listed in Table 1 (two-tail t-test, $\alpha = 0.05$, $n = 3$). A protein-protein interaction network was drawn by STRING 9.0 <http://string-db.org/> using proteins identified in panel A and data from SWISS-PROT function annotation as input.

Table 1

Effects of LFM-A13 in Signaling Pathways

Antibody	Fold Change	p-value
pBAD S136	0.41	0.02
pGSK-3B S9	1.68	0.02
c-Myc	1.32	0.00
GSK-3B	1.31	0.00
LC3B	0.61	0.00
pAKTS473	1.46	0.00
pAurora A/B/C T288/232/198	1.39	0.05
pGSK-3a/B S21/9	1.43	0.00
pMDM2 S166	1.67	0.00
pmTOR S2448	1.26	0.00
pp90RSK S380	0.59	0.01