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Dipyridamole as a new drug to prevent Epstein-Barr virus reactivation

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Abstract

Epstein-Barr virus (EBV) is a widely distributed gamma-herpesvirus that has been associated with various cancers mainly from lymphocytic and epithelial origin. Although EBV-mediated oncogenesis has been associated with viral oncogenes expressed during latency, a growing set of evidence suggested that antiviral treatments directed against EBV lytic phase may contribute to prevent some forms of cancers, including EBV-positive Post-Transplant Lymphoproliferative Diseases. It is shown here that dipyridamole (DIP), a safe drug with favorable and broad pharmacological properties, inhibits EBV reactivation from B-cell lines. DIP repressed immediate early and early genes expression mostly through its ability to inhibit nucleoside uptake. Considering its wide clinical use, DIP repurposing could shortly be evaluated, alone or in combination with other antivirals, to treat EBV-related diseases where lytic replication plays a deleterious role.

Keywords

Epstein-Barr virus; dipyridamole; drug repurposing; EBV reactivation

1. Introduction

Epstein-Barr virus (EBV) is a human gamma-herpesvirus that persistently infects more than 90% of the adult population worldwide. Primary infection occurs mostly during childhood without apparent symptoms but it can cause infectious mononucleosis in young adults. Following primary infection, EBV establishes a life-long persistent infection that combines lytic replication and latent phases, supported by its dual tropism for B lymphocytes and epithelial cells (Chiu and Sugden, 2016; Hutt-Fletcher, 2014). Whereas EBV infection is usually tightly controlled by the immune response, it has been associated with various malignancies including Burkitt's lymphoma, Hodgkin's disease, various B-cell lymphomas linked to immunosuppression, including Post-Transplant Lymphoproliferative Diseases (PTLD), some forms of T-cell lymphomas, undifferentiated nasopharyngeal carcinoma and gastric cancers (Jha et al., 2016).

Sporadic reactivations leading to the production of viral progeny may occur from latently infected B-lymphocytes in response to physiological factors that are still largely unknown *in vivo*. Reactivation can be induced *ex vivo* by phorbol esters (zur Hausen et al., 1978), calcium ionophores (Faggioni et al., 1986), transforming growth factor beta (TGF-β) (Renzo et al., 1994), sodium butyrate (Luka et al., 1979), hypoxia (Jiang et al., 2006) or following activation of B-cell receptor with antibodies directed against surface immunoglobulins (anti-sIg) (Takada, 1984).

Viral reactivation is a highly coordinated process that is initiated by the expression of two immediate early viral genes, namely BZLF1 (encoding for Zta, Z, Zebra, EB1) and BRLF1 (encoding for Rta, R, EB2) that encode transactivators required for early gene expression. Zta also activates the lytic origin of replication OriLyt. Lytic replication of the viral genomes precedes the expression of late viral proteins that eventually leads to virion assembly and release (Feederle et al., 2000; Murata, 2014). Although B-cell transformation by EBV is usually associated with latently expressed viral oncogenes, there is a need for non-toxic antiviral drugs that can inhibit EBV lytic replication, notably in immunosuppressed patients. Indeed, immunosuppression favors EBV reactivation, thereby increasing viral load and promoting B-cell reinfection and lymphomagenesis. In addition, it has been demonstrated that viral reactivation directly and indirectly contributes to

lymphomagenesis both in mouse models and in humans (Cohen, 2000; Hong et al., 2005; Ma et al., 2011; Manners et al., 2018). EBV lytic replication may also contribute to carcinogenesis in epithelial tumors (Wu et al., 2018). Whereas antiviral agents have a limited impact for the treatment of active infections such as infectious mononucleosis, their prophylactic administration resulted in a reduced incidence of PTLD (Hierro et al., 2008; Höcker et al., 2012; Malouf et al., 2002).

So far, antiviral molecules against EBV mainly target viral DNA replication. These include nucleoside analogues (acyclovir, ganciclovir) and their prodrugs (valaciclovir, valganciclovir, valomaciclovir), nucleotides (cidofovir) and pyrophosphate (foscarnet) analogs that are active against alpha- and/or beta-herpesviruses. These drugs have been assayed *in vitro* or used experimentally against EBV, but none received approval by the Food and Drug Administration nor the European Medicines Agency for the treatment of EBV related diseases (Andrei et al., 2019; Pagano et al., 2018). Therefore, identifying new and non-toxic antiviral drugs that inhibit EBV reactivation remains an important challenge.

Recent advances in drug screening technologies have helped identify new molecules. However, drug development is an uncertain process due to high attrition rates combined with the time and the cost necessary to bring new compounds to market. This makes new drug development a less desirable choice for pharmaceutical industries and investors. In that context, drug repurposing strategies propose new medical indications for either investigational or approved drugs that are outside the scope of the original targets (Pushpakom et al., 2018). This approach reduces the risk of failure because of safety considerations and the timeframe for drug development, thus requiring less investment. To be efficient, however, a drug repurposing strategy first requires identifying a candidate molecule for the indication of interest. This systematic approach mostly relies on previously published experimental data describing both pharmacological features and mechanism of action.

Dipyridamole (DIP) (2,6-bis [diethanolamino]-4,8-dipiperidino- pyrimido 5,4-d pyrimidine) is a safe drug with favorable and broad pharmacological properties. Initially, DIP was used as a coronary vasodilator and soon after it was recognized as

an antithrombotic agent. This allowed its long use as a prophylactic compound to prevent strokes and other vascular diseases (Chakrabarti and Freedman, 2008). DIP inhibits both vasodilatation and platelet aggregation through two complementary mechanisms: (1) the inhibition of phosphodiesterase (PDE), in particular PDE3 and PDE5 (Gresele et al., 2011), and (2) the blockade of nucleoside transport from the extracellular compartment to the cytoplasm of the cells (Harker and Kadatz, 1983; Knabb et al., 1984). The inhibition of nucleoside uptake into platelets, endothelial and red blood cells increases plasmatic adenosine levels, which in turn activates adenosine receptors, including the A2-receptor, thus stimulating adenylate cyclase and the production of cyclic-3′,5′-adenosine monophosphate (cAMP). This effect, concomitant with PDEs inhibition, promotes the elevation of cAMP and cGMP levels in cells, impairing platelet aggregation and promoting arteriolar smooth muscle relaxation (Chakrabarti and Freedman, 2008).

DIP antiviral activity was previously demonstrated against Herpes simplex virus *in vitro* (Tenser et al., 2001) and in a mouse model (Hay et al., 1996) and it was shown to inhibit Mengovirus RNA replication (Fata-Hartley and Palmenberg, 2005). Additionally, in a combined treatment, DIP potentiated the antiviral activity of nucleoside analogs against human immunodeficiency virus (HIV) (Patel et al., 1991).

Cellular nucleotides are essential components required for rapid viral genome replication (Sanchez and Lagunoff, 2015). *In vivo*, nucleotides can be synthesized *de novo* through enzymatic reactions or recycled through the salvage pathway of extracellular nucleosides, refueling the intracellular nucleotide pools (Nyhan, 2014). Since viral transcription and replication rely on the sources of nucleotides from the host cells, the present work aimed at targeting nucleoside uptake with DIP and to characterize its ability to inhibit EBV reactivation.

2. Material and methods

2.1. Antibodies and reagents

Anti-EBV ZEBRA (Zta) (sc-53904), anti-EBV EA-D (sc-58121), anti-β-actin (sc-47778) were purchased from Santa Cruz and horseradish peroxidase (HRP) linked-secondary antibody (#7076) was from Cell Signaling Technology. Dipyridamole (D9766), adenosine (A9251), thymidine (T9250), S-(4-Nitrobenzyl)-6-thioinosine (NBMPR) (N2255) and dimethyl sulfoxide (DMSO) (D2650) were purchased from Sigma-Aldrich and H89 was purchased from Biomol GmbH. DIP, NBMPR and H89 stock solutions were dissolved in DMSO; adenosine and thymidine were dissolved in water.

2.2. Cell lines and treatments

Akata, Mutu-I and B95-8 cells were cultured in RPMI 1640 medium (Gibco BRL) supplemented with 10 % heat-inactivated fetal bovine serum and 2 mM L-glutamine (Life Technologies) at 37°C in a humidified 5% CO_2 incubator. All cell culture media and supplements were obtained from Gibco (Thermo Fisher Scientific). EBV reactivation in Mutu-I cells was induced by treating cells for eight hours with 2 ng/ml TGF-β (Eurobio). EBV reactivation in Akata cells was triggered by the crosslinking of the surface immunoglobulins with 7.5 μg/mL polyclonal rabbit anti-human IgG (α-sIg) (A0423, Dako) for five, eight or twenty-four hours for viral RNA, proteins or genomic viral DNA (gDNA)/virion production analysis, respectively. The cells were treated with DIP and/or adenosine or thymidine for the indicated times and concentrations. The cells were treated with NBMPR at the indicated drug concentrations for four hours prior addition of α-sIg. H89 was used at 5 μM and added one hour prior the addition of DIP. The absence of DIP toxicity was assessed by Trypan blue exclusion counting in light microscopy and propidium iodide (Molecular Probes, Life Technology, Cergy Pontoise, France) staining followed by flow cytometry detection. Treatment with 0.01% Triton-X100 was used as a positive control to assess membrane permeabilization to propidium iodide.

2.3. RNA extraction and RT-qPCR analysis

Total RNA was extracted and purified from cell cultures using TRIzol reagent (Invitrogen; Thermo Fisher Scientific) following the manufacturer's instructions. Seven micrograms of purified RNA were submitted to DNAse treatment using TURBO DNA-free kit (Life technologies). Then, 1.5 micrograms of DNAse-treated RNA were reverse-transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Invitrogen; Thermo Fisher Scientific). Real-time PCR was performed using SensiFAST SYBR No-Rox Kit (Bioline). Primers are listed in Supplementary table 1. Cellular β-globin RNA levels were used as an internal control to normalize viral mRNA and fold-changes were calculated by using the ΔΔCT method.

2.4.Analysis of virion production and intracellular viral DNA

Total intracellular DNA was isolated from cell cultures using DNeasy Blood and Tissue Kit (Qiagen) as described previously (Masud et al., 2017). Intracellular viral DNA was quantified by real-time PCR using primers designed for detecting BHRF1. β-globin coding gene amplification was used as an internal control. Primers are listed in Supplementary table 1. Fold-changes were calculated by using the ΔΔCT method. For virion production analysis, cell cultures supernatants were collected and treated with DNase I (New England BioLabs) to eliminate naked free DNA. To estimate EBV virion production, real-time PCR was performed as described for intracellular DNA and the absence of amplification for β-globin was used as a control of external DNA elimination.

2.5.Western blot analysis

Cells were washed twice in PBS and lysed for 30 minutes at 4°C in denaturing lysis buffer [50 mM Tris HCl (pH 6.8), 2 % SDS, 2 % 2-mercaptoethanol]. Proteins were resolved on 12 % SDS-PAGE and blotted on to PVDF membranes (Amersham; GE Healthcare Life Sciences). Membranes were saturated by non-fat dried milk and antigens revealed by immunoblotting overnight at 4°C using the following primary antibodies: mouse monoclonal IgG anti-Zta (1:1000), mouse monoclonal IgG anti-EA-D (1:1000) and mouse monoclonal IgG anti-β-actin (1:5000). Primary antibodies were detected by an anti-mouse IgG, HRP-linked antibody

(1:10000) and antigens revealed using an enhanced chemiluminescence kit (Amersham; GE Healthcare Life Sciences) and X-ray film exposure (Carestream Kodak BioMax light film). Digital images were collected, and band intensities measured using ImageJ software and normalized to β-actin signal. Western blots are representative of 3 independent experiments.

2.6.Statistical analysis

Statistical analysis was performed using Prism 6 (GraphPad Software, La Jolla). The level of significance was determined with a one-way analysis of variance test (ANOVA) followed by Bonferroni's multiple comparisons test. P-value < 0.05 was considered as statistically significant.

3. Results

3.1. DIP inhibits spontaneous and induced EBV reactivation

Akata is a well-characterized EBV-positive Burkitt's lymphoma-derived cell line in which EBV reactivation can be induced with antibodies directed against surface immunoglobulins (anti-sIg/α-sIg), a process that mimics physiological reactivation following B cell receptor-mediated stimulation (Takada, 1984). Following this treatment, around 15% of the cells enter reactivation, as observed by flow cytometry immunodetection of the proteins Zta and EA-D (data not shown).

To investigate DIP antiviral activity, Akata cells were incubated with increasing concentration of DIP prior to reactivation. Preliminary experiments indicated that four hours pre-treatment was optimal (Supp. Fig. 1A and B). The production of virus particles was monitored by quantifying virion DNA in the cell culture supernatant after 24 hours reactivation. As illustrated in figure 1A, virion production was inhibited in a dose-dependent manner. Importantly, cell viability was not affected when Akata cells were grown in the presence of up to 20 µM DIP for 48 hours (Supp. Fig. 1C and 1D). Moreover, DIP concentrations used herein are in the range attained after oral administration in humans (Grem and Fischer, 1989).

B95-8 is an EBV positive marmoset B-cell line in which a minority of cells spontaneously enters into reactivation. Treatment of B95-8 cells with DIP also resulted in a significant decrease in virus production albeit to a lesser extent (Fig. 1B). This lower antiviral activity was expected since only 3 to 5% of cells expressed lytic antigens before treatment (data not shown).

3.2. DIP inhibits viral lytic replication as well as early and immediate early gene expression

To further characterize DIP antiviral activity, we evaluated its effect on viral DNA replication. For this purpose, intracellular viral DNA was extracted from Akata cells and quantified following reactivation in the presence or absence of DIP. Whereas DIP had no significant effect on latent genomes (i.e. non-reactivated cells), the treatment significantly repressed viral replication following reactivation (Fig. 1C). Accordingly, DIP also repressed viral replication in B95-8 cells (Fig. 1D).

During the lytic phase of the cycle, the replication of EBV genomes is placed under the control of immediate early and early viral proteins, including viral DNA polymerase, auxiliary factors and the viral transacting factor Zta. The expression of early (EA-D) and immediate early (Zta) proteins was analyzed by immunoblotting following 8 hours reactivation in the presence of increasing concentrations of DIP. As shown in figure 2A, a significant dose-dependent inhibition of both early and immediate early proteins was observed. Inhibition reached 80 and 90%, respectively, for the highest dose of DIP in Akata cells whereas a 50 and 40% decrease in EA-D and Zta protein expression, respectively, was observed in B95-8 cells at 48 hours for the highest concentration of DIP (Fig. 2B). Importantly, this effect of DIP was not limited to B cells in which EBV reactivation is induced by antibodies directed against surface immunoglobulins or observed spontaneously. DIP indeed showed a similarly strong inhibitory effect on the Zta protein expression levels that is induced by TGF-β in Mutu-I cells, an EBV-positive Burkitt's lymphoma-derived cell line expressing both TGF-β receptors I and II (Supp. Fig. 1E). These results suggest that DIP acts on EBV reactivation independently of the activation pathway.

We next wondered whether early and immediate early protein accumulation was inhibited at the transcriptional or translational level. For this purpose, a quantitative reverse transcription PCR-based assay was designed to measure the expression of viral (immediate early BZLF1, which encodes for Zta transactivator; early genes BMRF1 and BHRF1, which encode for EA-D and EA-R, respectively) and cellular genes (β-globin, β-actin and cyclophilin). As shown in figure 2C, DIP significantly repressed viral gene transcription following reactivation. This effect was specific since expression of control cellular genes was unaffected in the same conditions. It is notable that repression of early transcription was expected since early gene expression is under the control of immediate early proteins Zta and Rta, whose expression was strongly inhibited by DIP.

3.3. DIP antiviral activity is associated with its ability to inhibit nucleoside uptake

Our data suggested that DIP could act upstream of viral gene transcription. DIP is indeed a known inhibitor of phosphodiesterases (PDE) (Gresele et al., 2011), leading to an increase of intracellular concentration of cAMP with possible effect on the cAMP/PKA signaling pathway. However, an activation of the cAMP/PKA-dependent pathway by DIP was unlikely to explain DIP antiviral activity since, in our model, its effect on EBV reactivation was not affected by the PKA inhibitor H89 which acts downstream of DIP in this specific pathway (Supp. Fig. 1F).

Since DIP was reported to interfere with nucleotide metabolism by potently and directly inhibiting equilibrative nucleoside transporters (especially ENT1 and ENT2), we next wondered whether DIP inhibition of nucleoside uptake may explain its antiviral activity. To test this hypothesis, we first evaluated the impact of S-(4-Nitrobenzyl)-6-thioinosine (NBMPR), an inhibitor of ENT1 (Griffiths et al., 1997), on immediate early and early protein expression. As shown on figure 3A, NBMPR significantly repressed Zta and EA-D accumulation. The effect may be lower than DIP-mediated inhibition because NBMPR promotes the inhibition of ENT1 but not ENT2, which are both sensitive to DIP (Boswell-Casteel and Hays, 2017; Wang et al., 2013). If DIP antiviral activity was indeed associated with its ability to inhibit nucleoside transport, we assumed that this process could be reversed if the cells were reactivated in the presence of a large excess of either adenosine or thymidine, therefore promoting a competition for the binding to the transporters. To test this assumption, Akata cells were treated with DIP in the presence of 100 μM adenosine or thymidine for 4 hours prior to treatment with anti-sIg. Then, the expression of viral proteins was analyzed by immunoblotting following 8 hours reactivation. As shown on figure 3B, both adenosine and thymidine reversed DIP inhibition of Zta and EA-D expression, although this effect seemed to be less effective on Zta than on EA-D. Importantly, when given alone, neither adenosine nor thymidine induced EBV reactivation or interfered with it (Fig. 3C). Altogether, these data confirmed that DIP antiviral activity on EBV reactivation was related to its ability to prevent nucleoside import.

4. Discussion and conclusions

Although several drugs have been successfully evaluated for their ability to inhibit EBV replication *ex vivo*, their clinical use has been limited so far. It was initially suggested to use antivirals against EBV in clinical situations where active EBV replication was supposedly associated with the clinical symptoms, such as infectious mononucleosis (IM). However, antiviral therapy proved to have only a limited interest in this setting. Indeed, although acyclovir, a nucleoside analog, efficiently reduced EBV shedding in the saliva during IM, it was not associated with a clear clinical benefit (Pagano et al., 2018).

Although EBV-associated tumors are mainly linked to latently expressed oncogenes, a growing set of basic and clinical evidence suggested that EBV lytic replication might also directly and indirectly contribute to EBV-associated lymphomagenesis. This is notably supported by experimental data demonstrating that EBV mutants unable to undergo lytic viral replication were defective in promoting EBV-mediated lymphoproliferative disease in severe combined immunodeficient mice (Hong et al., 2005). This observation was, at least in part, explained by the increased production of B-cell growth factors, such as cellular IL-10, viral IL-10 and cellular IL-6 in response to EBV infection by wild-type viruses compared to Zta-knock out mutants. The contribution of lytic expression to the development of lymphomas was further confirmed in a humanized mouse model, in which both human fetal CD34 hematopoietic stem cells and thymus/liver tissue were transplanted (Ma et al., 2011).

The benefit of antivirals to prevent primary EBV infection in high-risk patients and/or PTLD patients is supported by several clinical investigations. Antiviral prophylaxis with ganciclovir or valganciclovir resulted in a significant decrease in EBV primary infections in a group of EBV-negative pediatric renal transplant patients receiving a graft from EBV-positive donors (Höcker et al., 2012). Malouf and colleagues reported a reduction in the incidence of PTLD in high-risk EBV-seronegative lung transplant recipients who received a continuous, specific anti-viral prophylaxis with acyclovir, valaciclovir or ganciclovir (Malouf et al., 2002). Another study reported that antiviral prophylaxis could prevent late onset PTLD (Ville et al., 2018). Antiviral therapy inhibiting lytic EBV replication may also

be advantageous for treating epithelial tumors, such as EBV-positive nasopharyngeal carcinoma, where lytic proteins have been proved to directly contribute to carcinogenesis (Wu et al., 2018; Yoshizaki et al., 2008). Importantly, antiviral therapies might be more effective if they target the expression of immediate early and early proteins. Indeed, Zta and Rta have been shown to promote IL-6 secretion, a cytokine that has been involved in a variety of hematological and epithelial cancers (Jones et al., 2007). Moreover, lytically infected B-cells secrete cytokines, including cellular IL-10, viral IL-10 and VEGF, that promote B-cell proliferation and angiogenesis (Beatty et al., 1997; Hong et al., 2005).

Most antiviral therapies target the virus directly, for example impairing the activity of virus-encoded enzymes and decreasing virus production. However, emerging adaptive mutations of the viral genome lead to drug resistance, remaining one of the major causes of reduced effectiveness (De Clercq and Li, 2016). Considering that viruses interplay with cellular signaling pathways (Altmann and Hammerschmidt, 2005; Boulant et al., 2015) and rely on the cellular supply of metabolites to ensure productive infection (Sanchez and Lagunoff, 2015), cellular mechanisms have gained increasing interest as targets for novel antiviral therapies (Planz, 2013; Scheuch et al., 2018). Hereof, EBV reactivation was shown to be repressed by the inhibition of Cyclin-Dependent Kinase in B cells (Kudoh et al., 2004) and by targeting p38 Mitogen-Activated Protein Kinase pathway (Gao et al., 2004; Matusali et al., 2009). Thereby, antiviral compounds targeting cellular pathways present the potential of broad-spectrum activity and are still welcome.

In that sense, we here report that DIP, a safe and widely used drug, could prevent EBV reactivation from B-cell lines and inhibit immediate early protein Zta expression. Based on the central role played by this protein in EBV reactivation, its transcriptional (and therefore translational) inhibition is indeed expected to have a major impact on the lytic cycle. However, the regulation of BZLF1 transcription is still puzzling, and several hypotheses can be proposed. First, DIP might act on the signaling pathways that activate BZLF1. Nonetheless, this hypothesis is unlikely since DIP antiviral activity was observed in three distinct situations: (1) spontaneous reactivation in B95-8 cells, (2) activation of B-cell receptor in Akata cells and (3) induction by TGF-β dependent pathway in Mutu-I cells (Kenney and

Mertz, 2014). Second, DIP may inhibit the regulation of BZLF1 promoter. It is indeed regulated through a complex interplay between cellular trans-activating factors (TF) and CpG methylation occurring on specific binding sites within the BZLF1 promoter (Li et al., 2016). In this model, DIP would inhibit BZLF1 promoter activation by modulating one or several TF binding, although there is no experimental evidence so far for such hypothesis. Third, DIP antiviral activity would be mostly due to its ability to block nucleoside import. This assumption is strongly supported by our data demonstrating that (1) DIP-mediated EBV inhibition could be reversed by an excess of extracellular adenosine or thymidine and (2) EBV reactivation could be reduced by NBMPR, an independent class of equilibrative nucleoside transporter inhibitor. DIP would so interfere with the high nucleotide demand that is required for viral transcription and replication as it was previously described for Herpes simplex virus (Sanchez and Lagunoff, 2015). Interestingly, DIP may directly act on viral RNA synthesis. Indeed, a previous report showed that DIP can reversibly inhibit RNA synthesis in a viral *in vitro* transcription system (Fata-Hartley and Palmenberg, 2005). Taken together, these reports and our data suggest that DIP antiviral activity is likely a multifactorial process, with nucleoside uptake a key determinant of its anti-EBV activity. The relative importance of salvage versus *de novo* nucleotide synthesis for viral and host nucleic acids metabolism is still unclear, and the precise characterization of the molecular mechanisms underlying DIP antiviral activity deserves a more detailed examination.

Compared to the several studies focused on the antioxidant (Ciacciarelli et al., 2015) and anticancer (Liu et al., 2014; Spano et al., 2013; Thomé et al., 2019) properties of DIP, its antiviral activity has been poorly explored. Of pharmacological importance is the fact that antiviral nucleoside analogs use different transporters to enter cells than those targeted by DIP that block nucleoside uptake (Pastor-Anglada et al., 2005). Therefore, DIP is unlikely to interfere with the import of this important class of antiviral compounds. On the contrary, combining nucleoside analogs with DIP might potentiate their antiviral activity by increasing the incorporation of their phosphorylated metabolites into the viral DNA. In that sense, it was described that DIP potentiates the antiviral activity of dideoxynucleoside drugs against HIV, probably by inhibiting the salvage of competing physiological nucleosides (Patel et al., 1991). Importantly, DIP

concentrations used in the present study were close to the range of the plasma concentration attained after oral administration in humans (Grem and Fischer, 1989). Remarkably, overdose case reports showed that higher DIP doses might be tolerated in humans, suggesting that even higher dosing could be used in new clinical settings (Lagas et al., 2011). As a drug repurposing strategy, another benefit of DIP is that it could be readily evaluated in clinical trials, alone or in combination with other classes of antiviral drugs. Finally, it is worthwhile to proceed with additional studies to determine whether DIP has a broad inhibitory effect on other virus families.

Conflicts of interest

The authors declare that they have no conflict of interest.

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References

Altmann, M., Hammerschmidt, W., 2005. Epstein-Barr Virus Provides a New Paradigm: A Requirement for the Immediate Inhibition of Apoptosis. PLoS Biol. 3. https://doi.org/10.1371/journal.pbio.0030404

Andrei, G., Trompet, E., Snoeck, R., 2019. Novel Therapeutics for Epstein–Barr Virus. Molecules 24, 997. https://doi.org/10.3390/molecules24050997

Beatty, P.R., Krams, S.M., Martinez, O.M., 1997. Involvement of IL-10 in the autonomous growth of EBV-transformed B cell lines. J. Immunol. 158, 4045–4051.

Boswell-Casteel, R.C., Hays, F.A., 2017. Equilibrative nucleoside transporters - A review. Nucleos Nucleot Nucl. 36, 7–30. https://doi.org/10.1080/15257770.2016.1210805

Boulant, S., Stanifer, M., Lozach, P.-Y., 2015. Dynamics of Virus-Receptor Interactions in Virus Binding, Signaling, and Endocytosis. Viruses 7, 2794–2815. https://doi.org/10.3390/v7062747

Chakrabarti, S., Freedman, J.E., 2008. Dipyridamole, cerebrovascular disease, and the vasculature. Vascul. Pharmacol. 48, 143–149. https://doi.org/10.1016/j.vph.2007.12.004

Chiu, Y.-F., Sugden, B., 2016. Epstein-Barr Virus: The Path from Latent to Productive Infection. Annu. Rev. Virol. 3, 359–372. https://doi.org/10.1146/annurev-virology-110615-042358

Ciacciarelli, M., Zerbinati, C., Violi, F., Iuliano, L., 2015. Dipyridamole: a drug with unrecognized antioxidant activity. Curr. Top. Med. Chem. 15, 822–829. https://doi.org/10.2174/1568026615666150220111942

Cohen, J.I., 2000. Epstein-Barr virus infection. N. Engl. J. Med. 343, 481–492. https://doi.org/10.1056/NEJM200008173430707

De Clercq, E., Li, G., 2016. Approved Antiviral Drugs over the Past 50 Years. Clin. Microbiol. Rev. 29, 695–747. https://doi.org/10.1128/CMR.00102-15

Faggioni, A., Zompetta, C., Grimaldi, S., Barile, G., Frati, L., Lazdins, J., 1986. Calcium modulation activates Epstein-Barr virus genome in latently infected cells. Science 232, 1554–1556. https://doi.org/10.1126/science.3012779

Fata-Hartley, C.L., Palmenberg, A.C., 2005. Dipyridamole Reversibly Inhibits Mengovirus RNA Replication. J. Virol. 79, 11062–11070. https://doi.org/10.1128/JVI.79.17.11062-11070.2005

Feederle, R., Kost, M., Baumann, M., Janz, A., Drouet, E., Hammerschmidt, W., Delecluse, H.J., 2000. The Epstein-Barr virus lytic program is controlled by the co-operative functions of two transactivators. EMBO J. 19, 3080–3089. https://doi.org/10.1093/emboj/19.12.3080

Gao, X., Wang, H., Sairenji, T., 2004. Inhibition of Epstein-Barr Virus (EBV) Reactivation by Short Interfering RNAs Targeting p38 Mitogen-Activated Protein Kinase or c-myc in EBV-Positive Epithelial Cells. J. Virol. 78, 11798–11806. https://doi.org/10.1128/JVI.78.21.11798-11806.2004

Grem, J.L., Fischer, P.H., 1989. Enhancement of 5-fluorouracil's anticancer activity by dipyridamole. Pharmacol. Ther. 40, 349–371. https://doi.org/10.1016/0163-7258(89)90084-3

Gresele, P., Momi, S., Falcinelli, E., 2011. Anti-platelet therapy: phosphodiesterase inhibitors. Br. J. Clin. Pharmacol. 72, 634–646. https://doi.org/10.1111/j.1365-2125.2011.04034.x

Griffiths, M., Beaumont, N., Yao, S.Y.M., Sundaram, M., Boumah, C.E., Davies, A.,

Kwong, F.Y.P., Coe, I., Cass, C.E., Young, J.D., Baldwin, S.A., 1997. Cloning of a human nucleoside transporter implicated in the Cellular uptake of adenosine and chemotherapeutic drugs. Nat. Med. 3, 89. https://doi.org/10.1038/nm0197-89

Harker, L.A., Kadatz, R.A., 1983. Mechanism of action of dipyridamole. Thromb. Res., Pharmacokinetic and Pharmacodynamic Approach to Antithrombotic Therapy 29, 39–46. https://doi.org/10.1016/0049-3848(83)90356-0

Hay, K.A., Gaydos, A., Tenser, R.B., 1996. Inhibition of herpes simplex virus reactivation by dipyridamole in a mouse model. J. Med. Virol. 50, 198–203. https://doi.org/10.1002/(SICI)1096-9071(199610)50:2<198::AID-JMV15>3.0.CO;2-I

Hierro, L., Díez-Dorado, R., Díaz, C., Vega, A.D. la, Frauca, E., Camarena, C., Muñoz-Bartolo, G., Zárate, A.G. de, Santamaría, M.L., Jara, P., 2008. Efficacy and safety of valganciclovir in liver-transplanted children infected with Epstein-Barr virus. Liver Transpl. 14, 1185–1193. https://doi.org/10.1002/lt.21498

Höcker, B., Böhm, S., Fickenscher, H., Küsters, U., Schnitzler, P., Pohl, M., John, U., Kemper, M.J., Fehrenbach, H., Wigger, M., Holder, M., Schröder, M., Feneberg, R., Köpf-Shakib, S., Tönshoff, B., 2012. (Val-)Ganciclovir prophylaxis reduces Epstein-Barr virus primary infection in pediatric renal transplantation. Transpl. Int. 25, 723–731. https://doi.org/10.1111/j.1432-2277.2012.01485.x

Hong, G.K., Gulley, M.L., Feng, W.-H., Delecluse, H.-J., Holley-Guthrie, E., Kenney, S.C., 2005. Epstein-Barr virus lytic infection contributes to lymphoproliferative disease in a SCID mouse model. J. Virol. 79, 13993–14003. https://doi.org/10.1128/JVI.79.22.13993-14003.2005

Hutt-Fletcher, L.M., 2014. Epstein–Barr virus replicating in epithelial cells. Proc. Natl. Acad. Sci. U. S. A. 111, 16242–16243. https://doi.org/10.1073/pnas.1418974111

Jha, H.C., Pei, Y., Robertson, E.S., 2016. Epstein–Barr Virus: Diseases Linked to Infection and Transformation. Front. Microbiol. 7. https://doi.org/10.3389/fmicb.2016.01602

Jiang, J.-H., Wang, N., Li, A., Liao, W.-T., Pan, Z.-G., Mai, S.-J., Li, D.-J., Zeng, M.-S., Wen, J.-M., Zeng, Y.-X., 2006. Hypoxia can contribute to the induction of the Epstein-Barr virus (EBV) lytic cycle. J. Clin. Virol. Off. Publ. Pan Am. Soc. Clin. Virol. 37, 98–103. https://doi.org/10.1016/j.jcv.2006.06.013

Jones, R.J., Seaman, W.T., Feng, W.-H., Barlow, E., Dickerson, S., Delecluse, H.-J., Kenney, S.C., 2007. Roles of lytic viral infection and IL-6 in early versus late passage lymphoblastoid cell lines and EBV-associated lymphoproliferative disease. Int. J. Cancer 121, 1274–1281. https://doi.org/10.1002/ijc.22839

Kenney, S.C., Mertz, J.E., 2014. Regulation of the latent-lytic switch in Epstein-Barr virus. Semin. Cancer Biol. 26, 60–68. https://doi.org/10.1016/j.semcancer.2014.01.002

Knabb, R.M., Gidday, J.M., Ely, S.W., Rubio, R., Berne, R.M., 1984. Effects of dipyridamole on myocardial adenosine and active hyperemia. Am. J. Physiol. 247, H804-810. https://doi.org/10.1152/ajpheart.1984.247.5.H804

Kudoh, A., Daikoku, T., Sugaya, Y., Isomura, H., Fujita, M., Kiyono, T., Nishiyama, Y., Tsurumi, T., 2004. Inhibition of S-Phase Cyclin-Dependent Kinase Activity Blocks Expression of Epstein-Barr Virus Immediate-Early and Early Genes, Preventing Viral Lytic Replication. J. Virol. 78, 104–115. https://doi.org/

10.1128/JVI.78.1.104-115.2004
Lagas, J.S., Wilhelm, A.J., Vos, R.M., van den Dool, E.-J., van der Heide, Y., Huissoon, S., Beijnen, J.H., Brandjes, D.P.M., 2011. Toxicokinetics of a dipyridamole (Persantin) intoxication: case report. Hum. Exp. Toxicol. 30, 74–78. https://doi.org/10.1177/0960327110368696
Li, H., Liu, S., Hu, J., Luo, X., Li, N., M.Bode, A., Cao, Y., 2016. Epstein-Barr virus lytic reactivation regulation and its pathogenic role in carcinogenesis. Int. J. Biol. Sci. 12, 1309–1318. https://doi.org/10.7150/ijbs.16564
Liu, X.-J., Zheng, Y.-B., Li, Y., Wu, S.-Y., Zhen, Y.-S., 2014. A Multifunctional Drug Combination Shows Highly Potent Therapeutic Efficacy against Human Cancer Xenografts in Athymic Mice. PLOS ONE 9, e115790. https://doi.org/10.1371/journal.pone.0115790
Luka, J., Kallin, B., Klein, G., 1979. Induction of the Epstein-Barr virus (EBV) cycle in latently infected cells by n-butyrate. Virology 94, 228–231. https://doi.org/10.1016/0042-6822(79)90455-0
Ma, S.-D., Hegde, S., Young, K.H., Sullivan, R., Rajesh, D., Zhou, Y., Jankowska-Gan, E., Burlingham, W.J., Sun, X., Gulley, M.L., Tang, W., Gumperz, J.E., Kenney, S.C., 2011. A new model of Epstein-Barr virus infection reveals an important role for early lytic viral protein expression in the development of lymphomas. J. Virol. 85, 165–177. https://doi.org/10.1128/JVI.01512-10
Malouf, M.A., Chhajed, P.N., Hopkins, P., Plit, M., Turner, J., Glanville, A.R., 2002. Anti-viral prophylaxis reduces the incidence of lymphoproliferative disease in lung transplant recipients. J. Heart Lung Transplant. 21, 547–554. https://doi.org/10.1016/S1053-2498(01)00407-7
Manners, O., Murphy, J.C., Coleman, A., Hughes, D.J., Whitehouse, A., 2018. Contribution of the KSHV and EBV lytic cycles to tumourigenesis. Curr. Opin. Virol., Engineering for viral resistance • Viruses and cancer 32, 60–70. https://doi.org/10.1016/j.coviro.2018.08.014
Masud, H.M.A.A., Watanabe, T., Yoshida, M., Sato, Y., Goshima, F., Kimura, H., Murata, T., 2017. Epstein-Barr Virus BKRF4 Gene Product Is Required for Efficient Progeny Production. J. Virol. 91. https://doi.org/10.1128/JVI.00975-17
Matusali, G., Arena, G., De Leo, A., Di Renzo, L., Mattia, E., 2009. Inhibition of p38 MAP kinase pathway induces apoptosis and prevents Epstein Barr virus reactivation in Raji cells exposed to lytic cycle inducing compounds. Mol. Cancer 8, 18. https://doi.org/10.1186/1476-4598-8-18
Murata, T., 2014. Regulation of Epstein-Barr virus reactivation from latency. Microbiol. Immunol. 58, 307–317. https://doi.org/10.1111/1348-0421.12155
Nyhan, W.L., 2014. Nucleotide Synthesis via Salvage Pathway, in: ELS. American Cancer Society. https://doi.org/10.1002/9780470015902.a0001399.pub3
Pagano, J.S., Whitehurst, C.B., Andrei, G., 2018. Antiviral Drugs for EBV. Cancers 10, 197. https://doi.org/10.3390/cancers10060197
Pastor-Anglada, M., Cano-Soldado, P., Molina-Arcas, M., Lostao, M.P., Larráyoz, I., Martínez-Picado, J., Casado, F.J., 2005. Cell entry and export of nucleoside analogues. Virus Res. 107, 151–164. https://doi.org/10.1016/j.virusres.2004.11.005
Patel, S. S., Szebeni, J., Wahl, L.M., Weinstein, J.N., 1991. Differential inhibition of 2'-deoxycytidine salvage as a possible mechanism for potentiation of the anti-human immunodeficiency virus activity of 2',3'-dideoxycytidine by dipyridamole. Antimicrob. Agents Chemother. 35, 1250–1253. https://doi.org/

10.1128/aac.35.6.1250
Planz, O., 2013. Development of cellular signaling pathway inhibitors as new antivirals against influenza. Antiviral Res. 98, 457–468. https://doi.org/10.1016/j.antiviral.2013.04.008
Pushpakom, S., Iorio, F., Eyers, P.A., Escott, K.J., Hopper, S., Wells, A., Doig, A., Guilliams, T., Latimer, J., McNamee, C., Norris, A., Sanseau, P., Cavalla, D., Pirmohamed, M., 2018. Drug repurposing: progress, challenges and recommendations. Nat. Rev. Drug Discov. https://doi.org/10.1038/nrd.2018.168
Renzo, L. di, Alttok, A., Klein, G., Klein, E., 1994. Endogenous TGF-β contributes to the induction of the EBV lytic cycle in two burkitt lymphoma cell lines. Int. J. Cancer 57, 914–919. https://doi.org/10.1002/ijc.2910570623
Sanchez, E.L., Lagunoff, M., 2015. Viral activation of cellular metabolism. Virology 479–480, 609–618. https://doi.org/10.1016/j.virol.2015.02.038
Scheuch, G., Canisius, S., Nocker, K., Hofmann, T., Naumann, R., Pleschka, S., Ludwig, S., Welte, T., Planz, O., 2018. Targeting intracellular signaling as an antiviral strategy: aerosolized LASAG for the treatment of influenza in hospitalized patients. Emerg. Microbes Infect. 7. https://doi.org/10.1038/s41426-018-0023-3
Spano, D., Marshall, J.-C., Marino, N., De Martino, D., Romano, A., Scoppettuolo, M.N., Bello, A.M., Di Dato, V., Navas, L., De Vita, G., Medaglia, C., Steeg, P.S., Zollo, M., 2013. Dipyridamole prevents triple-negative breast-cancer progression. Clin. Exp. Metastasis 30, 47–68. https://doi.org/10.1007/s10585-012-9506-0
Takada, K., 1984. Cross-linking of cell surface immunoglobulins induces Epstein-Barr virus in Burkitt lymphoma lines. Int. J. Cancer 33, 27–32. https://doi.org/10.1002/ijc.2910330106
Tenser, R.B., Gaydos, A., Hay, K.A., 2001. Inhibition of herpes simplex virus reactivation by dipyridamole. Antimicrob. Agents Chemother. 45, 3657–3659. https://doi.org/10.1128/AAC.45.12.3657-3659.2001
Thomé, M.P., Pereira, L.C., Onzi, G.R., Rohden, F., Ilha, M., Guma, F.T., Wink, M.R., Lenz, G., 2019. Dipyridamole impairs autophagic flux and exerts antiproliferative activity on prostate cancer cells. Exp. Cell Res. https://doi.org/10.1016/j.yexcr.2019.06.001
Ville, S., Imbert-Marcille, B.-M., Coste-Burel, M., Garandeau, C., Meurette, A., Cantarovitch, D., Giral, M., Hourmant, M., Blancho, G., Dantal, J., 2018. Impact of antiviral prophylaxis in adults Epstein-Barr Virus-seronegative kidney recipients on early and late post-transplantation lymphoproliferative disorder onset: a retrospective cohort study. Transpl. Int. Off. J. Eur. Soc. Organ Transplant. 31, 484–494. https://doi.org/10.1111/tri.13085
Wang, C., Lin, W., Playa, H., Sun, S., Cameron, K., Buolamwini, J., 2013. Dipyridamole analogues as pharmacological inhibitors of equilibrative nucleoside transporters. Identification of novel potent and selective inhibitors of the adenosine transporter function of human equilibrative nucleoside transporter 4 (hENT4). Biochem. Pharmacol. 86. https://doi.org/10.1016/j.bcp.2013.08.063
Wu, C.-C., Fang, C.-Y., Huang, S.-Y., Chiu, S.-H., Lee, C.-H., Chen, J.-Y., 2018. Perspective: Contribution of Epstein–Barr virus (EBV) Reactivation to the Carcinogenicity of Nasopharyngeal Cancer Cells. Cancers 10, 120. https://

doi.org/10.3390/cancers10040120
Yoshizaki, T., Wakisaka, N., Kondo, S., Murono, S., Shimizu, Y., Nakashima, M., Tsuji, A., Furukawa, M., 2008. Treatment of locally recurrent Epstein-Barr virus-associated nasopharyngeal carcinoma using the anti-viral agent cidofovir. J. Med. Virol. 80, 879–882. https://doi.org/10.1002/jmv.21165
zur Hausen, H., O'Neill, F.J., Freese, U.K., Hecker, E., 1978. Persisting oncogenic herpesvirus induced by the tumour promotor TPA. Nature 272, 373–375. https://doi.org/10.1038/272373a0

Figure legends

Figure 1. Dipyridamole inhibits virion release and EBV DNA replication in a dose-dependent manner. (A and C) Akata cells were exposed for 4 hours to the indicated DIP concentrations and then EBV reactivation induced or not by anti-sIg (α-sIg) treatment for 24 h. Column "C" corresponds to the control mock-treated cells. Column "C+" corresponds to the control mock-treated cells in which EBV reactivation was induced by the crosslinking of the surface immunoglobulins. (B and D) Spontaneously reactivating B95-8 cells were exposed for the indicated times to 10 or 20 μM DIP. Columns "C" correspond to the control mock-treated B95-8 cells. On panels A and B, cell culture supernatants were collected and subjected to real-time PCR for quantifying relative EBV production (virion release). On panels C and D, intracellular viral genomic DNA (gDNA) was collected and subjected to real-time PCR for quantifying viral replication. For Akata cells (A and C), treatment with anti-sIg alone was considered as the reference for EBV reactivation and normalized to 1 (columns "C+"). For the spontaneously reactivating B95-8 cells (B and D), mock-treated cells were considered as the reference for EBV reactivation and normalized to 1 (columns "C"). All graphics represent the average ± SEM of 3 independent experiments. On each panel, p values were calculated using a one-way analysis of variance test (ANOVA) followed by Bonferroni's multiple comparisons test. Statistical significance (p value) was indicated by * ($P<0.05$) and ** ($P<0.01$).

Figure 2. EBV immediate-early and early gene expression is impaired in a dose-depend manner. (A) Akata cells were exposed for 4 hours to the indicated DIP concentrations and then EBV reactivation induced or not by anti-sIg (α-sIg) treatment for 8 hours. Total protein extracts were prepared and analyzed by immunolabelling with antibodies directed against EA-D, Zta and β-actin. β-actin was used as a loading control. Column "C" corresponds to the control mock-treated cells. Column "C+" corresponds to the control mock-treated cells in which EBV reactivation was induced by the crosslinking of the surface immunoglobulins. (B) Spontaneously reactivating B95-8 cells were exposed for the indicated times to 10 or 20 μM DIP. Total protein extracts were prepared and analyzed by

immunolabelling with antibodies directed against EA-D, Zta and β-actin. β-actin was used as a loading control. Columns "C" correspond to the control mock-treated B95-8 cells. On A and B (right panels), digital images were collected, band intensities measured and normalized to β-actin signal. For Akata cells (A), treatment with anti-sIg alone was considered as the reference for EBV reactivation and the ratio of either EA-D or Zta versus β-globin signals were normalized to 1 (columns "C+"). For the spontaneously reactivating B95-8 cells (B), mock-treated cells were considered as the reference for EBV reactivation and the ratio of either EA-D or Zta versus β-globin signals were normalized to 1 (columns "C"). (C) Akata cells were exposed for 4 hours to 20 μM DIP concentrations and then EBV reactivation induced or not by anti-sIg (α-sIg) treatment for 5 hours. Total RNA were prepared and the expression levels of the immediate-early (BZLF1) and early (BMRF1 and BHRF1) EBV mRNAs as well as host genes (β-globin, β-actin and cyclophilin) analyzed by quantitative RT-PCR. For the EBV mRNA tested here (BZLF1, BMRF1, BHRF1), cells treated with anti-sIg alone were considered as the reference and the quantified mRNA levels normalized to 1 for each gene analyzed (columns "C+"). For the cellular host mRNA (β-globin, β-actin, cyclophilin), mock-treated cells were considered as the reference and the quantified mRNA levels normalized to 1 for each gene analyzed (columns "C"). All graphics represent the average ± SEM of 3 independent experiments. On each panel, p values were calculated using a one-way analysis of variance test (ANOVA) followed by Bonferroni's multiple comparisons test. Statistical significance (p value) was indicated by * ($P<0.05$) and ** ($P<0.01$).

Figure 3. Dipyridamole antiviral activity is related to its ability to block nucleoside uptake. (A) Akata cells were exposed for 4 hours to the indicated NBMPR concentrations and then EBV reactivation induced or not by anti-sIg (α-sIg) treatment for 8 hours. Total protein extracts were prepared and analyzed by immunolabelling with antibodies directed against EA-D, Zta and β-actin. β-actin was used as a loading control. Column "C" corresponds to the control mock-treated cells. Column "C+" corresponds to the control mock-treated cells in which EBV reactivation was induced by the crosslinking of the surface immunoglobulins. (B)

Akata cells were exposed for 4 hours to 20 μM DIP in the presence or absence of 100 μM adenosine (ADO) or thymidine (THY) and then EBV reactivation induced or not by anti-sIg (α-sIg) treatment for 8 hours. Total protein extracts were prepared and analyzed by immunolabelling with the indicated antibodies. β-actin was used as a loading control. Column "C" corresponds to the control mock-treated cells. Column "C+" corresponds to the control mock-treated cells in which EBV reactivation was induced by the crosslinking of the surface immunoglobulins. (C) Akata cells were incubated for 4 hours in the presence or absence of 100 μM adenosine (ADO) or thymidine (THY) and then EBV reactivation induced or not by anti-sIg (α-sIg) treatment for 8 hours. Total protein extracts were prepared and analyzed by immunolabelling with the indicated antibodies. β-actin was used as a loading control. Column "C" corresponds to the control mock-treated cells. Column "C+" corresponds to the control mock-treated cells in which EBV reactivation was induced by the crosslinking of the surface immunoglobulins. For A, B and C, digital images were collected from immunoblots, band intensities measured and normalized to β-actin signal (right panels). Treatment with anti-sIg alone was considered as the reference for EBV reactivation and the ratio of either EA-D or Zta versus β-globin signals were normalized to 1 (columns "C+"). All graphics represent the average ± SEM of 3 independent experiments. On each panel, p values were calculated using a one-way analysis of variance test (ANOVA) followed by Bonferroni's multiple comparisons test. Statistical significance (p value) was indicated by * ($P<0.05$) and ** ($P<0.01$).

Supplementary table 1
Real-time PCR primer sequences

Target	Forward primer 5'->3'	Reverse primer 5'->3'
BZLF1	TGAACAGTAGAATTGTCTCCAGGT	CCTCAGCTGTTCCCAGTCTC
BHRF1	GGAGATACTGTTAGCCCTG	GTGTGTTATAAATCTGTTCCAAG
BMRF1	AGGAGTGCTGCAGGTAAACC	GCTCTGGTGATTCTGCCACT
β-globin	TAGCAACCTCAAACAGACACCA	CAGCCTAAGGGTGGGAAAAT
Cyclophili	GCCTTAGCTACAGGAGAGAA	TTTCCTCCTGTGCCATCTC
β-actin	AGCACTGTGTTGGCGTACAG	GGACTTCGAGCAAGAGATGG

Supplementary Figure legend

Supplementary figure 1.

Panels A and B show the effect of varying the time of DIP pre-incubation on EBV reactivation. Akata cells were exposed for 0, 2 or 4 hours to 10 µM DIP and then EBV reactivation induced or not by anti-sIg treatment for 24 h. Cell culture supernatants were collected after 24 h reactivation and subjected to real-time PCR for quantifying relative EBV production (virion release, panel A). Treatment with anti-sIg alone was considered as the reference for EBV reactivation and normalized to 1 (column "C+"). Column "C" corresponds to the control mock-treated cells. Alternatively, total protein extracts were prepared after 8 h reactivation and analyzed by immunolabelling with antibodies directed against EA-D, Zta and β-actin. β-actin was used as a loading control (panel B). Digital images were collected, band intensities measured and normalized to β-actin signal. Treatment with anti-sIg alone was considered as the reference for EBV reactivation and the ratio of either EA-D or Zta versus β-globin signals were normalized to 1 (columns "C+"). Column "C" corresponds to the control mock-treated cells. Plots, on the right of panel B, represent the average ± SEM of 3 independent experiments. P values were calculated using a one-way analysis of variance test (ANOVA) followed by Bonferroni's multiple comparisons test. Statistical significance (p value) was indicated by * ($P<0.05$) and ** ($P<0.01$).

Panels C and D evaluate the toxicity of DIP on non-induced Akata cells. The viability of the Akata cells treated with increasing concentrations of DIP was evaluated by either propidium iodide staining followed by flow cytometry analysis (panel C) or

trypan blue exclusion test (panel D). Cells were incubated for 24 to 48 hours at the indicated DIP concentrations (1.2, 2.5, 5, 10 and 20 µM). On panel C, mock-treated cells served as a control and 0.01% Triton-X100-treated cells as a positive control for membrane dysfunction. On panel D, column "C" corresponds to the control mock-treated cells.

Panel E shows the effect of DIP on Zta and EA-D protein expression levels in Mutu-I cells reactivated by TGF-β. Mutu-I cells were exposed or not for 4 hours to 20 µM DIP and then EBV reactivation induced or not by 2 ng/ml TGF-β. Total protein extracts were prepared after 8 h reactivation and analyzed by immunolabelling with antibodies directed against EA-D, Zta and β-actin. β-actin was used as a loading control. Digital images were collected, band intensities measured and normalized to β-actin signal. Treatment with TGF-β alone was considered as the reference for EBV reactivation and the ratio of either EA-D or Zta versus β-globin signals were normalized to 1 (columns "C+"). Plots, on the right of panel E, represent the average ± SEM of 3 independent experiments. P values were calculated using a one-way analysis of variance test (ANOVA) followed by Bonferroni's multiple comparisons test. Statistical significance (p value) was indicated by * ($P<0.05$) and ** ($P<0.01$).

Panel F shows that DIP inhibition of EBV reactivation is not mediated through its phosphodiesterase inhibiting activity and cAMP/PKA signaling. As shown on the schematic representation, DIP inhibits phosphodiesterases activity and leads to the accumulation of cAMP, triggering PKA activation. The PKA inhibitor H89 should interfere with DIP if the antiviral activity of DIP is mediated through cAMP/PKA signaling. Akata cells were pre-incubated for 1 hour with 5 µM of the PKA inhibitor H89 (columns 2 to 4 and 6 to 8) and then exposed to either 0 (columns 2 and 6), 10 (columns 3 and 7) or 20 (columns 4 and 8) µM DIP for 4 hours. EBV reactivation was subsequently induced by anti-sIg treatment for 8 hours (columns 5 to 8) and cell extracts subjected to immunoblot analysis with the indicated antibodies. Column "C" corresponds to the control mock-treated cells. Column "C+" corresponds to the control mock-treated cells in which EBV reactivation was induced by the crosslinking of the surface immunoglobulins. H89 when given alone (column 6) does

not affect EBV reactivation (compare column 6 to column 5) as DIP did (columns 7 and 8). When combined with DIP, H89 does not interfere with its antiviral activity.

Figure 1

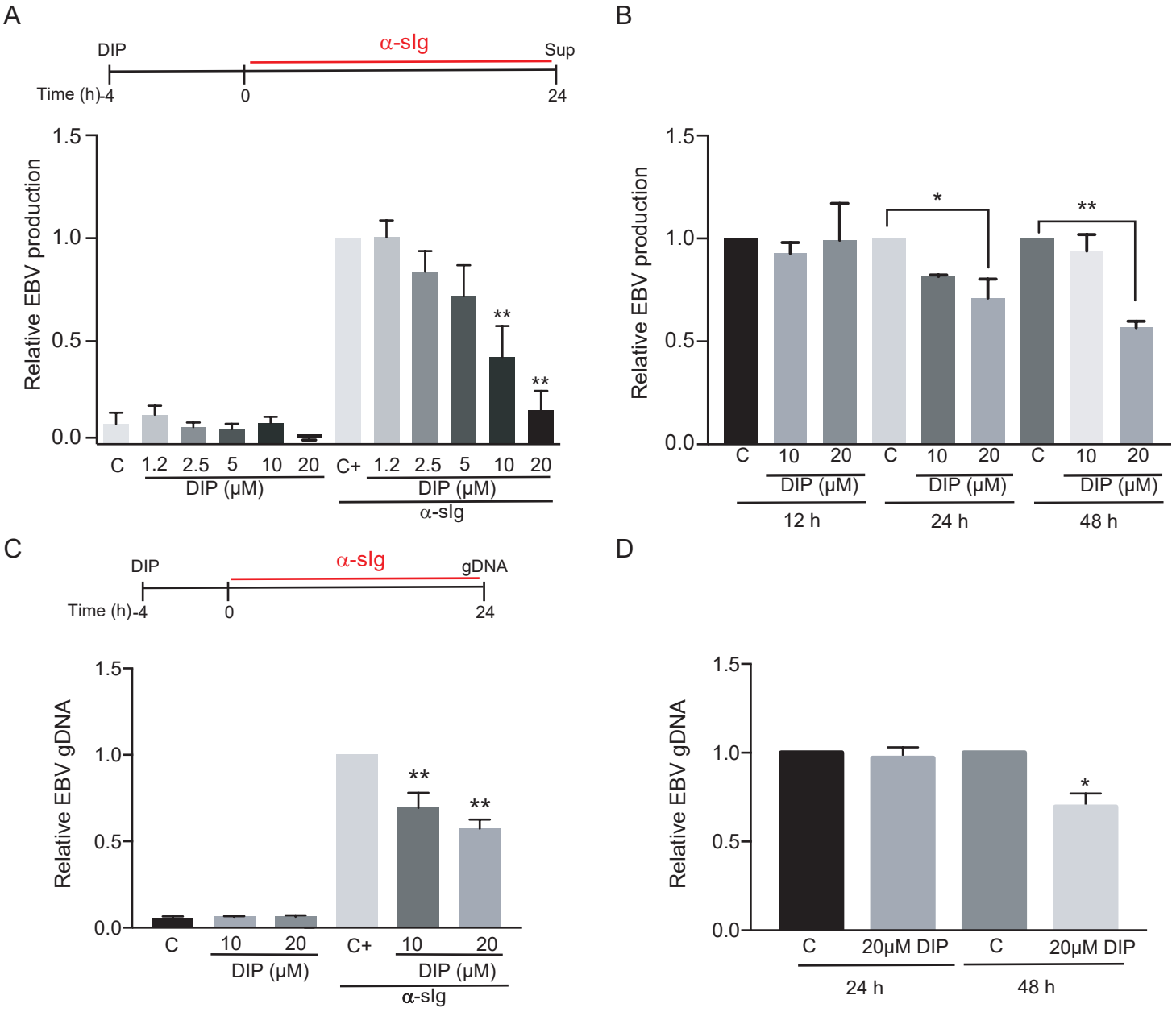
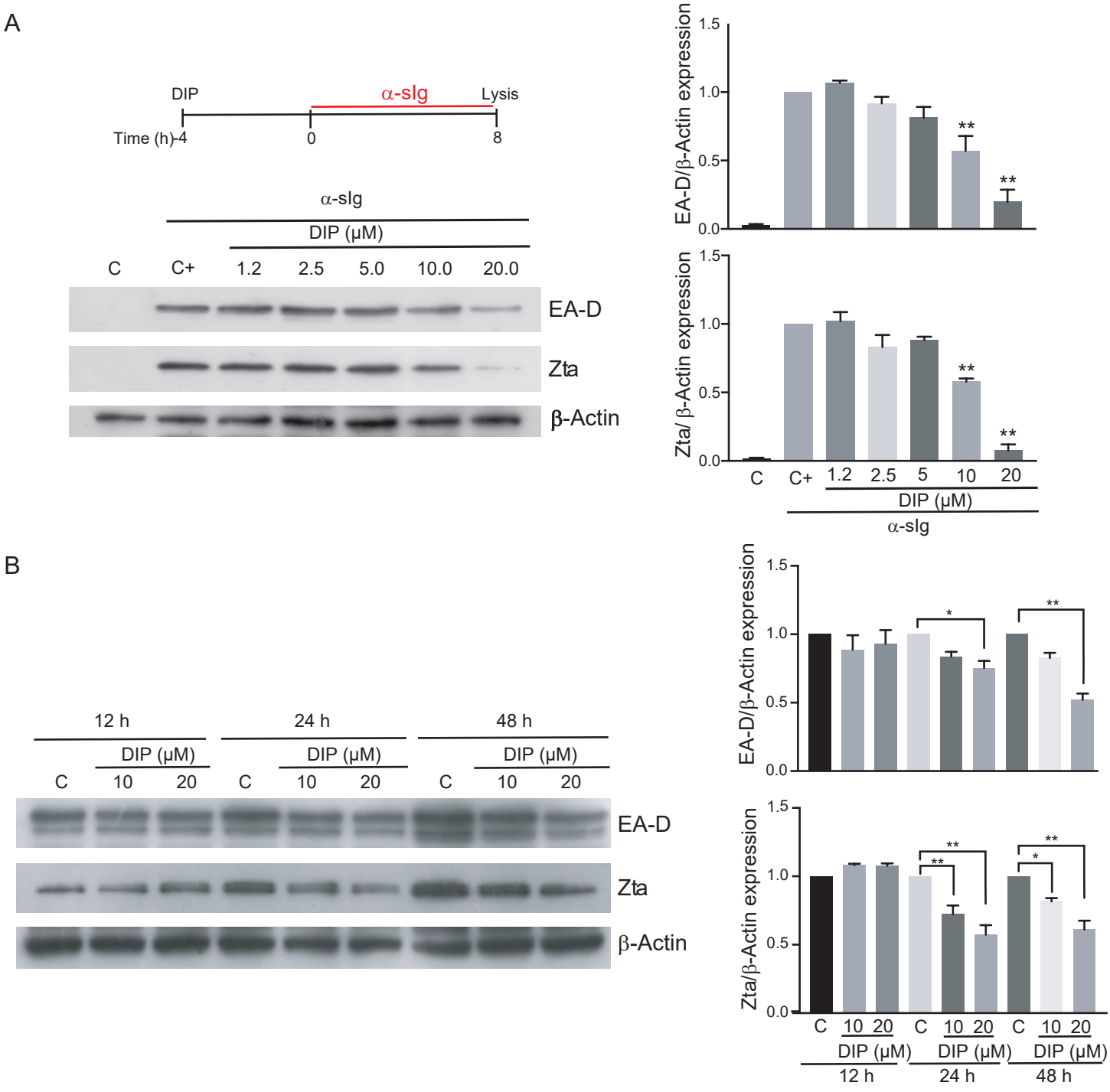

A
DIP
α-sIg
Sup
Time (h)-4
0
24
Relative EBV production
1.5
1.0
0.5
0.0
C
1.2
2.5
5
10
20
C+
DIP (μM)
α-sIg
**
B
Relative EBV production
*
**
12 h
24 h
48 h
C
DIP (μM)
C
gDNA
Relative EBV gDNA
20μM DIP

Figure 2


A
DIP
α-sIg
Lysis
Time (h)-4
0
8
α-sIg
DIP (μM)
C
C+
1.2
2.5
5.0
10.0
20.0
EA-D
Zta
β-Actin
EA-D/β-Actin expression
Zta/ β-Actin expression
1.5
1.0
0.5
0.0
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C
C+
1.2
2.5
5
10
20
DIP (μM)
α-sIg
B
12 h
24 h
48 h
DIP (μM)
C
10
20
EA-D
Zta
β-Actin
EA-D/β-Actin expression
Zta/β-Actin expression
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12 h
24 h
48 h

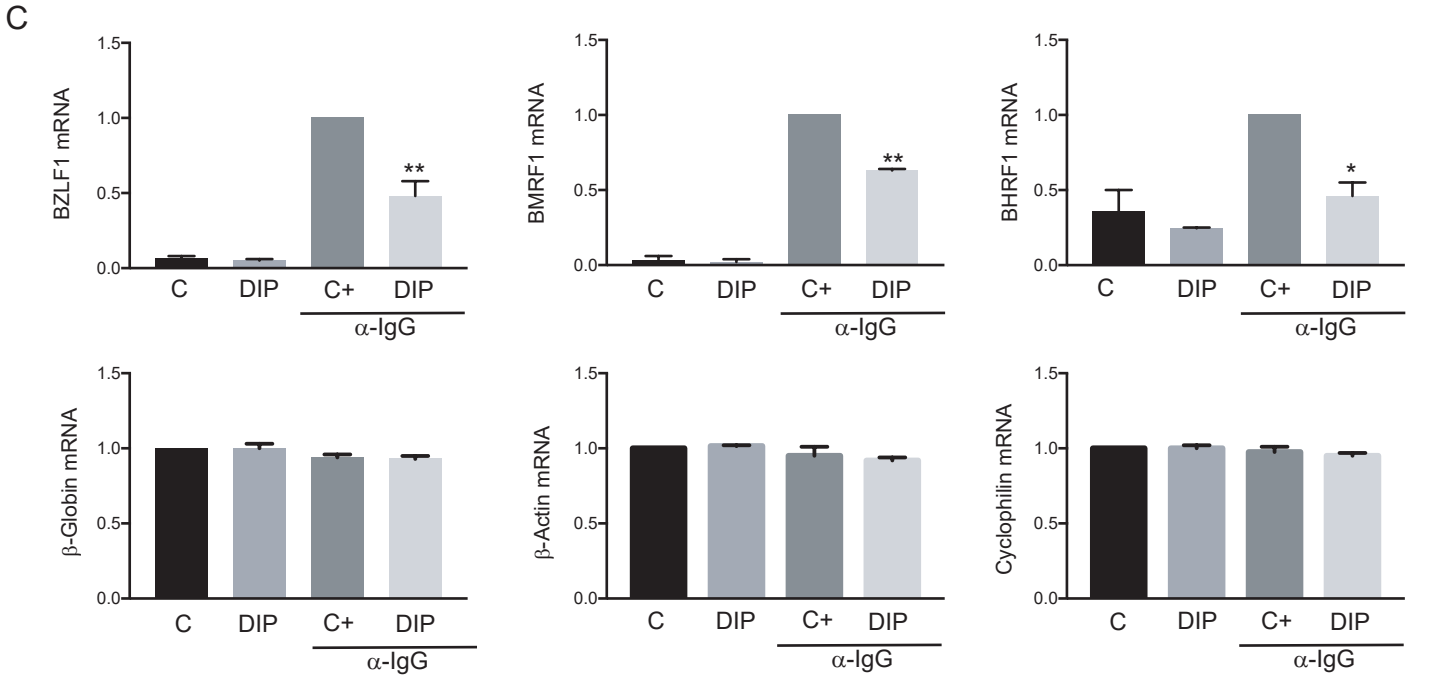
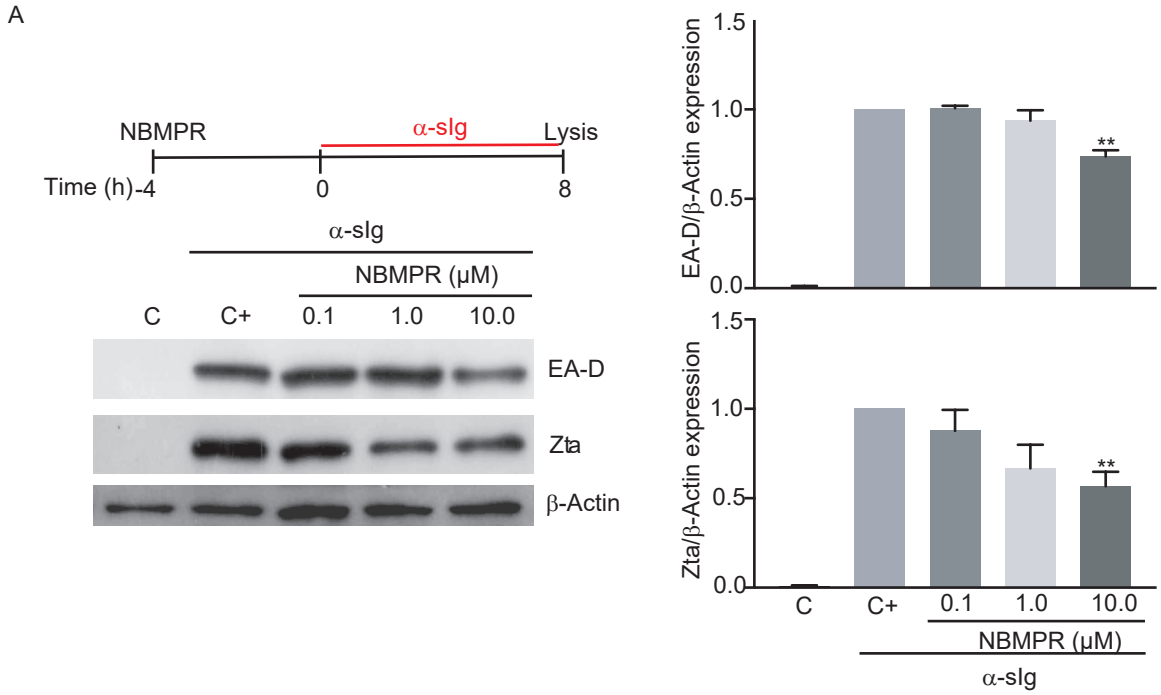

C
BZLF1 mRNA
BMRF1 mRNA
BHRF1 mRNA
β-Globin mRNA
β-Actin mRNA
Cyclophilin mRNA
1.5
1.0
0.5
0.0
**
*
C
DIP
C+
DIP
α-IgG

Figure 3


A
NBMPR
α-sIg
Lysis
Time (h)-4
0
8
α-sIg
NBMPR (μM)
C
C+
0.1
1.0
10.0
EA-D
Zta
β-Actin
EA-D/β-Actin expression
Zta/β-Actin expression
1.5
1.0
0.5
0.0
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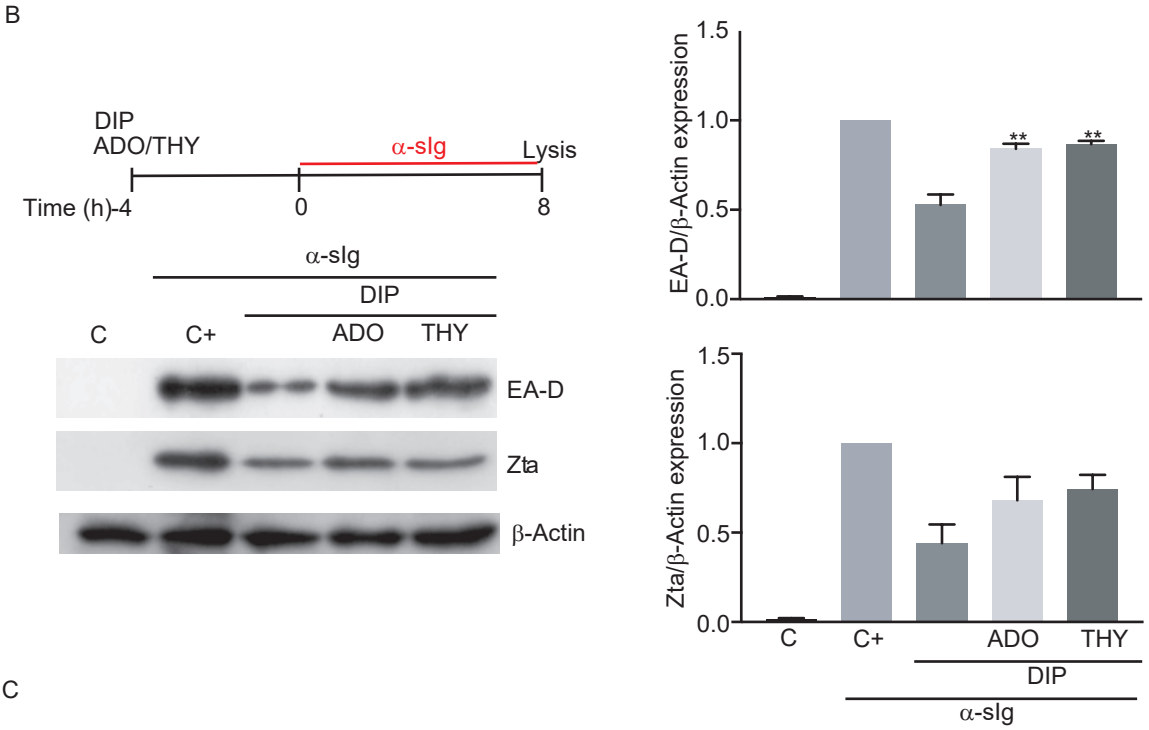
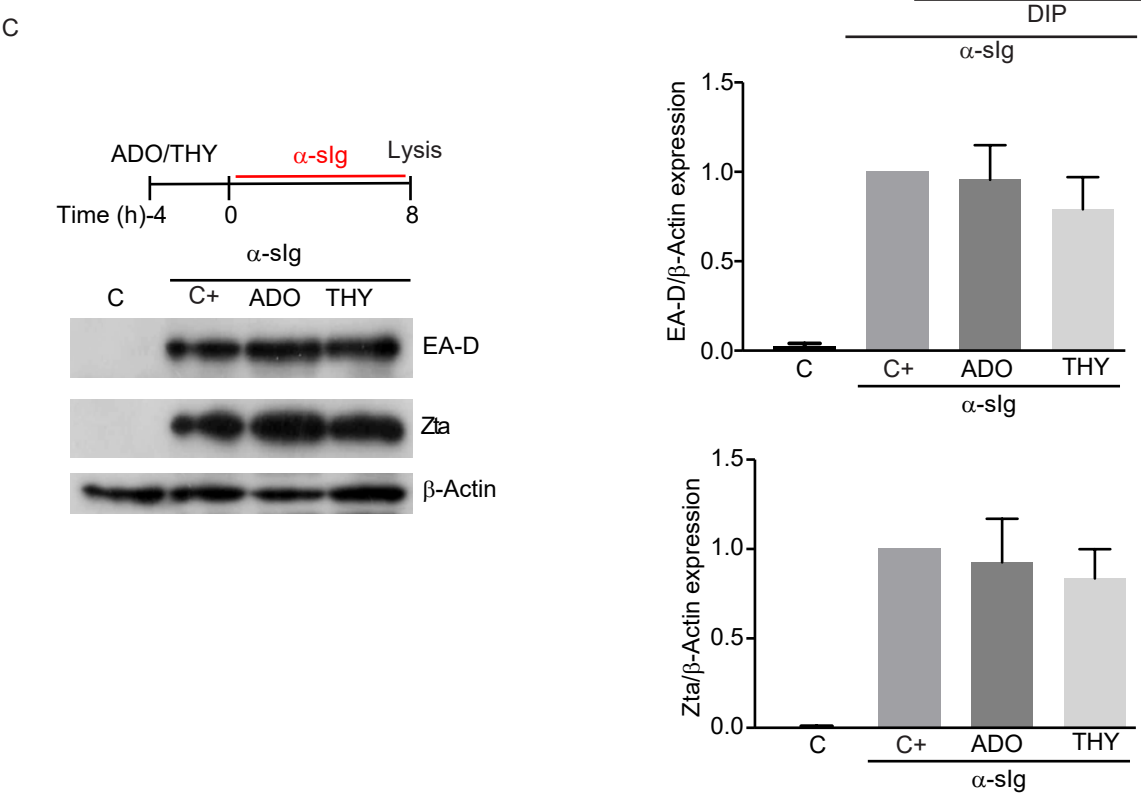
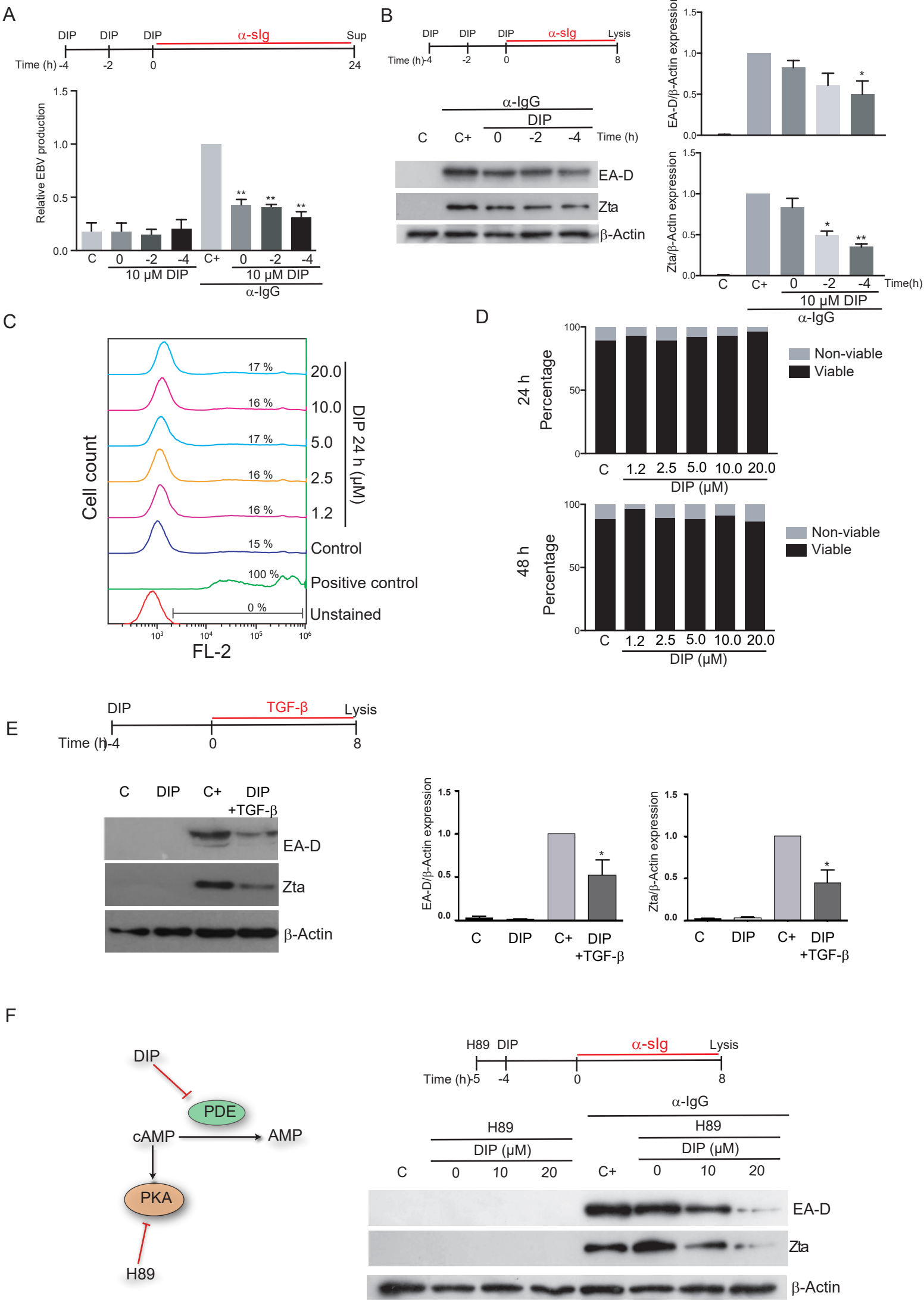
NBMPR (μM)
α-sIg


B
DIP
ADO/THY
α-sIg
Lysis
Time (h)-4
0
8
α-sIg
DIP
C
C+
ADO
THY
EA-D
Zta
β-Actin
EA-D/β-Actin expression
Zta/β-Actin expression
1.5
1.0
0.5
0.0
**
C
C+
ADO
THY
DIP
α-sIg


C
ADO/THY
α-sIg
Lysis
Time (h)-4
0
8
α-sIg
C
C+
ADO
THY
EA-D
Zta
β-Actin
EA-D/β-Actin expression
Zta/β-Actin expression
1.5
1.0
0.5
0.0
C
C+
ADO
THY
α-sIg

Sup. figure 1


A
DIP
DIP
DIP
α-sIg
Sup
Time (h) -4
-2
0
24
Relative EBV production
1.5
1.0
0.5
0.0
C
0
-2
-4
C+
0
-2
-4
10 μM DIP
10 μM DIP
α-IgG
B
DIP
DIP
DIP
α-sIg
Lysis
Time (h)-4
-2
0
8
α-IgG
DIP
C
C+
0
-2
-4
Time (h)
EA-D
Zta
β-Actin
EA-D/β-Actin expression
Zta/β-Actin expression
Time(h)
10 μM DIP
α-IgG
C
Cell count
17 %
16 %
17 %
16 %
16 %
15 %
100 %
0 %
20.0
10.0
5.0
2.5
1.2
DIP 24 h (μM)
Control
Positive control
Unstained
FL-2
D
24 h
48 h
Percentage
100
50
0
Non-viable
Viable
C
1.2
2.5
5.0
10.0
20.0
DIP (μM)
E
DIP
TGF-β
Lysis
Time (h)-4
0
8
C
DIP
C+
DIP
+TGF-β
EA-D
Zta
β-Actin
EA-D/β-Actin expression
Zta/β-Actin expression
F
DIP
PDE
cAMP
AMP
PKA
H89
H89
DIP
α-sIg
Lysis
Time (h)-5
-4
0
8
α-IgG
H89
DIP (μM)
C
0
10
20
C+
EA-D
Zta
β-Actin