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Protein kinase inhibitors that inhibit induction of lytic program and replication of Epstein–Barr virus

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ABSTRACT

Signaling pathways mediating Epstein–Barr virus (EBV) reactivation by Ag-bound B-cell receptor (BCR) were analyzed using a panel of 80 protein kinase inhibitors. Broad range protein kinase inhibitors Staurosporine, K252A, and PKC-412 significantly reduced the EBV genome copy numbers measured 48 h after reactivation perhaps due to their higher toxicity. In addition, selected inhibitors of the phosphatidylinositol-3-kinase (PI3K), protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) pathways, glycogen synthase kinase 3β (GSK-3β), platelet-derived growth factor receptor-associated tyrosine kinase (PDGFRK), and epidermal growth factor receptor-associated tyrosine kinase (EGFRK) significantly reduced the EBV genome copy numbers. Of those, only U0126 and Erbstatin analog, which inhibit MAPK pathway and EGFRK, respectively, did not inhibit viral reactivation assessed by expression of the EBV early protein, EA-D. None of the tested compounds, except for K252A, affected the activity of the EBV-encoded protein kinase *in vitro*. These results show that EBV reactivation induced by BCR signaling is mainly mediated through PI3K and PKC, whereas MAPK might be involved in later stages of viral replication.

1. Introduction

Over 90% of the world population is infected with Epstein–Barr virus (EBV) by adulthood. Like other herpesviruses, EBV has a biphasic life cycle which includes latent and lytic programs. Upon primary infection, the virus establishes a lifelong latency in memory B lymphocytes characterized by a highly restricted gene expression and lack of virion production (Kieff and Rickinson, 2007). A variety of EBV-associated malignancies are linked to the viral latency (Rickinson and Kieff, 2007). Various stimuli may activate viral lytic program in latently infected cells leading to a coordinated expression of majority of viral genes and production of progeny virions (Kieff and Rickinson, 2007). This phase of infection is clinically associated with infectious mononucleosis and oral hairy leukoplakia (Rickinson and Kieff, 2007).

Of the different ways to reactivate the lytic program *in vitro*, treatment with anti-Ig antibodies seems to be more physiologically relevant since it mimics B-cell Ag-receptor (BCR) signaling (Iwakiri and Takada, 2004; Takada, 1984; Tovey et al., 1978). Upon Ag binding, BCR aggregates and activates protein tyrosine kinases Lyn, a member of Src family of tyrosine kinases, and Syk. Simultaneously, Ag binding causes activation of phosphatidylinositol-3-kinase (PI3K) (through BCR co-receptor CD19), which, along with Lyn and Syk, is involved in activation of Bruton's tyrosine kinase (Btk). Once Lyn, Syk, and Btk are activated, the signal is further propagated through activation of phospholipase Cγ2 (PLCγ2). PLCγ2 hydrolyzes phosphatidylinositol diphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 generation causes the mobilization of Ca^{2+} ions required for the activation of several transcription factors (such as NF-κB), whereas DAG activates conventional protein kinase C (PKC) isotypes which regulate mitogen-activated protein kinase (MAPK) family. The family consists of three members: extracellular signal-regulated kinase

Abbreviations: BCR, B-cell receptor; EBV, Epstein–Barr virus; CDK, cyclin-dependent kinase; GSK-3β, glycogen synthase kinase 3β; PI3K, phosphatidylinositol-3-kinase; PCR, polymerase chain reaction; PDGFRK, platelet-derived growth factor receptor-associated tyrosine kinase; EGFRK, epidermal growth factor receptor-associated tyrosine kinase; NF-κB, nuclear factor κB; PLCγ2, phospholipase Cγ2; MAPK, mitogen-activated protein kinase; Btk, Bruton's tyrosine kinase; PIP2, phosphatidylinositol diphosphate; DAG, diacylglycerol; IP3, inositol triphosphate; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2 terminal kinase; CHPK, conserved herpesvirus protein kinase; HCMV, human cytomegalovirus; NGIC-I, non-glucosidic indolocarbazole I; BL, Burkitt's lymphoma; CDK, cyclin-dependent kinase; DMSO, dimethylsulfoxide; ACV, acyclovir; qPCR, quantitative real-time PCR; PKI, protein kinase inhibitor; GST, glutathione-S-transferase; MBP, myelin basic protein; DTT, dithiothreitol; MEK1/2, mitogen-activated protein kinase kinases 1 and 2; CKI, casein kinase I; CKII, casein kinase II; DRB, 5,6-dichloro-1β-D-ribofuranosilbenzimidazole.

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(ERK), c-Jun NH2-terminal kinase (JNK), and p38 MAPK (Johnson and Lapadat, 2002). Following activation, these kinases phosphorylate different sets of transcription factors, which then regulate expression of certain genes. Other similar 'downstream' effectors are Akt (or protein kinase B) and Ca^{2+}-calmodulin. Eventually, a complex interplay between the pathways induced in BCR signaling translates into a well-balanced system that ensures proper cell responses to a variety of conditions (Dal Porto et al., 2004; Kurosaki et al., 2010).

In addition to cellular protein kinases, herpesviruses and several other large DNA viruses (poxviruses, baculoviruses), encode their own protein kinases. These protein kinases appear to target multiple viral and cellular proteins coordinating different aspects of viral replication (Gershburg and Pagano, 2008; Jacob et al., 2011). Lymphotropic herpesviruses, such as EBV, encode only one protein kinase (the BGLF4 gene product), which is a member of conserved herpesvirus protein kinase (CHPK) family (Gershburg and Pagano, 2008; Kawaguchi and Kato, 2003). Kinases in this group are tegument proteins (Asai et al., 2006; Overton et al., 1992; van Zeijl et al., 1997) expressed during lytic program and play a crucial role in viral infection, because a knockdown of their expression levels or knockout of their corresponding genes results in significant replication defects (Gershburg et al., 2007; Murata et al., 2009; Prichard et al., 1999). Attempts to inhibit CHPKs using pharmacological inhibitors showed that while human cytomegalovirus (HCMV) pUL97 protein kinase autophosphorylation activity was inhibited by 0.5 μM of non-glycosidic indolocarbazole I (NGIC-I) (Marschall et al., 2002) and by 1 μM of maribavir (MBV) (Biron et al., 2002; Hamirally et al., 2009), these compounds did not inhibit the activity of the purified EBV-PK (Gershburg et al., 2004; Marschall et al., 2002). Noteworthy, however, the latter compound inhibits EBV replication (Gershburg et al., 2004; Wang et al., 2009; Zacny et al., 1999) and causes a loss of the hyperphosphorylated form of an EBV early protein EA-D (Gershburg and Pagano, 2002; Zacny et al., 1999).

Induction of the EBV lytic program in certain Burkitt's lymphoma (BL) cell lines after treatment with anti-Ig antibodies can be viewed as a 'side effect' of the BCR signaling, but these cell lines can serve as an invaluable tool to study pathways responsible for disruption of viral latency. A number of studies demonstrated that activation of PI3K, PKC, MAPK, and NF-κB pathways is crucial for activation of EBV lytic program (Daibata et al., 1990; Darr et al., 2001; Davies et al., 1991; Gao et al., 2001; Iwakiri and Takada, 2004; Lazdins et al., 1987; Mori and Sairenji, 2006; Oussaief et al., 2011; Satoh et al., 1999); however, other reports suggested that inhibition of PI3K or NF-κB pathways facilitates reactivation of lytic program of several gammaherpesviruses (Brown et al., 2003; Liu et al., 2008; Peng et al., 2010). The present study used a set of protein kinase inhibitors (PKIs) to verify contribution of several signaling pathways in EBV lytic program. The results indicate that inhibition of PI3K, MAPK, NF-κB pathways, and to some extent cyclin-dependent kinases (CDKs) significantly reduced EBV replication. An effect of these inhibitors on the activity of the EBV-encoded protein kinase was also analyzed, and this analysis showed that only K252A inhibited the EBV-PK activity *in vitro*.

2. Materials and methods

2.1. Reagents

Screen-Well™ Kinase Inhibitor Library was purchased from Enzo Life Sciences and contains 80 known kinase inhibitors supplied dissolved in dimethyl sulfoxide (DMSO) at 10 mM (a complete list of compounds can be found on the company's website). After initial screen, selected compounds were purchased from the following suppliers: LY294002, Ro-31-8220, 5-Iodotubercidin, Erbstatin analogue, and Kenpaullone from Cayman Chemicals; Wortmannin, Staurosporine and K252A from LC Laboratories; Rottlerin from Enzo Life Sciences; Bay-11-7082 from Calbiochem; and acyclovir (ACV) from Sigma. All compounds stocks were prepared in DMSO at concentration 10 mM and frozen in aliquots. A fresh aliquot was used for each experiment.

2.2. Cell culture

Akata is a Burkitt's lymphoma (BL) cell line latently infected with EBV (Takada et al., 1991). The cells were maintained in RPMI 1640 medium (Gibco, Invitrogen) supplemented with antibiotic/antimycotic mixture (Anti–Anti, Gibco, Invitrogen), MEM non-essential amino acids mixture (Gibco, Invitrogen), 1 mM sodium pyruvate (BioWhittaker, Cambrex Bio Science), 2 mM L-glutamate (Cellgro), and 10% fetal bovine serum. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. SF9 cells are a subclone of *Spodoptera frugiperda* (Fall Armyworm) IPLB-Sf21-AE cells (Invitrogen). The cells were maintained in Grace's insect medium (Invitrogen) supplemented with 2 mM L-glutamate (Cellgro) and 10% fetal bovine serum. For protein expression, the cells were adapted to serum-free suspension culture in Serum-Free (Sf) Insect Culture Medium (Allele Biotechnology) supplemented with 2 mM L-glutamate. The cells were cultured at 27 °C.

2.3. Viral reactivation and treatment with protein kinase inhibitors

To initiate viral lytic program, exponentially growing cells were pelleted by centrifugation at 500*g* and resuspended in fresh medium containing 50 μg/mL goat anti-human IgG ($F(ab')_2$) (Jackson Immunoresearch Laboratories) and 10 μM of the inhibitors. Cells treated only with DMSO served as a control for an uninhibited viral reactivation; cells treated with 50 μM of ACV served as a control for an efficient inhibition of viral replication.

2.4. Real-time PCR

The quantitative real-time PCR (qPCR) was performed using a TaqMan probe targeting the EBV BamHI fragment, which has previously been described in detail (Fan and Gulley, 2001; Gershburg et al., 2007). Briefly, total genomic DNA was isolated from Akata cells 48 h after viral reactivation using a DNeasy Blood and Tissue kit (Qiagen). One microliter of this DNA was analyzed by qPCR on a Step-One real-time thermocycler (Applied Biosystems) under the following cycle conditions: 50 °C for 2 min (1 cycle), 95 °C for 10 min (1 cycle), and 95 °C for 15 s and 60 °C for 1 min (40 cycles). Viral DNA copy numbers were calculated from external standards of known concentrations of EBV B95-8 DNA (Advanced Biotechnologies Inc., MD). The primers and the probe used for PCR are EBVW-1 (5′-GCAGCCGCCCAGTCTCT-3′), EBVW-2 (5′-ACAGACAGTGCA CAGGAGCCT-3′), and EBVW-FAM (5′-FAM [6-carboxyfluorescein]-AAAAGCTGGCGCCCTTGCCTG-TAMRA [6-carboxytetramethylrhodamine]-3′).

2.5. Cell viability assays

Effects of protein kinase inhibitors on the viability of latently infected Akata cells were measured by CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega). For these assays, the cells were seeded at 2×10^5 cells/well in 96-well plates (in triplicates) and treated with different concentrations (0.0005–30 μM) of the selected PKIs. Assay buffer was added 48 h after treatment for additional 4 h. The color development was measured on a PowerWave XS plate reader (Bio-Tek) at wavelength 490 nm.

2.6. Expression and purification of the GST-tagged EBV protein kinase

The coding sequence of EBV BGLF4 gene (B95-8 strain, Genebank V01555, nucleotides 122328–123692) was amplified by PCR and inserted into the NcoI and XhoI restriction sites of a modified pFastBacHT vector in which the His_6 tag was replaced with a glutathione S-transferase (GST) tag (pFastBacGST) (generously provided by J. Sondek, University of North Carolina at Chapel Hill). An inactive kinase variant was generated by using site-directed mutagenesis changing an invariant lysine-102 into isoleucine. Recombinant baculoviruses were prepared following the Bac-to-Bac method (Invitrogen).

Recombinant GST-BGLF4 was expressed in SF9 insect cells for 72 h at 27 °C after virus infection. The cells were lysed in 20 mM Tris (pH 7.5), 300 mM NaCl, 1 mM DTT, 0.5% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 10% (v/v) glycerol, and Halt protease inhibitor cocktail (Pierce Biotechnology) on a XL-2000 sonicator (Qsonica, LLC). GST-BGLF4 was purified using sequential affinity (GSTrap FF) and ion-exchange (HiTrap DEAE FF) (GE Healthcare) column chromatography steps. Fractions containing the GST-BGLF4 protein were pulled and concentrated in 20 mM Tris (pH 7.5), 200 mM NaCl, 1 mM DTT and 10% (v/v) glycerol for *in vitro* kinase assays.

2.7. In vitro kinase assays

The kinase assays were performed as previously described (Gershburg et al., 2010) with several modifications. Each reaction contained 1pmole of GST-BGLF4 kinase and 1 μg of myelin basic protein (MBP) as a substrate in a kinase buffer (75 mM Hepes, pH 7.3, 10 mM NaCl, 5 mM $MnCl_2$, 1 mM dithiothreitol (DTT) and 0.1% NP-40) complemented with 50 μM ATP (Sigma), 2.5 μCi of [γ-^{32}P]-ATP (specific activity 4500 Ci/mmol, MP Biomedicals), and 10 μM of each protein kinase inhibitor or DMSO. The reactions were incubated for 5 min at 37 °C in Thermomixer (Eppendorf) and then half of the volume (10 μl) of each reaction was spotted on a P81 phosphocellulose disc (Whatman). The discs were sequentially washed twice in 75 mM H_3PO_4 and once in 95% ethanol and air-dried. The discs were placed in 1 ml of ScintiSafe Econo-1 scintillation liquid (Fisher Scientific) and amounts of incorporated ^{32}P were measured on a scintillation counter (Beckman-Coulter).

2.8. Immunoblotting

EBV lytic program was reactivated in Akata cells as described above. At 24 h post reactivation, the cells were collected, washed with PBS, and whole-cell lysates were prepared in RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing Halt protease and phosphatase inhibitor cocktails (Pierce Biotechnology). Cell debris was removed by centrifugation at 14000 rpm for 15 min. Then, 5 μg of the lysates were denatured in Laemmli loading buffer (5 min at 95 °C), resolved by SDS–PAGE (10% gels), and transferred onto nitrocellulose membranes (Millipore). After incubation in blocking buffer [5% Difco skimmed milk (BD Biosciences) in TBST (Tris-buffered saline with 0.2% Tween 20)], the membranes were incubated for 16 h with anti-EA-D mAb (Bioworld Consulting Laboratories) diluted to 1:1000 in blocking buffer. The membranes were washed in TBST in incubated for 1 h with IRDye-800CW-conjugated goat anti-mouse secondary antibody (Li-Cor Biosciences) diluted 1:20000 in blocking buffer. After additional washes, the proteins of interest were visualized on an Odyssey® Infrared Imaging system (Li-Cor Biosciences). To verify the equal loading, membranes were re-probed with anti-β-actin mAb (Sigma) used at 1:2000 dilution.

3. Results

3.1. Protein kinase inhibitors (PKIs) that inhibit EBV reactivation in Akata cells

Our initial objective was to investigate whether inhibition of different protein kinases will affect EBV reactivation. To this end, we used a commercial set of protein kinase inhibitors (Screen-Well™ Kinase Inhibitor Library from Enzo Life Sciences) and examined their effect on EBV lytic program in Akata cells. Due to the large number of inhibitors, conditions of the assay were simplified: (a) only one concentration (10 μM) of PKIs was used, and (b) the inhibitors were added concurrently with the inducer, goat anti-human IgG. The cells were incubated for 48 h post-induction and EBV genome copy numbers were enumerated in total genomic DNA by qPCR. The data of 3 independent experiments performed in duplicates was processed as follows: (a) in each experiment, genome copy numbers were calculated based on standard curve built using a commercial standard EBV DNA, (b) the number of genomes per nanogram of total genomic DNA was calculated, (c) these concentrations were normalized and expressed as percentage relative to the positive control (cells treated with DMSO), (d) percent values were averaged and expressed as (100–[mean of the 3 experiments]) ± standard error of mean (sem), *p*-values were calculated using one sample *t*-test (Fig. 1). Based on degree of inhibition, the inhibitors were divided into active (over 80% inhibition), partially active (50–79% inhibition), and inactive (less than 50% inhibition); only compounds in the active group were selected for further studies. ACV, a known inhibitor of viral replication, was used at 50 μM and reduced viral replication by 88 ± 6%, which is comparable to the cells not treated with IgG (96 ± 2%).

Staurosporine and K252A are pan-specific protein kinase inhibitors, which affect a wide range of protein kinases and pathways. Therefore, while inhibition of viral replication by these compounds (88 ± 6% and 92 ± 6%, respectively) implies that protein kinases play a significant role in viral replication, no specific pathway could be identified. In addition, a number of compounds that inhibit certain pathways more specifically were also found to be effective in inhibiting the EBV replication. LY294002 and Wortmannin are PI3 kinase inhibitors (Arcaro and Wymann, 1993; Vlahos et al., 1994); U0126 inhibits mitogen-activated protein kinase kinases 1 and 2 (MEK1/2) (Favata et al., 1998); Tyrphostin 9 is a platelet-derived growth factor receptor (PDGFR)-associated tyrosine kinase inhibitor (Bilder et al., 1991); PKC412, a Staurosporine derivative, inhibits multiple kinases, but its main target is PKC (Fabbro et al., 2000); Ro 31-8220 inhibit PKC and GSK-3β (Gschwendt et al., 1994; Hers et al., 1999; McKenna and Hanson, 1993); rottlerin, which originally thought to specifically inhibit PKCδ, was later shown to inhibit multiple other kinases (Davies et al., 2000), 5-Iodotubercidin inhibits casein kinase I (CKI), PKC, and ERK2 (Fox et al., 1998; Massillon et al., 1994); erbstatin analog is an epidermal growth factor receptor (EGFR)-associated tyrosine kinase inhibitor (Umezawa et al., 1990); BAY-11-7082 is a nuclear factor kB (NF-κB) pathway inhibitor (Pierce et al., 1997); and Kenpaullone inhibits glycogen synthase kinase 3β (GSK-3β) and several cyclin-dependent kinases (CDKs) (Schultz et al., 1999; Zaharevitz et al., 1999). Thus, our screen identified or confirmed a number of protein kinase inhibitors that inhibit EBV replication thereby pointing out that pathways inhibited by these compounds play a role in EBV replication.

3.2. Selectivity of the PKIs inhibiting EBV replication

Next, we asked whether the antiviral effect of the selected PKIs is due to a decrease in cell viability. The cells were incubated with 0.0005–30 μM of PKIs, which were active in the EBV replication

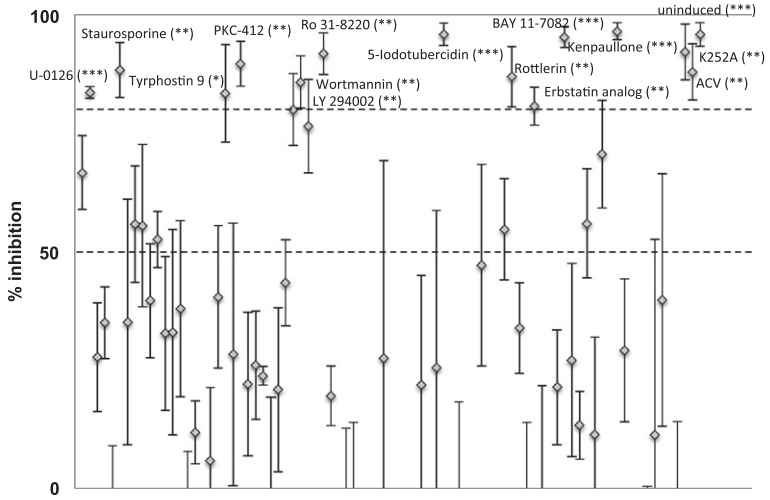

Fig. 1. EBV replication in Akata cells is inhibited by selected protein kinase inhibitors. EBV lytic program was induced in Akata cells by IgG treatment in the presence of 10 μM of each PKI. Forty-eight hours after induction, total genomic DNA was isolated and EBV genomes were enumerated by qPCR. Results are expressed as percentage of inhibition relative to the DMSO treatment. Means ± sem of 3 independent experiments are shown. Names of the compounds selected for further studies are shown. The selection cut-off values of 50% and 80% inhibition are marked by dashed lines. DMSO-treated uninduced cell and ACV-treatment served as negative controls. *p*-values are: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

inhibition assay, for 48 h at which point their metabolic activity was measured by a modified MTS assay (Promega) and EBV genome copy numbers were measured by qPCR. The cytotoxic and inhibitory concentrations inhibiting the cellular activity and viral replication by 50% (CC50 and IC50) were calculated in Prism 5 (GraphPad) using a four parameters sigmoidal dose–response curve and summarized in Table 1. A control treatment with ACV at 50 μM exhibited no significant effects on cell viability (89 ± 4% remaining viability relative to DMSO-treated cells) (not shown). As expected, Staurosporine and K252A have had a major effect on cell viability with CC50 values of 0.004 and 0.08 μM, respectively, because both compounds were previously shown to cause apoptosis (Bertrand et al., 1994; Chin et al., 1999). Likewise, Tyrphostin 9, PKC-412, rottlerin, Ro 31-8220, and 5-iodotubercidin were relatively toxic (CC50 ranging from 0.056 to 1.147 μM). These compounds (except for 5-iodotubercidin) also induce apoptosis in variety of systems (Bahlis et al., 2005; Han et al., 2000; Palumbo et al., 1997; Ringshausen et al., 2006) Moreover, majority of the compounds were non-selective and had a low therapeutic index (TI) ranging from 1 to 3. Only 3 compounds had a higher TI – U-0126, Wortmannin, and BAY 11-7082 (TI = 13); the TI for Kenpaullone and K252A were 8 and 6 respectively (Table 1). These results indicated that the compounds inhibiting MAPK, PI3K, and NF-κB pathways (and to some extent, CDKs) were able to inhibit EBV replication with certain degree of selectivity. It should also be noted that while the TI values even for more specific compounds were lower than values reported for other EBV inhibitors (Gershburg et al., 2004; Williams et al., 2003), these data identify pathways intimately involved in EBV reactivation.

Table 1
Selectivity of PKIs inhibiting EBV replication. EBV lytic program was induced in Akata cells by IgG treatment in the presence of 0.0005–30 μM of selected PKIs. Forty-eight hours after induction, EBV genomes were enumerated by qPCR and cell viability was measured by a modified MTS assay. Cytotoxic and inhibitory concentrations inhibiting the cellular activity and viral replication by 50% (CC50 and IC50) were calculated in Prism 5 (GraphPad) using a four parameters sigmoidal dose–response curve. Therapeutic index (TI) represents the CC50/IC50 ratio.

	CC50 (μM)	IC50 (μM)	TI
U-0126	7.376	0.552	13
Staurosporine	0.004	0.005	1
Tyrphostin 9	0.573	0.217	3
PKC-412	0.056	0.031	2
LY 294002	6.160	1.835	3
Wortmannin	4.686	0.367	13
Ro 31-8220	1.147	0.398	3
5-Iodotubercidin	1.121	0.890	1
Rottlerin	1.005	0.983	1
Erbstatin analog	3.896	2.373	2
BAY 11-7082	2.636	0.208	13
Kenpaullone	5.127	0.681	8
K252A	0.080	0.014	6

3.3. Inhibition of the EBV reactivation vs replication

MAPK, PI3K, NF-κB pathways have previously been shown to play a significant role in EBV infection (Daibata et al., 1990; Darr et al., 2001; Davies et al., 1991; Gao et al., 2001; Iwakiri and Takada, 2004; Lazdins et al., 1987; Mori and Sairenji, 2006; Oussaief et al., 2011; Satoh et al., 1999). However, no clear distinction has been made as to whether these pathways play a role in viral reactivation or in later steps of the infection. In other words, does the inhibition of these pathways only prevent signal transduction that leads to lytic program initiation or these pathways also function at later steps in viral infection? To distinguish these two possibilities, steady-state levels of an early EBV protein, EA-D, in the presence of 1 and 10 μM of selected PKIs were analyzed by immunoblotting. This protein is usually detected 6–8 h after lytic program reactivation and its expression is regulated by EBV immediate-early proteins BZLF1 and BRLF1 (Adamson and Kenney, 1998; Feederle et al., 2000). In the presence of 1 μM of Staurosporine, K252A, PKC-412, and Wortmannin no EA-D expression was detected (Fig. 2B). In contrast, the same concentration of U-0126, Tyrphostin 9, Ro 31-8220, 5-Iodotubercidin, rottlerin, ebstatin analog, and kenpaullone did not affect the expression of EA-D (Fig. 2B). The effect of BAY 11-7082 and LY294002 was intermediate and mainly affected the levels of a hyperphosphorylated form of the EA-D, whereas treatment with Ro 31-8220 lead to an increased level of this form (top band, Fig. 2B). At 10 μM the inhibitory effect of all compounds (perhaps with exception of Erbstatin analog) is more pronounced (Fig. 2A). The EA-D expression levels remain relatively high in U-0126 treatment (although visibly lower than at 1 μM treatment), whereas they are hardly detectable for other

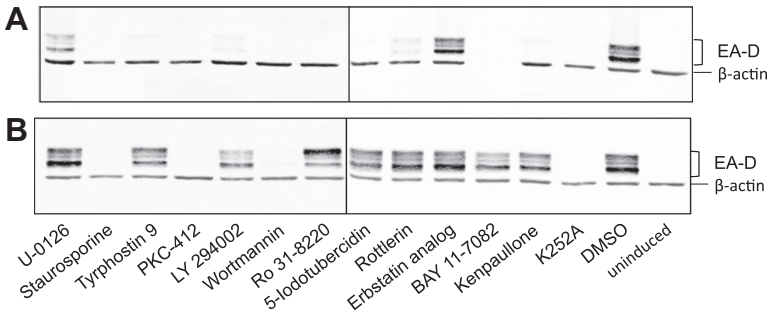

Fig. 2. Effect of PKIs inhibiting EBV replication on the EBV EA-D expression. EBV lytic program was induced in Akata cells by IgG treatment in the presence of 10 μM (A) or 1 μM (B) of selected protein kinase inhibitors. Twenty-four hours after induction, whole-cell lysates were prepared and subjected to SDS–PAGE. EA-D expression was assessed by immunoblotting.

treatments. Moreover, at 10 μM all compounds exhibit significant toxicity (Table 1) as exemplified by the Bay 11-7082, which even affected expression levels of housekeeping genes (loss of β-actin, Fig. 2A). These results suggest that an active PI3 kinase is essential for reactivation of the EBV lytic program, because its inhibition by an irreversible inhibitor (Wortmannin) completely blocked viral reactivation, while the treatment with suboptimal concentrations of the reversible PI3 kinase inhibitor LY294002 resulted only in partial inhibition (Fig. 2B). The results also suggest that pan-specific inhibitors, such as K252A, also block viral reactivation likely by inhibiting multiple pathways.

3.4. Properties of GST-BGLF4 in vitro activities

Treatment with LY294002 and BAY 11-7082 had a more pronounced effect on the levels of the hyperphosphorylated form of EA-D (Fig. 2B). This form is only detected in the presence of an active EBV-encoded protein kinase (EBV-PK) (Gershburg and Pagano, 2002), which also has previously been shown to play a critical role in viral replication (Gershburg et al., 2007; Murata et al., 2009). Therefore, we inquired whether the inhibition of viral replication by selected PKIs involves a direct inhibition of the EBV-PK activity. To this end, the EBV-PK was expressed as a fusion with a glutathione S-transferase (GST) gene in insect cells infected with a recombinant baculovirus. The purification procedure included an affinity and ion-exchange chromatography steps and resulted in near homogeneous preparation of GST-tagged EBV-PK used in the consequent experiments (not shown). Next, we tested the activity of the GST/EBV-PK at various reaction conditions (Fig. 3). We found that the optimal conditions for the GST/EBV-PK slightly differ from previously reported (Chen et al., 2000; Daikoku et al., 1997; He et al., 1997). We found that the rate of phosphorylation was linear for ~7 min and complete by 20 min. To determine optimal conditions, the reactions were stopped at 5 min and phosphorylation rates were measured by filter-binding assays. Phosphorylation was greatest at physiological pH, with maximal incorporation at about pH 8.0 (Fig. 3D) and significantly reduced at higher pH. The activity was inhibited by salt, with an optimum at 100 mM NaCl and with about 25% activity remaining at 0.5 M NaCl (Fig. 3C). The activity absolutely required Mn^{2+}, which could not be replaced by Mg^{2+} (Fig. 3B), Ca^{2+}, Zn^{2+}, or Co^{2+} (not shown) at concentrations up to 10 mM. The final conditions used in

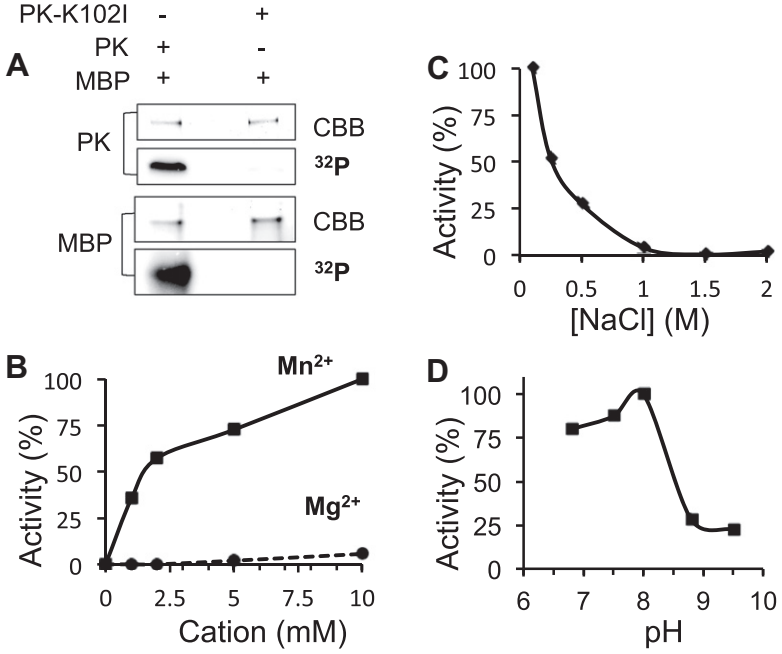

Fig. 3. Optimization of reaction conditions for GST-tagged EBV-PK. (A) One pmole of wild-type or inactive EBV-PK (PK and PK-K102I, respectively) were incubated with 1 μg of MBP as described in Section 2 and subjected to SDS–PAGE. The gel was stained with Coomassie R-250 (CBB) and the ^{32}P incorporation (^{32}P) was assessed on a phosphor imager. (B–D) EBV-PK was incubated with MBP as described above except that divalent cation concentrations (B), NaCl concentrations (C), and pH (D) varied as indicated. The ^{32}P incorporation was measured by filter binding assays.

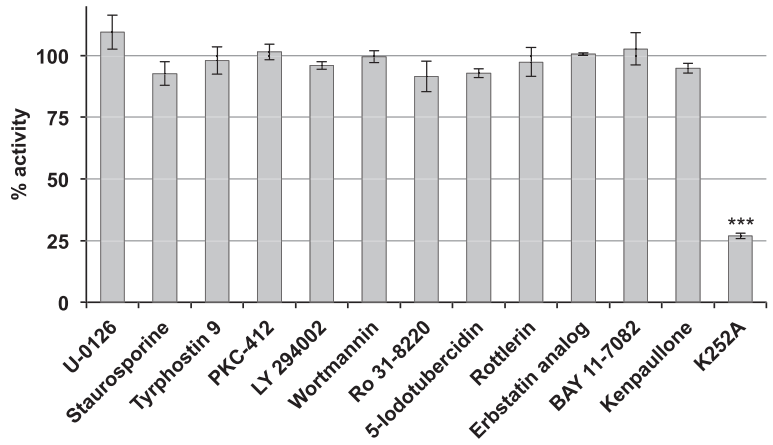

Fig. 4. Effect of the PKIs inhibiting EBV replication on the EBV-PK activity *in vitro*. EBV-PK was incubated with MBP at optimal conditions established earlier and in the presence of DMSO or 10 μM of selected inhibitors. The ^{32}P incorporation was measured by filter binding assays and is expressed as percentage of kinase activity relative to the DMSO control. Means ± sem of 3 independent experiments are shown.

subsequent assays were: 75 mM Hepes, pH 8.0, 100 mM NaCl, 5 mM $MnCl_2$, 1 mM dithiothreitol (DTT), 0.1% NP-40, and 50 μM ATP. To verify that the observed kinase is due to the GST/EBV-PK and not due to a co-purified cellular kinase, an inactive mutant of the EBV-PK, in which an invariable lysine-102 has been mutated into isoleucine, was tested in the optimized conditions and exhibited no detectable activity, whereas the wild type EBV-PK efficiently phosphorylated MBP and itself (Fig. 3A).

3.5. Effects of selected PKIs on GST-BGLF4 kinase activity in vitro

Protein kinase inhibitors that exhibited a significant (more than 80%) anti-EBV activity were tested *in vitro* to assess their direct effect on the EBV-PK activity. Purified GST/EBV-PK was incubated with 10 μM of inhibitors or DMSO at previously optimized conditions, and its activity was measured by filter binding assays. Incubation with K252A served as a positive control for the assays, because we have previously shown that this compound inhibits the EBV-PK activity in a similar assay (Gershburg et al., 2004). Indeed, K252A efficiently inhibited the GST/EBV-PK activity by about 75%. In contrast, other tested PKIs exhibited only negligible inhibitory effects on the GST/EBV-PK activity (Fig. 4). To verify the activity of used compounds, 5,6-dichloro-1-β-D-ribofuranosilbenzimidazole (DRB), apigenin, and 5-Iodotubericin were incubated with their known target, CKII, and almost completely blocked its activity at significantly lower concentrations (not shown). These results (a) suggest that the anti-EBV activity of PKIs is not mediated through the EBV-PK, and (b) imply that despite encoding its own protein kinase the virus relies on several host cell protein kinases (and potentially several signaling pathways) to attain an efficient infection.

4. Discussion

4.1. BCR-mediated induction of the EBV lytic program and its inhibition by PKIs

Ag binding to BCR activates signaling cascade that involves multiple pathways. In certain EBV-positive BL cell lines, BCR signaling reactivates EBV lytic program, but the contribution of different pathways in viral reactivation is not clearly understood. The present study aimed to analyze the contribution of these different pathways by using a set of protein kinase inhibitors that inhibit components of the several major pathways involved in BCR signaling as well as additional pathways not apparently involved in BCR signaling. A simplified flowchart of the BCR signaling and some inhibitors used in the study are presented in Fig. 5.

Out of 80 PKIs used in the study 32 primarily target tyrosine kinases including Syk and Btk, which play a significant role in BCR signaling (Dal Porto et al., 2004; Kurosaki et al., 2010). Only two compounds, Tyrphostin 9 and Erbstatin analog, which inhibit platelet-derived growth factor receptor-associated tyrosine kinase (PDGFRK) and epidermal growth factor receptor-associated tyrosine kinase (EGFRK), respectively, inhibited EBV replication in cells. However, the selectivity of both compounds in our assays was low (TI = 3 and 2, respectively). This lack of selectivity may indicate that the antiviral effect is simply a result of cytotoxicity, in particular for Tyrphostin 9, which has been shown to inhibit cdk2 activity, block cell cycle, and cause apoptosis in addition to inhibiting PDGFRK (Palumbo et al., 1997). Such an assumption is strengthened by the fact that none of the other EGFRK or PDGFRK inhibitors demonstrated any anti-EBV activity. Most surprising however was lack of the effect of Syk and Btk inhibitors. None of the three inhibitors targeting these kinases inhibited EBV replication. Two possible explanations for this phenomenon are (1) the effective inhibitory concentration was not reached (the reported IC50 for one of the Btk inhibitors, LFM-A13, is 17.2 μM), and (2) these kinases are not essential in pathways leading to viral reactivation and the activity of Lyn and participation of other Src family kinases may be sufficient for reactivation of EBV lytic program (Dal Porto et al., 2004).

Broad range PKIs such as Staurosporine, its derivative PKC-412, and K252A expectedly inhibited viral replication, but this effect is likely due to their cytotoxicity (TI = 1, 2, and 6, respectively). The compounds target multiple protein kinases including PKC that has previously been shown to play role in EBV reactivation (Daibata et al., 1990; Davies et al., 1991; Gao et al., 2001; Lazdins et al., 1987) and, in agreement with such a possibility, all three compounds block expression of EBV early protein EA-D (Fig. 2).

The only other compound that inhibited EA-D expression (and likely EBV reactivation) is a PI3K inhibitor wortmannin. PI3K plays an essential role in BCR signaling (Dal Porto et al., 2004; Kurosaki et al., 2010) and EBV reactivation (Iwakiri and Takada, 2004) and hence the effect of wortmannin is not surprising particularly when another PI3K inhibitor, LY294002, similarly showed high anti-EBV activity. The activity of the latter was somewhat less pronounced likely due to the differences in mechanisms of action (Vlahos et al., 1994; Wymann et al., 1996) and the fact that concentration of LY294002 was below its IC50 (Fig. 2B and Table 1). More surprising however was lack of activity of quercetin (on basis of which the

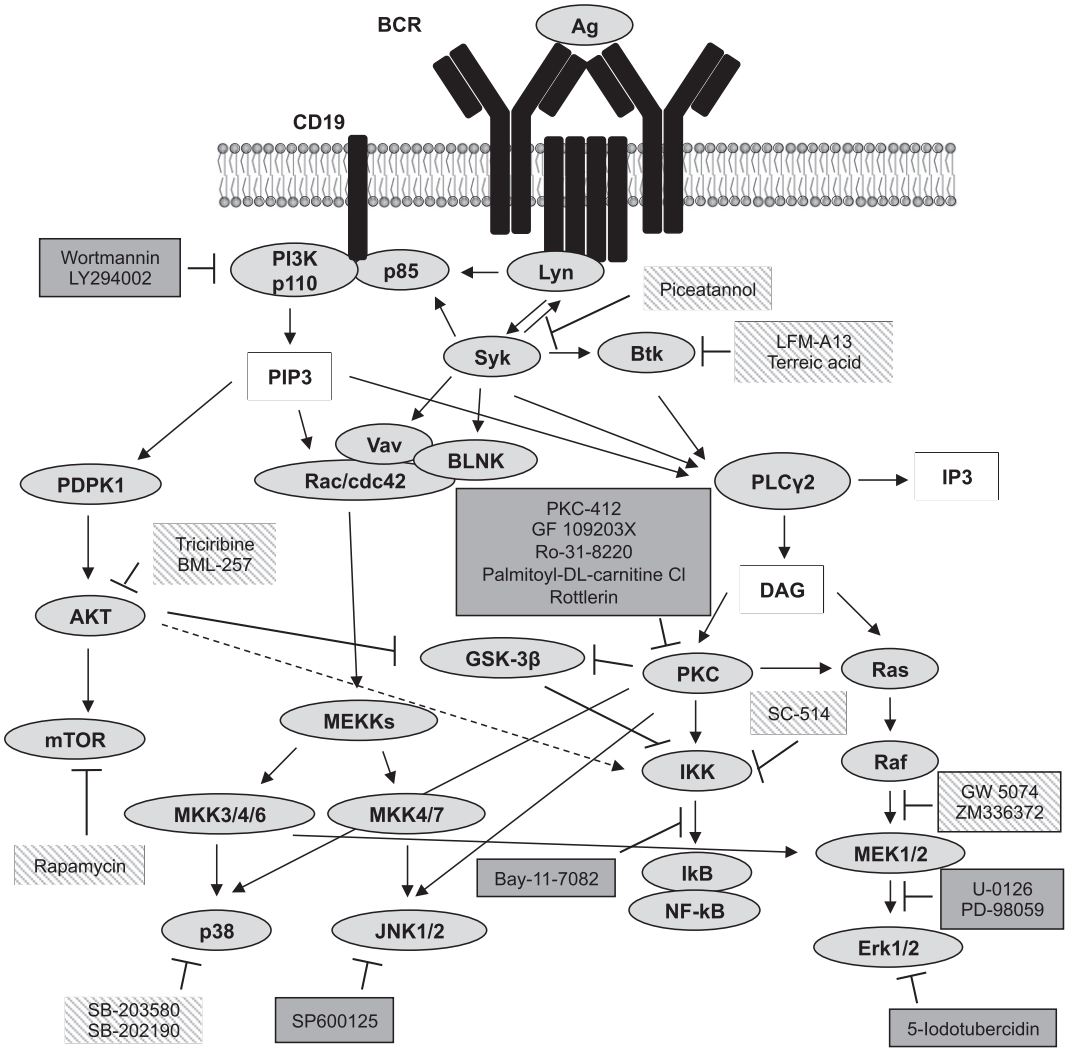

Fig. 5. Diagram of pathways activated in BCR signaling and selected inhibitors affecting these pathways. Protein components of the pathways are shown as light grey ovals, non-protein component are shown as white rectangles. PKIs that inhibited EBV replication are shown in dark grey rectangles; PKIs that had no effect on EBV replication are shown in patterned rectangles.

LY294002 was synthesized). A traditional PI3K/Akt/mTOR pathway seems to be dispensable for the EBV reactivation since inhibitors of mTOR and Akt had no effect on viral replication.

The study confirmed that PI3K, PKC and MAPK are the key components in a pathway (or pathways) leading to EBV reactivation. Several inhibitors of PKC, NF-κB, and MAPK pathways inhibited EBV replication with different degree of selectivity. More importantly, however, we identified several components that did not affect EBV reactivation or replication: (a) inhibition of p38MAPK by three specific inhibitors had no effect on viral replication; (b) inhibition of Raf by two inhibitors had no effect on viral replication (even though inhibitors of MEK1/2 and Erk1/2 inhibited it); (c) inhibition of IKKβ by SC-514 had no effect, whereas less specific Bay 11-7082 inhibited viral replication suggesting the leading role of IKKα in this process. Taken together, these results suggest that EBV reactivation induced by BCR signaling is achieved by a coordinated activity of several interconnected pathways in which PI3K, PKC, and MAPK pathways play a dominant role.

4.2. In vitro activity of the EBV-PK and its inhibition by PKIs

Optimal conditions required for the EBV-PK were somewhat different from other CHPKs tested to date (Daikoku et al., 1997; He et al., 1997). The enzyme's activity peaked at physiological pH and low salt concentrations, and was highly selective in terms of its needs in bivalent ions (Fig. 3). All these are in contrast to preferences to high salt concentrations, relatively high pH, and a relative ion substitution tolerance (Mn^{2+} substitution for Mg^{2+}) previously shown for a GST-tagged purified pUL97 of HCMV (He et al., 1997), or lack of pH sensitivity and full activity in Mg^{2+} (other ions were not tested) shown for purified UL13 of HSV-2 (Daikoku et al., 1997). Our data also differs from a previously published analysis of EBV-PK immunoprecipitated from mammalian cells transiently expressing an EBV-PK protein tagged with an EBNA-1-based tag (Chen et al., 2000). The discrepancies between the two datasets are most likely due to the degree of purification; the enzyme used in our studies is highly purified through several chromatography steps and doesn't seem to carry contaminants that possess a detectable kinase activity, whereas the immunoprecipitated complexes used in the other study most likely contained such contaminants which is illustrated by the fact that a mutant with an invariable lysine-102 changed into an alanine (as well as several other similar mutants) was still fully active [Figs. 10 and 11 in (Chen et al., 2000)]. While the biological relevance of the differences in optimal conditions for different herpesviral conserved protein kinases is not clear, the study establishes experimental conditions at which the highly purified EBV protein kinase exhibits optimal activity.

Another aspect of the study dealt with the effect of tested protein kinase inhibitors on the activity of EBV-PK *in vitro*. None of the anti-EBV compounds (except for K252A) inhibited the viral protein kinase (Fig. 4). Moreover, we eventually tested all 80 compounds and only few of them exhibited marginal inhibitory effect (10–15% relative to DMSO-treated control) (not shown). These observations reiterate the structural "uniqueness" of this kinase (and likely the other CHPKs) in comparison to cellular kinome and raise the possibility of developing a highly specific inhibitor against this protein kinase.

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