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Proteasome inhibitors block HIV-1 replication by affecting both cellular and viral targets

Liang Yu [a,c,*,1], Venkatramanan Mohanram [b,1], Oscar E. Simonson [a], C.I. Edvard Smith [a], Anna-Lena Spetz [b,2], Abdalla J. Mohamed [a,*,2]

[a] Department of Laboratory Medicine, Clinical Research Center, Karolinska Institutet, Karolinska University Hospital Huddinge, Stockholm, Sweden
[b] Department of Medicine, Center for Infectious Medicine, Karolinska Institutet, Karolinska University Hospital Huddinge, Stockholm, Sweden
[c] Department of Hematology, Huaian No. 1 Hospital, Nanjing Medical University, Huaian 223300, Jiangsu, China

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ABSTRACT

HIV-1 has proved to be notoriously difficult to tackle despite the availability of more than 20 clinically approved drugs. The majority of these drugs, however, target viral genes and their continued use will select for drug-resistant strains. Since NF-κB signaling is critical for viral replication, we wanted to investigate the effect of proteasome inhibitors on viral gene expression. We herein demonstrate that proteasome and NF-κB inhibitors effectively shut down transcription from the HIV-1 LTR-promoter. We further show that replication of HIV-1 in PBMC was severely compromised following treatment with proteasome inhibitors alone or in combination with other antiretroviral drugs. Finally, incubation of PBMC with these drugs reduced expression of IL-2 inducible T cell kinase (Itk), a Tec-family kinase, recently shown to be required for HIV-1 replication. These results suggest that proteasome inhibitors suppress LTR-promoter activity by interfering with cellular targets required for viral replication.

Introduction

Since the identification of HIV-1 as the causative agent of AIDS, there have been remarkable advances in the treatment of HIV-1 infection. Nonetheless, there is at present no effective drug capable of eradicating the virus in HIV-1 infected individuals, and the emergence of highly drug-resistant viral strains remains a challenge [1,2].

More recently, several additional targets of the HIV-1 life cycle have been identified as potential intervention sites for antiviral chemotherapy [3]. The LTR-promoter is pivotal in controlling HIV-1 replication not only during acute viral infection but also in chronically infected cells. Consequently, inhibitors of HIV-1 transcriptional regulation may have the potential to significantly inhibit virus replication.

Regulation of HIV-1 transcription is a complex, multistage process requiring cooperative action of both viral and cellular proteins. The enhancer region of HIV-1 LTR-promoter contains two functional active NF-κB binding sites [4]. NF-κB is a ubiquitous transcription factor critical in inflammation and immunity. Previously, various reagents targeting the NF-κB signaling pathway have been examined for their inhibitory effects on HIV-1 [5].

NF-κB activation and signaling is dependent on the activity of the 26S proteasome. Protein ubiquitination is a posttranslational modification event critical in regulating many cellular processes [6,7]. The fact that the ubiquitin–proteasome-pathway is involved in key biological processes validates it as a novel therapeutic target. To date one proteasome inhibitor Bortezomib has made its way into the clinic, being the first FDA-approved proteasome inhibitor for treating multiple myeloma [8].

We have recently demonstrated that proteasome inhibitors can repress transcription of Bruton's tyrosine kinase through inhibition of the NF-κB signaling pathway [9]. In the present work, we demonstrate, both *in vivo* and *in vitro*, that proteasome inhibitors dramatically reduce transcription from the HIV-1 LTR-promoter. In addition, these inhibitors, including the clinical drug Bortezomib, markedly inhibit HIV-1 replication in human PBMC.

The IL-2 inducible T cell kinase (Itk) is the major Tec-family tyrosine kinase that controls T cell activation and development [10]. It was recently shown that inhibition of Itk blocks HIV-1 replication at multiple stages of the viral life cycle [11]. We previously reported that proteasome inhibitors reduce steady-state levels of Itk in Jurkat cells [9]. Here, we show that these drugs potently

* Corresponding authors. Address: Department of Laboratory Medicine, Clinical Research Center, Karolinska Institutet, Karolinska University Hospital Huddinge, SE-141 86 Stockholm, Sweden. Fax: +46 8 58583650.
E-mail addresses: liang.yu@ki.se, liangkarolinska@yahoo.com (L. Yu), abdalla.jama@ki.se (A.J. Mohamed).
[1] These authors have contributed equally to this work.
[2] These authors have contributed equally to this work.

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block induction of Itk expression upon activation of primary PBMC. Our results suggest that proteasome inhibitors reduce HIV-1 replication by targeting both the LTR-promoter and the endogenous transcriptional machinery.

Materials and methods

Reagents. Anti-GFP antibody was from BD Biosciences. Anti-Itk antibody and proteasome/NF-κB inhibitors have been described [9]. Azidothymidine (AZT) from GlaxoSmithKline (Brentford, Middlesex, United Kingdom), and Indinavir (IND) from MERCK (NJ, USA).

Cell culture and transfections. COS7, HEK293T, A20, and Jurkat cells were grown and transfected as described [9,12]. PBMC from healthy blood donors were purified and cultured as described [13].

HIV-1 infection of primary cells. The viral isolate used was a chemokine receptor 5-using HIV-1_{BaL} isolate (NIH AIDS Research and Reference Reagent Program, NIH, USA).

PHA stimulated PBMC were seeded in 96-well flat bottom plates (2×10^5 cells/well) and exposed to HIV-1_{BaL} at a tissue culture infective dose ($TCID_{50}$) of 3000 or mock. Twenty-four hours later, cells were washed and fresh RPMI medium was added with or without drugs (Bortezomib, MG132, IND, AZT), and further cultured for 3 days, then washing to remove the drugs. Finally, cells were cultured for three additional days. Supernatants were harvested at day 7 and RT-activity was quantified using the Lenti-RT-activity kit (Cavidi Tech, Uppsala, Sweden). Cell viability was assessed by the WST-1 kit (Roche Applied Science, Mannheim, Germany).

Absolute quantification of HIV-LTR in PBMC. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). HIV-LTR sense, anti-sense primer sequences and probes were previously described [14] (Applied Biosystems). For absolute quantification of HIV-LTR, a control plasmid was constructed by cloning the 121 bp HIV-LTR amplicon into pCR 4-TOPO (Invitrogen). Plasmid copy number was quantified as described [15].

Plasmid constructs and luciferase assay. To create plasmid pLTR-GFP, a 450-bp fragment corresponding to the HIV-1 LTR-promoter region was isolated from the plasmid pLTR/CAT (AIDS reagent project/program EVA centralized facility) and sub-cloned into the promoter-less plasmid pEGFP-1. Similarly, the HIV-1 LTR-luciferase reporter (pLTR-Luc) was generated by cloning the LTR-encoding promoter fragment into plasmid pGL3-basic vector (Promega, WI, USA). Plasmid pcDNA1-p65 and the Luciferase measurement were previously described [9].

Immunoprecipitation and immunoblotting. Cells were routinely analyzed 48 h post-transfection. Immunoprecipitation and immunoblotting were performed essentially as described [12].

Hydrodynamic transfection. Hydrodynamic transfections of plasmids were carried out as previously described [9].

Ethical permission. All research work involving animals was approved by the Local Committee for Animal Ethics in Stockholm, Sweden and performed in accordance.

Statistical analysis. Statistical significance was assessed by Mann–Whitney test and was considered significant at $P < 0.05$.

Results

Proteasome inhibitors suppress HIV-1 LTR-promoter

We have reported that proteasome inhibitors reduce the expression of nuclear NF-κB in B cells [9]. Because the HIV-1 promoter contains two NF-κB binding sites, we decided to investigate the effect of proteasome inhibitors on HIV-1 gene expression. We found that in HEK293T cells, HIV-1 LTR-promoter (pLTR-Luc) was induced by overexpressing p65 (Fig. 1A), and identical results were obtained in both B and T cell lines (Fig. S1).

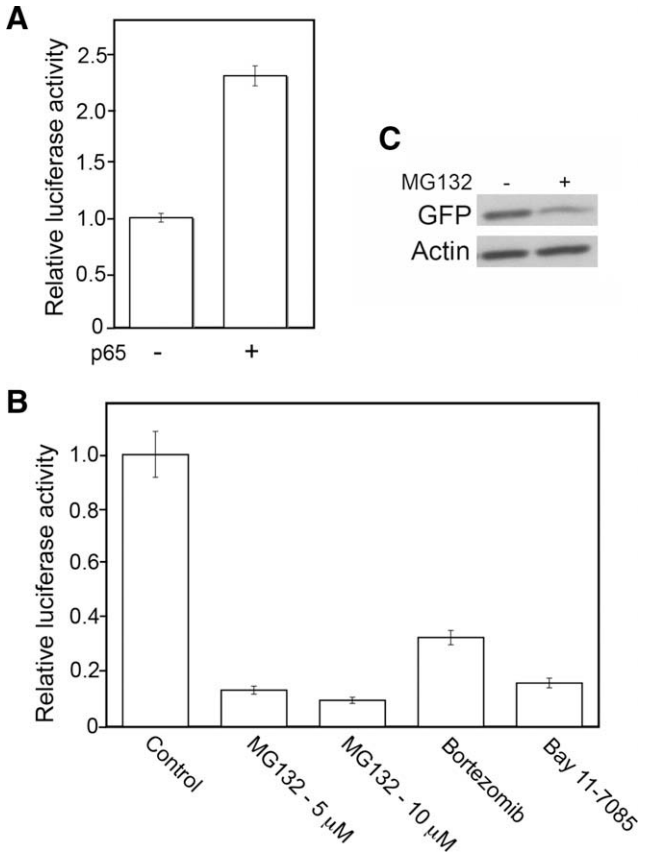

Fig. 1. Proteasome and NF-κB inhibitors suppress HIV-1 gene expression. (A) Plasmid pLTR-Luc was introduced into HEK293T cells together with p65. Forty-eight hours later, cell lysates were processed for luciferase activity measurement. (B) pLTR-Luc was transfected into Jurkat cells. Thirty-two hours post-transfection, cells were grown for 16 h in the presence of reagents (MG132 5 or 10 μM, Bortezomib 20 nM, Bay 11-7085 10 μM). (C) HEK293T were transfected with pLTR-GFP and 32 h later cells treated with MG132 (10 μM) for 16 h.

To further determine whether proteasome inhibitors are capable of blocking HIV-1 LTR-mediated gene expression, transiently transfected A20 cells expressing the reporter construct were incubated overnight with MG132 or Bortezomib. Both drugs led to downregulation of the HIV-1 LTR-promoter (Fig. S2A). Moreover, proteasome inhibitors effectively blocked LTR gene expression even after 100-fold induction by HIV-1 Tat (data not shown). Because T cells are one of the primary host cells for HIV-1 and their activation is critical to viral infection and replication [16], we replicated the same experiment in T cell line Jurkat. As seen in Fig. 1B, proteasome inhibitors also reduced transcription from the HIV-1 LTR-promoter in Jurkat cells, and the NF-κB specific inhibitor BAY 11-7085 has the same effects (Fig. 1B). Similar results were obtained in HEK293T cells (Fig. S2B). Next, we utilized a related reporter construct where the HIV-1 LTR-promoter drives expression of GFP. As expected, expression of GFP was reduced in the MG132-treated cells (Fig. 1C). Thus, proteasome and NF-κB inhibitors block gene expression mediated by the HIV-1 LTR-promoter.

In vivo transcriptional repression of the HIV-1 LTR-promoter by proteasome inhibitors

To address whether proteasome inhibitors can influence the HIV-1 LTR-promoter *in vivo*, we employed the hydrodynamic infusion method, which is an *in vivo* gene delivery technique that we previously used to functionally dissect promoter activities in mice

[9]. To this end, we introduced the pLTR-Luc into the liver of mice. Result show that the HIV-1 LTR-promoter was functional and highly active in hepatocytes. Next, we injected the animals with Bortezomib. As seen in Fig. 2, Bortezomib treatment led to reduction of reporter gene expression, which is consistent with the *in vitro* data. Thus, we conclude that proteasome inhibitors suppress the HIV-1 LTR-promoter both *in vitro* and *in vivo*.

Inhibition of Itk expression by proteasome inhibitors

Itk is important for T cell activation and development. Recent report shows that inhibition of Itk activity or its depletion in PBMC had a negative impact on several aspects of the HIV-1 life cycle, including infection/replication, gene expression, and virion assembly/release [11]. Previously, we have reported that proteasome inhibitors reduce Itk expression in Jurkat cells, [9]. Here, we asked whether proteasome inhibitors could inhibit Itk expression during activation of primary PBMCs. First, PBMC from healthy human donors were treated with MG132. As depicted in Fig. 3A, base line expression of Itk in resting PBMC was reduced by MG132 in a dose-dependent manner. Similar results were observed when PBMC were treated with Bortezomib (Fig. 3B). Second, we demonstrate that proteasome inhibitors blocked activation-dependent Itk induction in PBMC (Fig. 3C). However, when PHA- and IL-2 activated PBMC were treated with proteasome inhibitors, expression levels of Itk were only moderately affected (Fig. 3D). On the other hand, steady-state levels of NF-κB p65 fell sharply following treatment of PHA activated PBMC with these drugs (Fig. 3D). These results demonstrate that proteasome inhibitors effectively suppress induction of Itk expression upon PHA activation and downregulates steady-state levels of Itk in resting PBMC.

Proteasome inhibitors block HIV-1 replication

The above results show that proteasome inhibitors repress transcriptional activity of the HIV-1 LTR-promoter. To further assess whether these agents affect viral replication, PHA-activated PBMC from healthy individuals were infected with HIV-1_{BaL} in the presence of proteasome inhibitors or the anti-HIV drugs Indinavir (IND) and azidothymidine (AZT). The *in vitro* concentrations of drugs used here (IND, AZT, and Bortezomib) are comparable to plasma levels in patients receiving therapy [17].

To assess viral replication, the RT-activity in supernatant from infected cultures was quantified. As shown in Fig. 4A, there was a significant inhibition of viral replication by both Bortezomib and MG132 ($P < 0.001$). Moreover, both reagents inhibited viral replication in a concentration not markedly compromising cellular survival. Inhibition of viral replication by Bortezomib and MG132 was similar as IND and AZT. To gain further insight into potential synergistic effects, different concentrations of Bortezomib were combined with AZT or IND. We did not detect clear synergistic effects allowing reduction of compound concentrations although combined treatment with Bortezomib and AZT or IND led to effective inhibition of viral replication without severely compromising cell viability (Figs. S3A, B and S4). In contrast, an earlier report showed increased infection of a HIV-1 reporter virus pseudotyped with the vesicular stomatitis virus envelope after short-term (1 h) treatment with high-dose MG132 (50 μM) [18]. However,

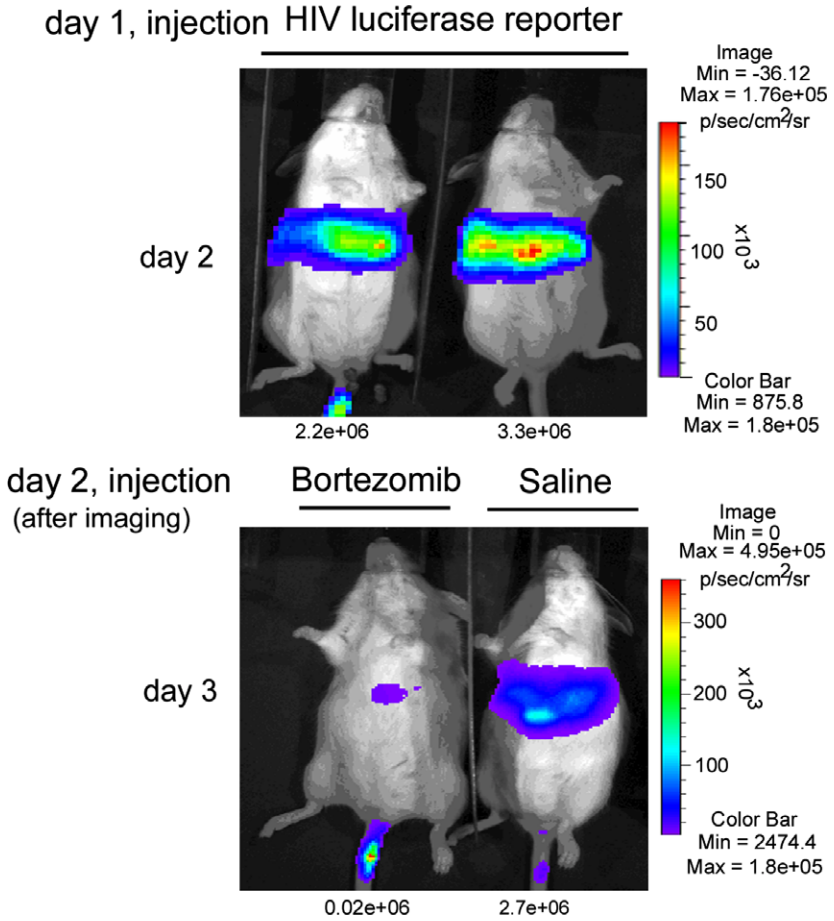

Fig. 2. Proteasome inhibitors block HIV-1 LTR-promoter *in vivo*. NMRI mice were injected with 10 μg pLTR-Luc using the hydrodynamic procedure. At day 2 after imaging, the mice were treated with Bortezomib (1 mg/kg) or saline. *In vivo* biophotonic imaging was performed using the IVIS imaging system.

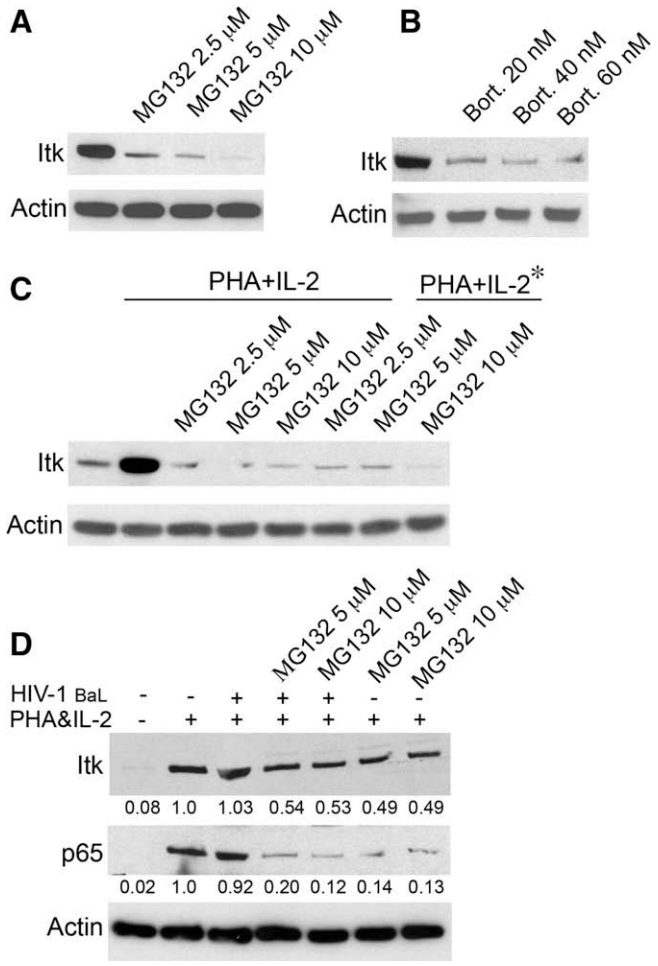

Fig. 3. Proteasome inhibitors suppress Itk expression in PBMC. PBMC were treated overnight with MG132 (A) or Bortezomib (B). (C) PBMC were left untreated or stimulated with PHA (2.5 μg/ml) and IL-2 (75 U/ml). Cells were then cultured overnight in the presence of MG132. (*Cells were treated with MG132 for 1 h prior to the addition of PHA and IL-2). (D) PBMC were stimulated with PHA and IL-2 for 3 days, and then infected with HIV-1_{BaL} in the presence of MG132 for 16 h. Relative amount of Itk and p65 levels is demonstrated at the bottom.

increased infectivity after MG132 treatment was variable depending upon tumor cell line analyzed [18]. We therefore investigated the effects of short-term treatment with proteasome inhibitors in primary PBMC. We did not detect any increased viral replication after short-term treatment with Bortezomib or MG132 (Figs. S5 and S6). Kinetic analyses showed that 96 h treatment with Bortezomib almost completely blocked RT-activity (Fig. S5). High-dose treatment with MG132 (10 μM) was cytotoxic to primary PBMC (Fig. S6B). However, we did not detect any increased viral replication in cultures treated for 1 or 5 h with MG132 (10 or 0.2 μM) (Fig. S6A). Altogether, these results clearly show that proteasome inhibitors can effectively suppress HIV-1 replication in PBMC.

We next assessed whether treatment with proteasome inhibitors prior to PBMC activation could block HIV-1 replication. We therefore set up three different treatment scenarios and measured viral infectivity by QPCR and RT-activity (Fig. 4B). In the first condition, Itk expression was induced by PHA prior to HIV-1 infection and treatment with ARVs, resulting in effective inhibition of viral replication by 2.5 nM Bortezomib (Fig. 4C and D, Treatment 1). In the second setting, a short pulse with Bortezomib was provided concomitantly with PHA activation in order to inhibit Itk expression and Bortezomib was washed away from the culture at the time of HIV-1 infection (Treatment 2). Depletion of Itk prior to HIV-1 infection led to reduced RT-activity using either 1.25 or 2.5 nM of Bortezomib (Fig. 4C, Treatment 2). However, a dose of 2.5 nM was required to reduce viral copy numbers, regardless of which treatment setting used (Fig. 4D). Moreover, continuous treatment with Bortezomib (2.5 nM) throughout the experiment resulted in effective inhibition of RT-activity and suppression of HIV-1 infection (Fig. 4C and D, Treatment 3).

Discussion

In this study we report that proteasome inhibitors, including the clinically approved drug Bortezomib, can block HIV-1 replication in PBMC. By inhibiting the NF-κB signaling pathway, these and similar drugs significantly reduced transcription from the HIV-1 LTR-promoter. Second, in addition to suppressing HIV-1 LTR-promoter activity, proteasome inhibitors also affected Itk expression which is critical for viral infection and replication. In a recent report, Readinger et al. showed that Itk is essential during several steps of the HIV-1 life cycle and its inhibition or depletion compromises viral replication [11]. Current ARVs are subject to the emergence of resistant viruses. Because inhibitors directed against cellular proteins required for HIV-1 replication may be less prone to this problem [19], the use of such inhibitors could offer an addition to current regimens [20]. Here we show that a clinically available drug could have multiple effects by targeting not only viral, but also key cellular antiviral targets.

NF-κB signaling is indispensable for T cell activation and HIV-1 preferentially replicates in proliferating $CD4^+$ T cells, partly by taking advantage of the activated host transcription machinery [21]. In this report we confirmed that the NF-κB subunit p65/RelA induces the HIV-1 LTR-promoter in different cell lines. Interestingly, this finding is consistent with recent data showing that NF-κB shuttling in T cells regulates HIV-1 LTR-dependent gene expression [13]. Moreover, p65/RelA, but not other subunits of the NF-κB family, has been shown to bind and trans-activate the HIV-1 LTR-promoter in $CD4^+$ T cells following ligation of the co-stimulatory receptor CD28 [22].

To fully establish productive infection, viruses have to avoid and/or tackle cellular barriers. The cellular cytidine deaminase APOBEC3G is an apolipoprotein B mRNA editing enzyme inhibiting HIV-1 replication in non-permissive cells. HIV-1, however, utilizes one of its accessory proteins – the viral infectivity factor (*vif*) – to neutralize the antiviral activity of APOBEC3G by promoting proteasome-dependent degradation [23,24]. Thus, treatment regimens employing drugs that inactivate proteasomes may restore physiological levels of these antiviral enzymes.

It was previously reported that inhibition of proteasome activity by high doses of MG132 or Lactacystin decreases budding, maturation and infectivity of HIV-1 by inhibiting processing of the Gag polyprotein [25]. Conversely, other reports showed that short-term treatment with proteasome inhibitors may enhance HIV-1 infection in certain cell lines [18,26]. We herein show that treatment with proteasome inhibitors effectively inhibit viral replication in PBMC. Pretreatment prior to PBMC activation and subsequent treatment during HIV-1 infection effectively abrogated HIV-1 replication as revealed by RT-activity and QPCR analyses (Fig. 4C and D). The doses required to obtain antiviral activity of PBMC *in vitro* correspond to concentrations measured in plasma in patients undergoing therapy against cancer.

Earlier, we showed that treatment with proteasome inhibitors, leads to reduction of the nuclear levels of NF-κB (p65), causing transcriptional repression of NF-κB regulated genes [9] in B cell lines. Herein we present evidence that proteasome inhibitors suppress HIV-1 transcription, following downregulation of NF-κB (p65) expression. This result is corroborated by the fact that the

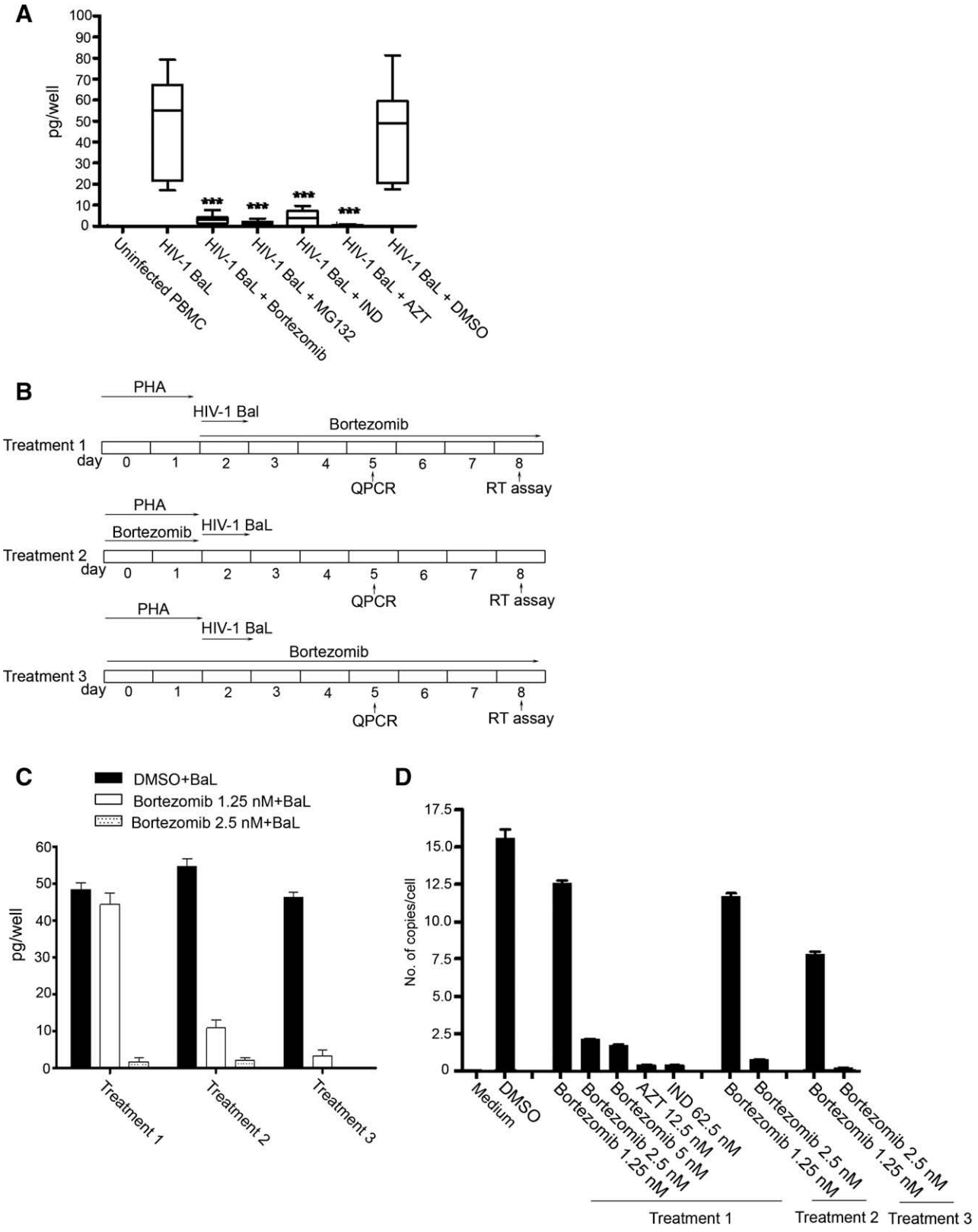

Fig. 4. Proteasome inhibitors block HIV-1 replication in human PBMC. (A) PBMC were stimulated with PHA and IL-2 for 2 days. Cells were either left uninfected or infected with HIV-1_{BaL} in the presence of drugs (Bortezomib 2.5 nM, MG132 200 nM, IND 1 μM, AZT 1 μM in triplicates. Box plots (range and median) represent level of active RT in supernatants at day 7 following infection. Significant difference between HIV-1_{BaL} exposed PBMC ($n = 12$) treated with or without drugs is indicated as *** ($P < 0.001$). (B) Schematic representation of three treatment strategies. PBMC from three healthy individuals were continuously treated with Bortezomib, AZT, IND after PHA activation (Treatment 1) or with Bortezomib during the PHA activation for 48 h (Treatment 2) or with Bortezomib during PHA activation and throughout the experiment (Treatment 3). (C) Graph represents level of active RT present in supernatants at day 7 following infection (as pg/well). (D) Graph represents number of HIV-1 DNA copies per cell at day 4 following infection quantified using QPCR.

specific NF-κB inhibitor BAY 11-7085 blocked HIV-1 gene expression. Furthermore, we demonstrate that proteasome inhibitors were able to reduce steady-state levels of Itk protein in primary PBMC and to block induction of Itk expression upon PHA activation of primary PBMCs. The ability of proteasome inhibitors to interfere with T cell activation processes and thereby also suppressing HIV-1 replication has the potential to become a new avenue to combat HIV-1. Natural SIV infection of African non-human primates is asymptomatic and one emerging key factor in protecting hosts from AIDS is lower levels of $CD4^+$ and $CD8^+$ T cell immune activation [27]. Although, it may be beneficial to limit $CD4^+$ T cell activation and thereby reduce viral replication, the potential risk of inhibiting $CD8^+$ cytotoxic T cells or other immunosuppressive effects by proteasome inhibitors should be taken into account.

Our results suggest that proteasome inhibitors are able to reduce HIV-1 replication by targeting multiple stages of the viral life cycle due to its effects on both the viral LTR-promoter and cellular targets with key functions during T cell activation. Given that proteasome inhibitors regulate large numbers of biological processes, it is likely that their effect on HIV-1 involves multiple pathways. A recent study using RNAi-based screens [20], identified more than 200 mRNAs, whose downregulation affected susceptibility to HIV-1. Using bioinformatics tools we found that a large number of corresponding proteins have been implicated as proteasome- and/or NF-kB regulated (unpublished data). It is therefore likely that in addition to the LTR-promoter and Itk, several other targets influencing HIV-infectivity are also affected [24,28]. However, irrespective of the precise contribution by each of these components, we have demonstrated that the FDA-approved drug Bortezomib, and other proteasome inhibitors, severely compromise HIV-1 replication in primary human cells. Importantly, these drugs were also successfully combined with other anti-HIV drugs. These features could make this novel class of compounds interesting for inhibition of HIV-1 replication.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.04.156.

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