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The Isoquinoline Alkaloid Berberine Inhibits Human Cytomegalovirus Replication by Interfering with the Viral Immediate Early-2 (IE2) Protein Transactivating Activity

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Abbreviations: BBR, berberine; IE2, Immediate-Early 2; IE3, Immediate-Early 3; GCV, ganciclovir; CDV, cidofovir; FOS, foscarnet; NTZ, nitazoxanide.

Abstract

The identification and validation of new small molecules able to inhibit the replication of human cytomegalovirus (HCMV) remains a priority to develop alternatives to the currently used DNA polymerase inhibitors, which are often burdened by long-term toxicity and emergence of cross-resistance. To contribute to this advancement, here we report on the characterization of the mechanism of action of a bioactive plant-derived alkaloid, berberine (BBR), selected in a previous drug repurposing screen expressly devised to identify early inhibitors of HCMV replication. Low micromolar concentrations of BBR were confirmed to suppress the replication of different HCMV strains, including clinical isolates and strains resistant to approved DNA polymerase inhibitors. Analysis of the HCMV replication cycle in infected cells treated with BBR then revealed that the bioactive compound compromised the progression of virus cycle at a stage prior to viral DNA replication and Early (E) genes expression, but after Immediate-Early (IE) proteins expression. Mechanistic studies in fact highlighted that BBR interferes with the transactivating functions of the viral IE2 protein, thus impairing efficient E gene expression and the progression of HCMV replication cycle. Finally, the mechanism of the antiviral activity of BBR appears to be conserved among different CMVs, since BBR suppressed murine CMV (MCMV) replication and inhibited the transactivation of the prototypic MCMV *E1* gene by the IE3 protein, the murine homolog of IE2. Together, these observations warrant for further experimentation to obtain proof of concept that BBR could represent an attractive candidate for alternative anti-HCMV therapeutic strategies.

Keywords: Berberine chloride; Human Cytomegalovirus; Murine Cytomegalovirus; IE2; IE3; Promoter transactivation.

1. Introduction

Human cytomegalovirus (HCMV) represents one of the most important opportunistic human pathogens. It belongs to the *Beta-herpesvirinae* subfamily and rarely causes symptomatic clinical manifestations in immunocompetent individuals (Griffiths et al., 2015; Luganini et al., 2016; Britt & Prichard, 2018). However, HCMV induces severe morbidity and mortality in the immunocompromised population following reactivation or primary infection, leading to gastro-intestinal diseases, pneumonia, retinitis, and other organs' infections (Griffiths et al., 2015; Luganini et al., 2016). Moreover, HCMV is also the viral leading cause of congenital defects in newborn children, causing deafness and other neurological disorders in approximately 0.1% cases of congenital infections (Britt, 2018). Understanding the molecular mechanisms of HCMV replication and identifying essential viral and host factors involved in productive infection is mandatory for the development of new effective antiviral agents and therapeutic strategies that could fill the gap left by the currently approved anti-HCMV therapies. In fact, the drugs available for the treatment of HCMV infections suffer from several drawbacks, including long-term toxicity and poor bioavailability (Meesing and Razonable, 2018). In addition, since the anti-HCMV drugs all share a common target (i.e., the viral DNA polymerase), the possibility may occur that drug-resistant viruses selected during long-term therapy are also cross-resistant to all the available drugs, leaving the patient bereft of therapeutic options (Haidar and Singh, 2017; Razonable, 2018). A step forward was the very recent approval of letermovir, a viral terminase inhibitor, albeit only as a prophylactic agent for preventing HCMV disease in patients undergoing hematopoietic stem cells transplantation (Bray et al., 2018; Marty et al., 2017). For all these reasons, there is an evident medical need for the development of new anti-HCMV drugs with a favorable pharmacological profile and directed against different viral targets.

The multifunctional and essential viral Immediate-Early 2 (IE2) protein is thought one of the most promising new targets for anti-HCMV drug discovery, and over time targeting IE2 expression

or activity has been proven to represent an effective antiviral strategy ((Scholz et al., 2001; Mercorelli et al., 2014a). Indeed, ISIS 2922 (fomivirsen), an antisense phosphorothioate oligonucleotide complementary to the mRNA encoding IE2 and thus able to prevent IE2 protein expression, is a drug approved for treatment of HCMV diseases, although infrequently used compared to ganciclovir (GCV) (Britt & Prichard, 2018). More recently, several small molecules, including already approved drugs, have been reported to interfere with the expression or activity of IE2 (Mercorelli et al., 2014b; Gardner et al., 2015; Mercorelli et al., 2016; Beelontally et al., 2017; Mercorelli et al., 2018a). In detail, we focused on the gene transactivating activity of IE2 (Stinski & Petrik, 2008) as a HCMV-specific target for a drug-repurposing cell-based screening of a library of both approved drugs and natural bioactive compounds (Mercorelli et al., 2016). Among the 38 identified hits, the anti-parasitic drug nitazoxanide (NTZ) was observed to inhibit HCMV replication, as well as viral Early (E) and Late (L) gene expression and DNA synthesis. Mechanistic studies then revealed that NTZ hindered the IE2-dependent transactivation of essential viral E genes (Mercorelli et al., 2016). NTZ represents therefore the prototype of a novel class of anti-HCMV agents that can act by interfering with the transactivating activity of IE2. The natural compound berberine hydrochloride (BBR) was another of the molecules selected by the screening that showed a potent inhibitory activity on HCMV replication at low-micromolar concentrations (Mercorelli et al., 2016).

BBR is an isoquinoline alkaloid that can be isolated from different plants belonging to the *Berberis* genus and is a traditional component of Chinese and Ayurvedic medicine used since millennials for its antimicrobial and antiparasitic effects (Imenshahidi and Hosseinzadeh, 2016; Kumar et al., 2015). Currently, BBR is under preclinical and clinical investigation for its broad-spectrum pharmacological properties, including anti-cancer, anti-diabetes, and anti-hypertensive activities (Imenshahidi and Hosseinzadeh, 2016; Kumar et al., 2015). An inhibitory activity of BBR have been already reported against different viruses, such as herpesviruses (herpes simplex virus and HCMV) (Song et al., 2014; Hayashi et al., 2007), influenza virus (Cecil et al., 2011),

respiratory syncytial virus (RSV) (Shin et al., 2015), alphavirus (Varghese et al., 2016a; Varghese et al., 2016b), enterovirus (Wang et al., 2017), and flavivirus (Robinson et al., 2018). Moreover, BBR is used as a common dietary supplement all over the world and thus its repurposing potential is high.

However, in the case of HCMV, the mechanism of the antiviral activity of BBR has not been investigated. Here, we report the characterization of the mechanism of action of BBR against both HCMV and the murine cytomegalovirus (MCMV) and provide strong evidence that BBR targets the activity of the HCMV transcription factor IE2, as well as of its murine homolog IE3.

2. Materials & Methods

2.1 Compounds

Berberine chloride (BBR), nitazoxanide (NTZ), foscarnet (FOS), and ganciclovir (GCV) were purchased from Sigma-Aldrich. Cidofovir (CDV) was from Gilead Sciences. Fomivirsen (ISIS 2922) was synthesized by Metabion International AG. The anti-HCMV 6-aminoquinolone compound WC5 was previously described (Mercorelli et al., 2009).

2.2 Cells and viruses

Human Foreskin Fibroblasts (HFF), low-passage number human embryonic lung fibroblasts (HELF), U373-MG, and NIH 3T3 cells were cultured in Dulbecco modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Euroclone), 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate (P/S, both from Euroclone) at 37°C in a humidified atmosphere supplemented with 5% CO_2. Quiescent HELFs were obtained by culturing the subconfluent cultures for 48 h in DMEM supplemented with 0.5% serum (low-serum medium).

HCMV laboratory strain AD169 was purchased from American Type Culture Collection (ATCC; VR-538). HCMV TB40-UL32-EGFP (kindly provided by C. Sinzger, University of Ulm,

Germany) was previously described (Sampaio et al., 2005). HCMV VR1814 (kindly provided by G. Gerna, IRCCS Policlinico San Matteo, Pavia, Italy) was recovered from a cervical swab from a pregnant woman (Revello et al., 2001). HCMV 388438U, a clinical isolate of HCMV, was collected from a urine sample at the Microbiology and Virology Unit of Padua University Hospital (Italy) and was at passage 4 after primary isolation. HCMV strains resistant to antiviral drugs were obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD) and were previously described (Mercorelli et al., 2009). Murine cytomegalovirus (MCMV) strain Smith was purchased from the ATCC (ATCC VR-194). Recombinant adenoviral vectors expressing HCMV IE2 or *E. coli* β-galactosidase (LacZ) were previously described (Mercorelli et al., 2014).

2.3 Plaque reduction assays

For plaque reduction assays (PRA) with HCMV and MCMV, HFF and NIH 3T3 cells, respectively, were seeded in 24-well plates. The next day, cell monolayers were infected at 37°C with 100 Plaque Forming Units (PFU) per well of the different viral strains. At 2 h post-infection (p.i.), viral inocula were removed, cells were washed, and media containing increasing concentrations of each compound, 5% FBS, and 0.6% methylcellulose were added. All compound concentrations were tested at least in duplicate. After 10 days for HCMV and 6 days for MCMV, cell monolayers were fixed, stained with crystal violet, and viral plaques were microscopically counted.

2.4 Cytotoxicity assays

The cytotoxicity of BBR and reference compounds were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) method, as described previously (Loregian and Coen, 2006).

2.5 Quantitative Real-Time PCR

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356
357 To analyze the effects of BBR on HCMV DNA synthesis, HFFs were seeded at a density of
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359 1.5×10^5 per well in 24-well plates. The next day, cell monolayers were infected with HCMV
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361 AD169 at a multiplicity of infection (MOI) of 0.1 PFU/cell and, following virus adsorption (2 h at
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363 37°C), were incubated with 50 μM BBR or 25 μM GCV as a control. At different times p.i., cells
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365 were collected and total DNA was extracted. The levels of viral DNA were then determined by
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367 quantitative Real-time PCR (qPCR) and normalized to the cellular *β-globin* gene copies as
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369 previously described (Loregian et al., 2010). The oligonucleotide sequences used for the qPCR
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371 experiments were previously reported (Mercorelli et al., 2016).
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377 *2.6 Immunoblotting*
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379 Sub-confluent HFF cells cultured in 6-well plates were infected with HCMV AD169 at an
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381 MOI of 0.5 PFU/cell. Following virus adsorption, cells were treated with 50 μM BBR, 30 μM NTZ,
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383 or with DMSO (0.1% v/v) as a control. Whole-cell protein extracts were prepared at different times
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385 p.i. as previously described (Cavaletto et al., 2015), fractionated through 8% SDS-PAGE, and then
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387 transferred to PVDF membranes (BioRad). After blocking with 5% nonfat dry milk in TBS-Tween
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389 0.05%, membranes were incubated overnight at 4°C with the following mAbs: anti-IEA (IE1 and
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391 IE2) (1:2000, clone CH160, Virusys), anti-UL44 (1:2000, clone CH16, Virusys), anti-UL99
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393 (1:2000, clone H19, Virusys), and anti-tubulin (1:2000, clone TUB 2.1, Sigma) as a control for
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395 protein loading. Immunocomplexes were then detected with a goat anti-mouse Ig Ab conjugated to
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397 horseradish peroxidase (Life Technologies) and visualized by enhanced chemiluminescence
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399 (Western Blotting Luminol Reagent, Santa Cruz).
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404 *2.7 Plasmids*
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406 The pUL54-luciferase indicator plasmids pUL54-0.4, bearing the entire HCMV *UL54*
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408 promoter, and pUL54-0.15, containing *UL54* promoter sequence from −150 to +15 relative to the
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410 transcription start site, were previously described (Gariano et al., 2012). pRL-TK and pSV-Rluc
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416 vectors expressing *Renilla* luciferase were purchased from Promega. The indicator plasmids pE(-
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418 207)Luc and pME(-207)Luc containing the firefly luciferase reporter gene under control of wt and
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420 mutated cellular cyclin E promoter, respectively, were kindly provided by E.A. Thompson (Mayo
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422 Clinic Florida, FL) and were previously described (Bresnahan et al., 1998). Generation of the
423
424 pGL3-MIEP-crs wt plasmid expressing firefly luciferase under the control of HCMV Major
425
426 Immediate Early promoter was previously described (Mercorelli et al., 2014). The pGL4.10-luc2-
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428 LTR construct expressing the luciferase indicator gene under the control of HIV-1 LTR was
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430 previously described (Perrone et al., 2013). pRPneo/SL3/Tat expressing HIV-1 Tat protein was
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432 previoulsy described (Gibellini et al., 1995). The firefly luciferase indicator construct phTS-
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434 243/+30 contains a portion of the promoter of the human thymidylate synthase gene (TS) (-243 and
435
436 +30 relative to the transcription start codon) cloned into pGL3-basic vector (Promega) (Gribaudo et
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438 al., 2002). The pSGIE72 and pSGIE86 constructs, expressing the HCMV IE1-72 kDa or IE2-86
439
440 kDa proteins respectively, were generated as described previously (Klucher et al., 1993). The empty
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442 pSG5 expression vector was purchased from Agilent Technologies. The pGL3-E1 vector, harboring
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444 the MCMV early gene *E1* promoter responsive to IE3 protein (the MCMV homolog of HCMV
445
446 IE2), was generated as previously reported (Mercorelli et al., 2014). The pIE3 plasmid for the
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448 expression of IE3 was described previously (Messerle et al., 1992). pBSK plasmid was purchased
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450 from Agilent Technologies.
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457 *2.9 Cell transfection and adenoviral transduction*
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459 All transient transfection experiments in HELF cells were performed using Lipofectamine
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461 3000 (Life Technologies). For transfection/transduction experiments, HELF grown on 24-well
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463 plates were transfected with the luciferase reporter plasmid driven by either the UL54-0.4 or UL54-
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465 0.15 gene promoter, and the pRL-TK plasmid expressing *Renilla* luciferase to normalize variations
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467 in transfection efficiency (Mercorelli et al., 2016). At 24 h post-transfection, cells were transduced
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469 with AdVIE2, or AdVLacZ as a control, at an MOI of 20 PFU/cell for 2 h at 37°C, and then treated
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476 with 50 μM BBR, 30 μM NTZ, 5 μM ISIS 2922, or 0.1% DMSO (v/v). At 48 h post-transduction,
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478 cell extracts were prepared and firefly and *Renilla* luciferase activities were measured.
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480 To evaluate the effect of BBR on the TS gene promoter activity, HELF cells were co-
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482 transfected with phTS-243/+30, pSGIE72, or the empty pSG5 vector, along with pRL-TK plasmid.
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484 At 18 h post-transfection, cells were washed with warm medium and incubated in low-serum
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486 medium (0.2% FBS) containing 50 μM BBR, 30 μM NTZ, or 0.1% DMSO (v/v) for 48 h before
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488 measuring firefly and *Renilla* luciferases activities (Gribaudo et al., 2002; Mercorelli et al., 2018).
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490 Transient transfections in U373-MG cells were performed using calcium phosphate precipitation
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492 method (CellPhect Transfection Kit, GE Healthcare). Briefly, U373-MG were seeded in 24-well
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494 plates and the next day were transiently co-transfected with 0.5 μg of plasmids containing different
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496 promoters (*cyclin E* both wt and mutated, HCMV MIEP, and HIV-1 LTR) and, where indicated,
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498 pSGIE86 or Tat-expressing plasmids in 1:3 ratio, along with 0.25 μg of pSV-Rluc plasmid as a
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500 control for transfection efficiency. Total DNA amount was equalized with pSG5 empty vector.
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502 After incubation for 4 h at 37°C, the transfection mixtures were removed and medium containing
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504 either BBR or DMSO (0.1%) as a control was added to the cells. To investigate whether BBR could
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506 affect the transactivation of MCMV *E1* gene promoter by the IE3 protein, NIH 3T3 cells grown on
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508 24-well plates were co-transfected using Lipofectamine 3000 with 0.75 μg of luciferase reporter
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510 plasmid pGL3-E1 and, where indicated, with 1.5 μg of pIE3 plasmid and with 0.15 μg pRL-TK
511
512 plasmid. In control transfections, the total amount of DNA was equalized with pBSK. After 3 h at
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514 37°C, transfected cells were treated with 50 μM BBR, or 50 μM WC5 as a positive control, or 0.1%
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516 DMSO (v/v) as a negative control. At 48 h post-transfection, cells were harvested for the
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518 measurement of firefly and *Renilla* luciferase activities. For all transfection experiments, firefly and
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520 *Renilla* luciferase activities were measured using the Dual-Luciferase reporter assay system kit
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522 (Promega).
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529 *2.10 Statistical analysis*
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535 All statistical analyses were performed using GraphPad Prism version 5.00 for Windows
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537 (www.graphpad.com; GraphPad Software, San Diego, CA).
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541 ## 3. Results
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543 ### *3.1 Berberine exerts broad-spectrum anti-HCMV activity*
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545 In a drug repurposing screening specifically designed to identify inhibitors of the HCMV
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547 transcription factor IE2, we identified the bioactive plant-derived BBR as a hit compound
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549 (Mercorelli et al., 2016). The observation of the anti-HCMV activity of BBR in our experimental
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551 setting prompted us to characterize deeper its mechanism of action. To this end, we first confirmed
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553 the anti-HCMV activity and cytotoxicity of BBR in HFF cells by PRAs and MTT assays,
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555 respectively (Table 1). According to our previous observations (Mercorelli et al., 2016), BBR
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557 specifically inhibited HCMV AD169 replication in a dose-dependent manner (Fig. 1) with an
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559 Effective Concentration (EC_{50}) of 2.65 μM. This value was very similar to that measured for GCV
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561 in our experimental conditions (Table 1). The Cytotoxic Concentration (CC_{50}) determined by MTT
562
563 assays was 390 μM, thus resulting in a favorable Selectivity Index (SI) of 147 (Table 1). In this
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565 regard, a similar SI value was previously observed for BBR in MRC5 cells (Hayashi et al., 2007).
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567 To further characterize the anti-HCMV activity of BBR, we performed PRAs with several HCMV
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569 strains, including different low-passage clinical isolates (TB40-UL32-EGFP, VR1814, and
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571 388438U). Results reported in Table 2 indicated that the anti-HCMV activity of BBR is not
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573 dependent on the viral strain used for the studies, since the EC_{50} values obtained with different
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575 HCMV strains were all comparable.
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581 ### *3.2 Activity of BBR against drug-resistant HCMV strains*
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583 Next, we evaluated the activity of BBR against HCMV strains resistant to the available viral
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585 DNA polymerase inhibitors, as the emergence of drug resistance is an increasing cause of transplant
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587 failure associated with HCMV infections, in particular after prolonged antiviral therapy (Razonable,
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2018). BBR fully inhibited the replication of viruses with mutations in *UL54* gene conferring cross-resistance to GCV and CDV and to FOS and acyclovir (strains GDG^{r}P53 and PFA^{r}D100, respectively, Table 3). These results suggested that BBR may have a mechanism of action that differs from that of the currently available anti-HCMV drugs that target the viral DNA polymerase.

3.3 BBR inhibits HCMV DNA replication

To investigate the effect of BBR on the replication of HCMV genome, we performed a quantitative analysis of viral DNA production at different times p.i.. As depicted in Fig. 2, a progressive increase of viral DNA levels was detected by qPCR in a time-dependent manner up to 120 h p.i. in untreated infected cells. In contrast, already at 72 h p.i. (i.e., after a completed cycle of replication), BBR, like the specific HCMV DNA polymerase inhibitor GCV, significantly inhibited the replication of viral genome. The inhibitory activity of BBR was confirmed later at 96 and 120 h p.i. (Fig. 2). Thus, most likely BBR affects a stage of the HCMV replication cycle that is located prior to the onset of viral DNA replication and this interference resulted in a significant impairment of viral DNA synthesis.

3.4 BBR interferes with the HCMV replication cycle after the IE phase

To determine whether the observed inhibition of HCMV DNA synthesis (Fig. 2) was a consequence of a BBR-mediated effect on viral IE and/or E gene expression that foreruns viral genome replication, HFFs were infected with HCMV AD169 and treated with BBR for different times p.i. Then, total protein cell extracts were assayed by immunoblotting for the content of representative Immediate-Early (IE1 and IE2), Early (UL44), and Late (UL99) viral proteins (Figure 3). For each time p.i. analyzed, control samples were: (i) extracts prepared from AD169-infected cells treated with the appropriate DMSO amount, and (ii) AD169-infected cells treated with NTZ, as a positive control for inhibition of the expression of Early and Late HCMV proteins

(Mercorelli et al., 2016). Figure 3 shows that BBR did not seemingly affect IE1 and IE2 protein accumulation at each time analyzed. In contrast, compared to the untreated cells, BBR determined a clear reduction in UL44 and UL99 levels, although less markedly than NTZ, which was used as a positive control. Taken together, these results indicated that BBR affects HCMV replication cycle after the expression of IE proteins and, together with those reported in Fig. 2, suggested that BBR likely interferes with a molecular event involved in the expression of HCMV E genes.

3.5 BBR inhibits the IE2-dependent transactivation of both viral and cellular promoters

To investigate whether BBR might affect the transactivation of E genes of HCMV, luciferase reporter plasmids containing full-length or a minimal *UL54* gene promoter were transfected in HELFs. The *UL54* promoter is a prototypic HCMV E gene promoter that is transactivated by IE2, thus its activation marks out the E phase of HCMV replicative cycle (Stinski & Petrik, 2008). Two luciferase reporter plasmids containing different segments of the *UL54* gene 5'-flanking region were used: the UL54-0.4 construct includes the full-length gene promoter, while the UL54-0.15 plasmid carries a segment of 150 bp upstream from the transcription start site (Gariano et al., 2012). This shorter portion of the *UL54* 5'-flanking region mediates the IE2-dependent transactivation of the *UL54* gene at a level comparable to that of the full-length promoter. Furthermore, it includes an 8-bp inverted repeat element 1 (IR-1, located between -55 and -48 nt relative to the transcription start site) which has been shown to be required for both HCMV- and IE2-mediated transactivation (Kerry et al. 1996). Thus, to identify the minimal portion of the *UL54* gene promoter sufficient to mediate the sensitivity to BBR, the UL54-0.15 construct was also included in transfection-transduction experiments. At 24 h post-transfection, HELFs were transduced with the adenoviral vectors AdVIE2 or AdVLacZ as a negative control of transcriptional activation, and, after additional 2h, treated with BBR, ISIS 2922, or NTZ. The latter two compounds were employed as positive controls for inhibition of the IE2-dependent *UL54* gene promoter transactivaction (Mercorelli et al., 2016). As shown in Fig. 4A, ectopic expression of IE2

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710
711 significantly increased the transcriptional activity of both *UL54* reporter constructs compared to
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713 cells expressing LacZ. As expected, both NTZ and ISIS 2922, both used as a positive control,
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715 inhibited the IE2-dependent transactivation of the UL54-0.4 promoter by 87% and 95%,
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717 respectively (Mercorelli et al., 2016). Interestingly, also treatment with BBR decreased the
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719 transcriptional activity of the full-length UL54-0.4 promoter by 77% and that of the minimal
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721 promoter *UL54*-0.15 by about 65% (Fig. 4A). These data indicated the ability of at BBR to interfere
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723 with the IE2-dependent transactivation of the promoter of an essential viral E gene, and that a
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725 segment of 150 bp upstream from the *UL54* transcription initiation site is sufficient to mediate the
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727 inhibitory activity of BBR.
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729
730 The above observation then prompted the investigation on the effect of BBR on the activation of
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732 other gene promoters that can be activated by IE2. Cellular cyclin E (*CycE*) gene expression is up-
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734 regulated upon HCMV infection in quiescent cells to promote G0/G1 transition and its
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736 transcriptional activation can be directly stimulated by IE2 (Bresnahan et al., 1998). To assess the
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738 effect of BBR on the IE2-mediated transactivation of *CycE* gene promoter, we co-transfected
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740 permissive U373-MG cells with a construct expressing IE2 together with reporter constructs
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742 containing either a portion of wt *CycE* promoter (pCycE) or a mutated version wherein the binding
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744 sites for the cellular transcription factor E2F at -16 and +7 have been mutated (mpCycE). As shown
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746 in Fig. 4B, BBR efficiently inhibited the IE2-dependent transactivation of the pCycE. Similar
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748 results were obtained in cells transfected with mpCyE construct, thus indicating that disruption of
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750 E2F binding sites of the *CycE* gene promoter did not affect the inhibitory activity exerted by BBR
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752 (Fig. 4C).
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755 Taken together, these results suggest that BBR affects the transactivation of both viral and
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757 cellular promoters, which are known to depend on IE2 for their activation.
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762 *3.6 BBR is not a general inhibitor of viral and cellular transcription*
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771 To investigate whether the inhibitory effect of BBR was specific for IE2 and not due to a
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773 general inhibition of transcription, the activities of two other viral promoters, i.e., the MIEP of
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775 HCMV and the LTR of HIV-1, which contain multiple binding sites for general cellular
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777 transcription factors, were examined for their sensitivity to BBR. Although an inhibitory activity of
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779 BBR on the basal activity of both viral promoters was observed (Fig. 5A), it was not as solid or
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781 dose responsive as that measured on the IE2-dependent transactivation of both *UL54* and *CycE* gene
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783 promoters (Fig. 4). Moreover, since the HIV-1 LTR can be specifically transactivated by the virus-
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785 encoded Tat transcription factor (Ne et al., 2018), we also investigated the effect of BBR on the Tat-
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787 mediated transactivation of LTR. As shown in Figure 5B, BBR did not affect the Tat-mediated
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789 transactivation of HIV-1 LTR. These results thus suggest that most likely BBR is not a general
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791
792 inhibitor of transcription.
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794 Then, to further strengthening the specificity of IE2 inhibition by BBR, we evaluated its
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796 effect on the activity of the other major HCMV-encoded IE transcription factor, IE1. IE1 and IE2
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798 proteins originate from a single precursor RNA through alternative splicing, therefore IE1 shares
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800 the same first three exons (85 aa) with IE2 (Stinski and Petrik, 2008). We previously reported that
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802 IE1 transactivates the human thymidylate synthase (TS) gene promoter to stimulate cellular TS
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804 activity that is required for HCMV DNA replication in quiescent cells (Gribaudo et al., 2002).
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806
807 Based on this premise, we investigated the effects of BBR on the IE1-dependent transactivation of
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809 the human *TS* gene promoter. To this end, HELFs cells were co-transfected with phTS-243/+30 and
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811 an IE1 expression vector. As reported in Fig. 5C, IE1-dependent transactivation of the human *TS*
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813 promoter was not affected by the treatment with BBR. NTZ, included as a control of a specific
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815 inhibitor of the IE2-mediated transactivation, did not affect the IE1-mediated activation of cellular
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817 *TS* gene promoter as previously observed (Mercorelli et al., 2016).
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819 Altogether, these results indicate that the observed BBR-mediated inhibition of IE2-
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821 dependent transactivating activity is not due to a general inhibitory effect on transcription.
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824 Moreover, together with those reported in Fig. 4, they sustain the hypothesis that the overall
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829 inhibitory activity of BBR against HCMV likely stems from its ability to interfere with the
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832 expression of critical E genes required for viral DNA synthesis and progression of HCMV
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834 replication cycle.
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838 *3.7 BBR prevents murine CMV replication and inhibits the IE3-dependent transactivaction of the*
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840 *Early E1 gene promoter*
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842 Finally, we evaluated the activity of BBR also against murine CMV (MCMV). To this end,
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844 PRAs were performed in NIH 3T3 fibroblasts infected with the Smith strain of MCMV. As reported
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846 in Table 4, BBR fully retained antiviral activity against MCMV and the measured EC_{50} values were
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848 comparable to those obtained for the HCMV strains (Tables 1 to 3). Moreover, BBR did not show
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850 any significant cytotoxicity in uninfected cells, confirming that the inhibition of the virus replication
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852 was due to a specific antiviral effect.
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855 Having established that BBR is also active against MCMV, we wondered whether the
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857 mechanism of action of BBR against MCMV may be the same observed for the human virus. To
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859 this end, NIH 3T3 cells were transfected with an indicator plasmid containing the luciferase gene
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861 driven by the prototypic MCMV Early *E1* gene promoter (pGL3-E1), together with an IE3-
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863 expressing vector (pIE3). The MCMV IE3 protein is the structural and functional homolog of
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865 HCMV IE2 (Messerle et al., 1992). Transfected NIH 3T3 cells were then treated with BBR or
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867 WC5, used as a control for inhibition of MCMV E1 promoter (Mercorelli et al., 2014). As shown in
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869 Fig. 6, the expression of IE3 increased the activity of the *E1* gene promoter of more than 50-fold.
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871 Treatment with BBR reduced the IE3-dependent transactivation of the *E1* gene promoter by 60%
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873 compared to that of DMSO-treated cells. As expected, WC5 reduced the luciferase activity by 71%
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875 (Mercorelli et al., 2014). These results thus indicated that the mechanism of action of BBR against
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877 HCMV is conserved also against MCMV.
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883 **4. Discussion**
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889 The identification and validation of new antiviral molecules to prevent or limit HCMV
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891 replication remains a priority for the clinical management of HCMV infections. Since IE2 plays a
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893 critical role in the progression of HCMV replication, as well in virus pathogenesis and reactivation
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895 from latency (Scholz et al., 2001; Stinski and Petrik, 2008), we and others have addressed the
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897 identification and characterization of small molecules that could block IE2 synthesis or activities
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899 (Mercorelli et al., 2014a, b; Gardner et al., 2015; Mercorelli et al., 2016; Beelontally et al., 2017;
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901 Mercorelli et al., 2018a). In a drug repurposing screen devised to select early inhibitors of HCMV
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903 replication, we previously identified 38 different molecules active in the inhibition of HCMV E
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905 gene expression (Mercorelli et al., 2016). To date, some of them have been confirmed to selectively
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907 inhibit the IE2-mediated transactivation of essential viral E genes and hence HCMV replication
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909 (Mercorelli et al., 2016; Mercorelli et al., 2018a). Here, we add another piece of knowledge about
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911 this novel class of IE2 inhibitors by reporting the characterization of the mechanism of action of
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913 BBR, one of the compounds selected in the drug repurposing screen for its anti-HCMV activity.
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916 BBR is an isoquinoline alkaloid present in several medicinal plants including *Berberis*
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918 *vulgaris*, *Coptis chinensis*, *Hydrastis canadensis*, and *Rhizoma coptidis,* and over the past years
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920 several biological effects of BBR have been reported, including antimicrobial activities against
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922 bacteria, fungi, and viruses (Imenshahidi and Hosseinzadeh, 2016; Kumar et al., 2015). In this
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924 regard, an inhibitory activity of BBR against the replication of HCMV (strain not specified) was
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926 previously reported (Hayashi et al., 2007); however, the mechanism by which BBR impaired
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928 HCMV replication was not defined. In the present study, we have demonstrated that BBR is a
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930 broad-spectrum inhibitor of HCMV replication, including strains resistant to drugs that target viral
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932 DNA polymerase, thus suggesting that its mechanism of action differs from that of the currently
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934 used DNA polymerase inhibitors. To support further this hypothesis, we showed that BBR
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936 selectively reduced the expression of representative E and L viral proteins, without affecting that of
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938 IE proteins, and inhibited the synthesis of viral DNA as well, thus suggesting that these inhibitory
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940 effects of BBR most likely account for its marked overall antiviral activity on HCMV replication.
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Based on these facts, we hypothesized that BBR could target a molecular event involved in the switch from IE to E phase of virus replication cycle. Indeed, BBR strongly reduced the ability of IE2 to transactivate two different versions of a prototypic E gene promoter, i.e., the *UL54* gene promoter. Although other molecular mechanisms cannot be totally ruled out, the effect of BBR seems to be specific for the IE2 transactivating activity, since we observed that: i) it inhibited the activation of gene promoters of both viral and cellular origin that depend on IE2 for their transcriptional activity (Fig. 4); ii) it did not significantly affect neither the transcriptional activity of viral gene promoters responsive to general cellular transcription factors, nor the Tat-dependent transactivation of HIV-1 LTR (Fig. 5); and iii) it did not exert any interference with the transactivating activity of the closely related IE1 protein (Fig. 5), which shares 85 N-terminal residues with IE2 (Stinski and Petrik, 2008).

Among molecules targeting IE2, BBR acts differently from fomivirsen, the only IE2 inhibitor that had been approved for the treatment of HCMV infections so far (Britt and Prichard, 2018), since unlike the latter it does not inhibit the expression of IE2 (Fig. 3). BBR in fact interferes with the transactivating properties of IE2 (Fig. 4), and therefore, along with NTZ (Mercorelli et al., 2016), it belongs to a novel class of anti-HCMV agents that can act by interfering with the transactivating activity of IE2.

It is known that BBR can modulate multiple host cell signaling pathways, including NF-κB (Pandey et al., 2008) and mitogen activated protein kinases (MAPK) (Cui et al., 2009). Since these pathways are also activated by HCMV infection and contribute to the efficient viral gene expression and progression of HCMV replicative cycle (Johnson et al., 2000; DeMeritt et al., 2004; Caposio et al., 2007; Caposio et al., 2010), it is possible that BBR may hinder the full activation of these transduction pathways in HCMV-infected cells, thus leading to a significant impairment of viral replication. In this regard, Song *et al*. observed that BBR inhibits HSV IE protein expression when added on cells *before* HSV infection (Song et al., 2014). This inhibition was associated to an impairment of HSV-induced NF-κB activation, indicating that BBR compromises a very early stage

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1007 of the HSV replication cycle, such as the IE gene expression. The NF-κB pathway is activated very
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1009 early also by HCMV infection and is required to kick-start the viral IE gene expression (Caposio et
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1011 al., 2007). However, it is unlikely that BBR could affect NF-κB activation in HCMV-infected cells,
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1013 since we did not observe any significant effect on the accumulation of IE proteins (Fig. 3), whose
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1015 expression is driven by the Major IE Promoter (MIEP) of HCMV that contains four NF-κB sites
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1017 (Stinski and Meier, 2007). Moreover, in different virus models, such as those of influenza and Zika
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1019 viruses, it has been observed that BBR affects virus replication after the initial attachment and entry
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1021 (Cecil et al., 2011; Robinson et al., 2018).
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1023
1024 BBR is also a known inhibitor of MAPKs pathway, in particular ERK1/2 and p38 kinases (Cui et
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1026 al., 2009; Shin et al., 2015; Varghese et al., 2016b). Relevant to this activity of BBR, it is well
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1028 established that HCMV activates several MAPKs, including ERK1/2 and p38 (Johnson et al., 2000;
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1030 Caposio et al., 2010), and that an appropriate phosphorylation of MAPKs downstream partners in
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1032 HCMV-infected cells is required for efficient progression of the viral replicative cycle (Johnson et
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1034 al., 2001). Given that IE2 can be phosphorylated by MAPKs *in vitro* (Harel and Alwine, 1998), and
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1036 the phosphorylation status modulates IE2-dependent transcriptional activation of gene promoters
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1038 (Barrasa et al., 2005), one could hypothesize that the inhibitory activity of BBR on the IE2-
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1040 dependent transactivation of E genes may descend from its ability to hamper MAPKs activation.
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1042 Noteworthy, the block of MAPKs pathway was recently identified as the mechanism of the antiviral
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1044 activity of BBR against RSV and alphaviruses (Shin et al., 2015; Varghese et al., 2016b). Relevant
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1046 to the above hypothesis, it is worth mentioning that the p38 kinase inhibitor FHPI was reported to
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1048 halt HCMV replicative cycle after IE gene expression and prior to the onset of viral DNA
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1050 replication (Johnson et al., 1999), thus in the same time frame of the HCMV replication cycle that
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1052 we identified as the main target of the antiviral activity of BBR (Fig. 3). Clearly, further
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1054 experimentation is required to confirm this hypothesis and it will be object of future studies.
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1058 BBR is active against a broad range of different microbial pathogens (Kumar et al., 2015), thus it
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1060 may offer advantages over other current anti-HCMV drugs, since HCMV infection can increase the
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risk in immunosuppressed patients of other opportunistic infections, such as bacterial and fungal infections. Moreover, BBR shows very low toxicity and side effects (Pang et al., 2015) and these features, together with the other pharmacological properties, point out BBR as an interesting candidate to develop alternative anti-HCMV therapeutic strategies.

5. Conclusion

In conclusion, the results of this study suggest BBR as another attractive candidate for a new class of anti-HCMV drugs that exert their effects via novel pathways that target IE2 functions, and warrant further investigations to evaluate whether BBR may be effective in animal models of CMV infection. Indeed, BBR is active also against MCMV replication and prevents the transactivation of a prototypic MCMV E gene mediated by the IE3 protein, the murine homolog of HCMV IE2. Given this high similarity between HCMV IE2 and MCMV IE3, the investigation of the therapeutic potential of BBR in the murine model of CMV infection is worth pursuing.

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Declaration of interest

All authors declare no conflicts of interest.

Acknowledgments

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Figure legends

Figure 1. BBR exerts a dose-dependent inhibition of HCMV AD169 replication. HFFs were infected with HCMV AD169 and then treated with different concentrations of BBR (from 0.01 to 100 μM), and incubated at 37°C for 10 days. Data shown are expressed as a percentage of the plaque number determined in treated samples with respect to the DMSO-treated and mock-infected controls and represent the means ± SD of three independent experiments performed in duplicate.

Figure 2. Berberine inhibits viral DNA synthesis in HCMV-infected cells. Infected HFFs were treated with 50 μM BBR, 25 μM GCV, or 0.1% DMSO as a control. At 24, 72, 96, and 120 h p.i., total DNA was extracted and qPCR was performed with appropriate *IE2* and *β-globin* primers. HCMV genomic copies were normalized to the cellular *β-globin* copies. Data shown are the means

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1420 ± SD of four independent experiments performed in duplicate. *p < 0.05;**p < 0.01 versus
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1422 calibrator sample (DMSO).
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1424 **Figure 3. Berberine reduces the expression of E and L HCMV proteins.** HFFs were mock-
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1426 infected or infected with AD169 (MOI of 0.5 PFU/cell) and after 2 h of viral adsorption, cells were
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1428 treated with 50 μM BBR, 30 μM NTZ, or with 0.1% DMSO (added in mock-infected and virus-
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1430 infected samples). Total cell protein extracts were prepared at the indicated times p.i., fractionated
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1432 by SDS-PAGE, and analyzed by immunoblotting. Protein markers sizes are indicated in kilodaltons.
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1434 **Figure 4. BBR inhibits the IE2-dependent transactivation of viral and cellular promoters.** (A)
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1436 HELF cells were transfected with the luciferase reporter plasmids pUL54–0.4 or pUL54–0.15.
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1438 Twenty-four hours later, transfected cells were transduced with AdVIE2 or AdVLacZ at a MOI of
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1440 20 PFU/cell and then treated with 50 μM BBR, 30 μM NTZ, 5 μM ISIS 2922, or 0.1% DMSO.
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1442 Luciferase reporter activity was measured 48 h later to determine promoter activation under the
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1444 different conditions. Data represent the means ± SD from three independent experiments in
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1446 triplicate. The results were analyzed by one-way ANOVA with Bonferroni post-test correction for
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1448 multiple comparisons. ***p <0.0001; versus calibrator sample (AdVIE2 + DMSO).
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1450 (B) (C) U373-MG cells were transfected with either (B) pCycE wt or (C) mutated mpCycE
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1452 indicator constructs along with either the empty pSG5 plasmid or the IE2-expressing pSGIE86
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1454 (IE2) plasmid and treated for 48 h with 25 or 50 μM BBR, or 0.1% DMSO as a control. Then,
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1456 activation of CycE promoters under the different conditions was determined. Data represent the
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1458 means ± SD from four independent experiments in duplicate. The results were analyzed by one-way
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1460 ANOVA with Bonferroni post-test correction for multiple comparisons. **p <0.005; *p<0.05;
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1462 versus calibrator sample (pCycE or mpCycE + DMSO).
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1464 **Figure 5. BBR is not a general inhibitor of transcription.** U373-MG cells were transfected with
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1466 either (A) HCMV MIEP or (B) HIV-1 LTR indicator constructs along with either the empty pSG5
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1468 plasmid or a Tat-expressing plasmid and treated for 48 h with 25 or 50 μM BBR, or 0.1% DMSO as
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1470 a control. Then, promoter activation under the different conditions was determined. Data represent
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the means ± SD from four independent experiments in duplicate. The results were analyzed by one-way ANOVA with Bonferroni post-test correction for multiple comparisons versus the calibrator sample (pMIEP + DMSO for panel A; LTR + DMSO or LTR + Tat + DMSO for panel B).

(C) HELF cells were co-transfected with phTS-243/+30 reporter vector along with empty pSG5 vector or an IE1-expressing vector. At 18 h after transfection, cells were washed and treated with 50 μM BBR, 30 μM NTZ, or 0.1% DMSO (v/v) for 48 h. Then, phTS promoter activation under the different conditions was determined. The data shown are the mean ± SD of two experiments, each performed in triplicate. Data were analyzed by an unpaired t-test. **$p <0.001$; versus calibrator sample (phTS-243/+30 + pIE1 + DMSO).

Figure 6. BBR abrogates the IE3-dependent transactivation of the Early *E1* gene promoter of MCMV. NIH3T3 cells were transfected with a plasmid containing luciferase reporter gene under the control of the *E1* promoter (pGL3-E1) or co-transfected with an IE3-expressing plasmid (pIE3) and then treated with 50 μM BBR, 50 μM WC5, or 0.1% DMSO. After 48 h, *E1* promoter activation under the different conditions was determined. Data shown are the means ± SD from two independent experiments in triplicate and were analyzed by an unpaired t-test. *$p < 0.01$; **$p< 0.001$; versus calibrator sample (pGL3-E1 + pIE3 + DMSO).

Table 1. Antiviral activity of BBR against HCMV AD169.

Compound	EC_{50}[a] (μM)	CC_{50}[b] (μM)	SI[c]
BBR	2.65 ± 0.35	390 ± 10	147
GCV	2.5 ± 0.5	>500	>200

[a]50% Effective Concentration, the compound concentration that inhibits 50% of plaque formation, as determined by PRAs against HCMV AD169 in HFF cells. Reported values represent the means ± the SD of data derived from three independent experiments in triplicate.
[b]50% Cytotoxic Concentration, the compound concentration that results in 50% of cytotoxicity, as determined by MTT assays in HFF cells. Reported values represent the means ± the SD of data derived from at least three independent experiments performed in quadruplicate.
[c]SI, Selectivity Index (determined as CC_{50}/EC_{50}).

Table 2. Activity of BBR against different HCMV strains.

HCMV Strain	BBR EC_{50}[a] (μM)	CC_{50}[b] (μM)	SI[c]
TB40-UL32-EGFP	2.70 ± 1.13	390 ± 10	144
VR1814	4.00 ± 0.71	390 ± 10	98
388438U	1.30 ± 0.42	390 ± 10	300

[a]50% Effective Concentration, the compound concentration that inhibits 50% of plaque formation, as determined by PRAs in HFF cells. Reported values represent the means ± the SD of data derived from at least three independent experiments in triplicate.
[b]50% Cytotoxic Concentration, the compound concentration that results in 50% of cytotoxicity, as determined by MTT assays in HFF cells. Reported values represent the means ± the SD of data derived from at least three independent experiments performed in quadruplicate.
[c]SI, Selectivity Index (determined as CC_{50}/EC_{50}).

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Table 3. Comparison of the activity of BBR against AD169 and drug-resistant HCMV strains.

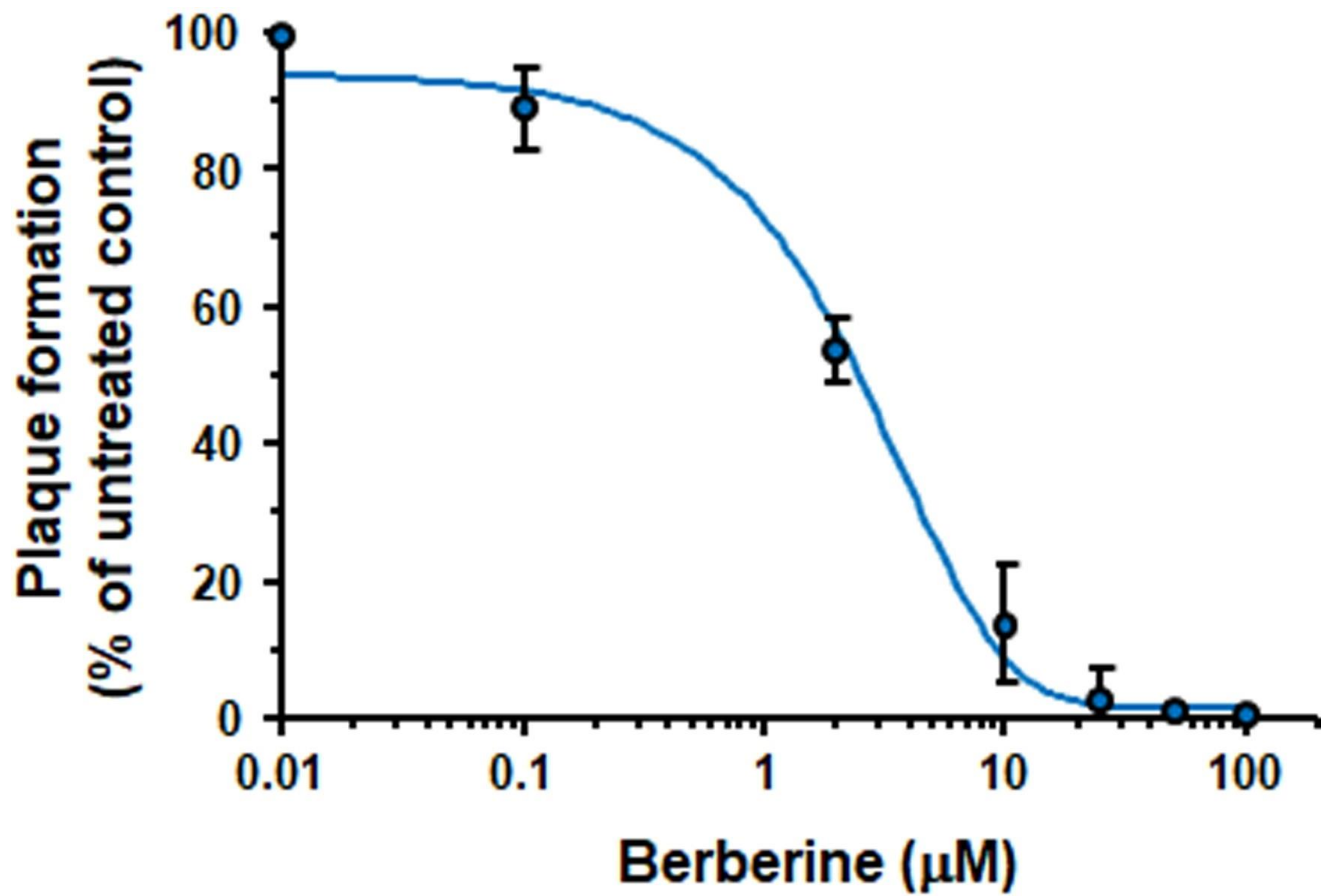
HCMV Strain	Drug resistance	BBR EC_{50}^{a} (μM)	Control[b] EC_{50} (μM)
AD169	None	2.35 ± 0.35	N.D.
GDG[r]P53	GCV, CDV	1.45 ± 0.08	75 ± 5
PFA[r]D100	FOS, ACV	1.40 ± 0.57	305 ± 18

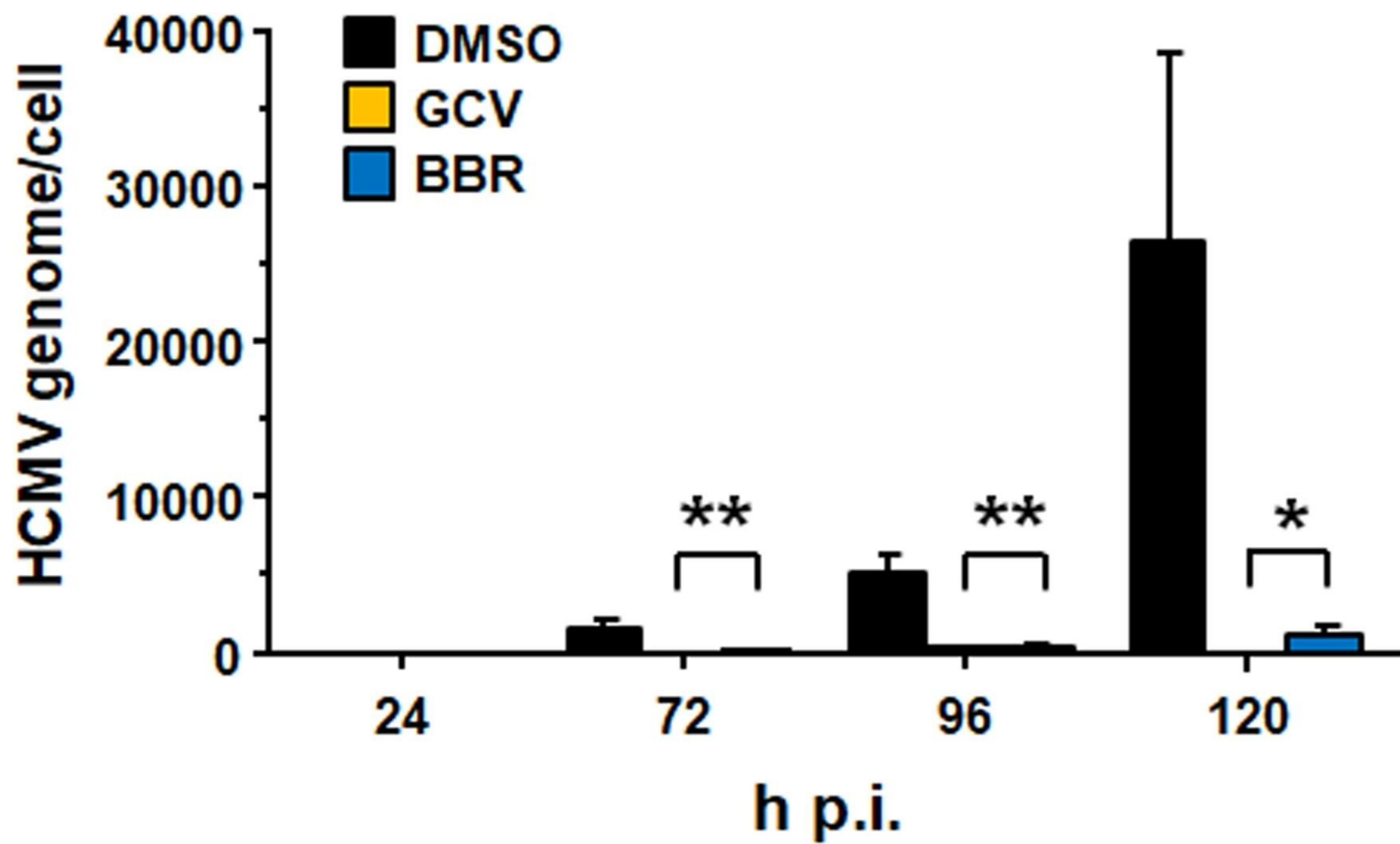
[a]50% Effective Concentration, the compound concentration that inhibits 50% of plaque formation, as determined by PRAs in HFF cells. Reported values represent the means ± the SD of data derived from at least three independent experiments in triplicate.
[b] GCV was used for GDG[r]P53 and FOS for PFA[r]D100.
N.D., not determined.

Table 4. Antiviral activity of BBR against MCMV.

Compound	EC_{50}^{a} (μM)	CC_{50}^{b} (μM)	SI[c]
BBR	1.95 ± 0.35	192 ± 12	98
CDV	0.53 ± 0.18	> 250	>472

[a]50% Effective Concentration, the compound concentration that inhibits 50% of plaque formation, as determined by PRAs against MCMV Smith in NIH 3T3 cells. Reported values represent the means ± the SD of data derived from three independent experiments in triplicate.
[b]50% Cytotoxic Concentration, the compound concentration that results in 50% of cytotoxicity, as determined by MTT assays in NIH 3T3 cells. Reported values represent the means ± the SD of data derived from at least three independent experiments performed in quadruplicate.
[c]SI, Selectivity Index (determined as CC_{50}/EC_{50}).


Plaque formation
(% of untreated control)
100
80
60
40
20
0
0.01
0.1
1
10
100
Berberine (µM)


DMSO
GCV
BBR
HCMV genome/cell
40000
30000
20000
10000
0
**
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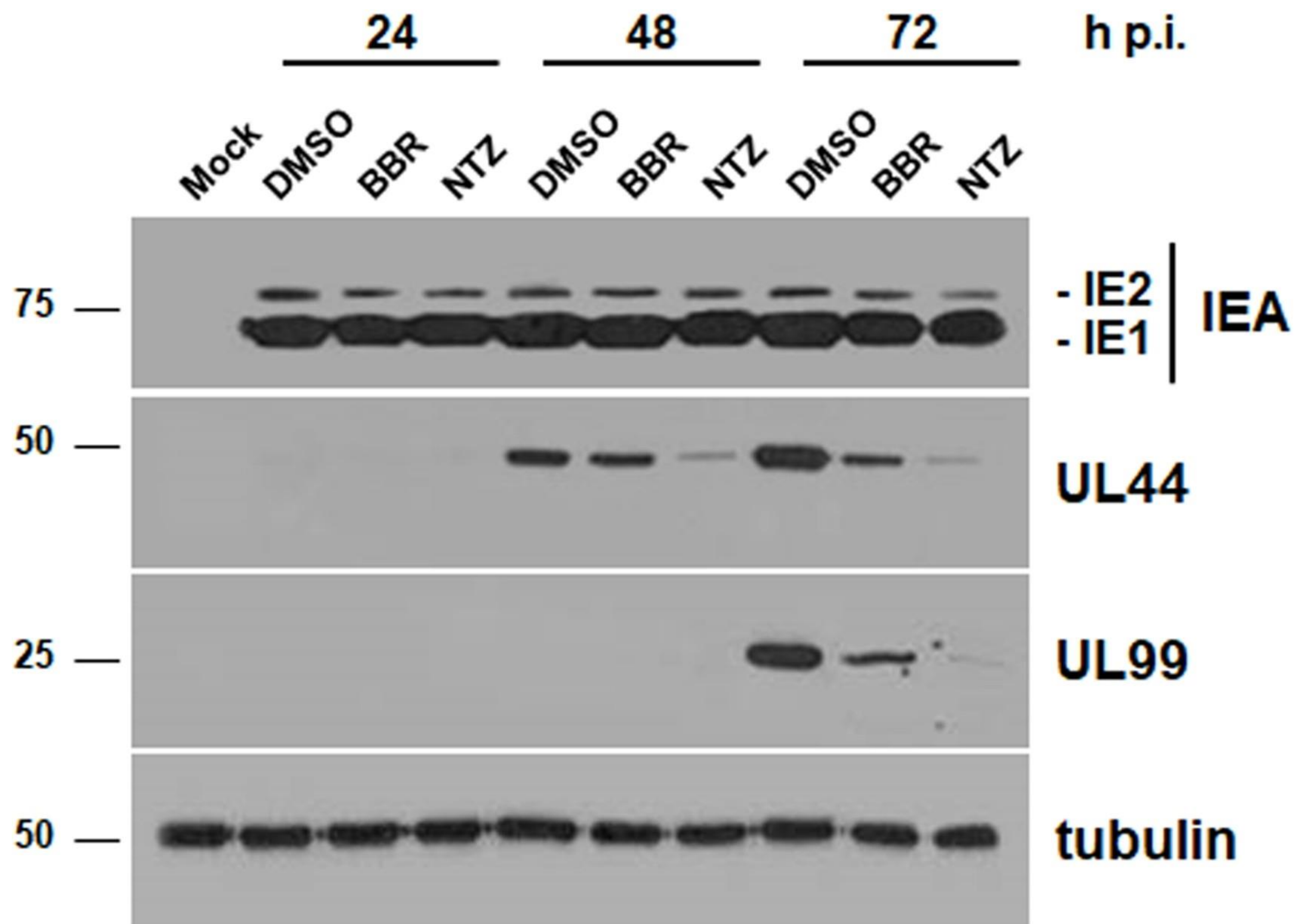
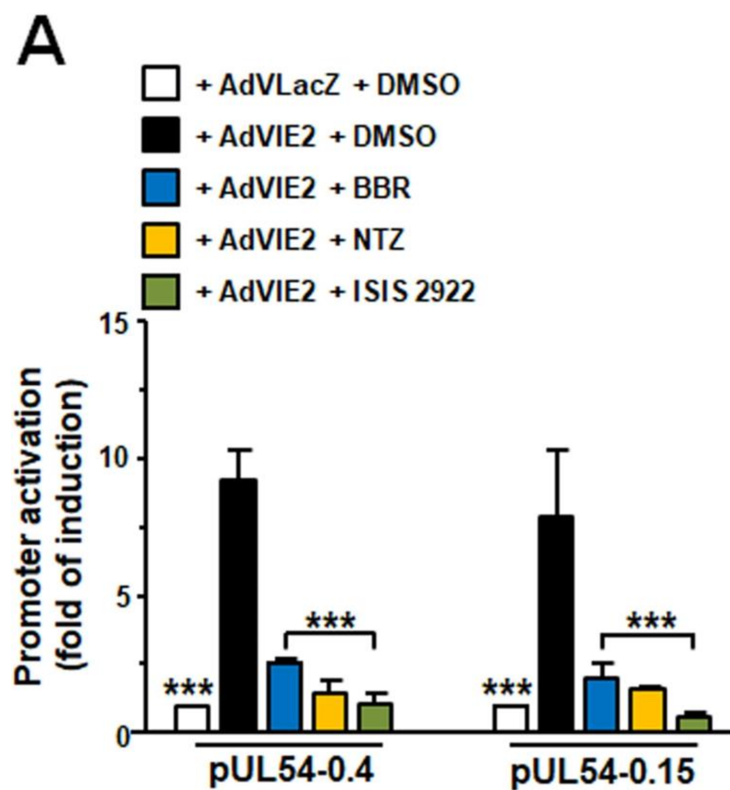
*
24
72
96
120
h p.i.



24
48
72
h p.i.
Mock
DMSO
BBR
NTZ
DMSO
BBR
NTZ
DMSO
BBR
NTZ
75
- IE2
- IE1
IEA
50
UL44
25
UL99
50
tubulin


A
+ AdVLacZ + DMSO
+ AdVIE2 + DMSO
+ AdVIE2 + BBR
+ AdVIE2 + NTZ
+ AdVIE2 + ISIS 2922
Promoter activation (fold of induction)
15
10
5
0
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***
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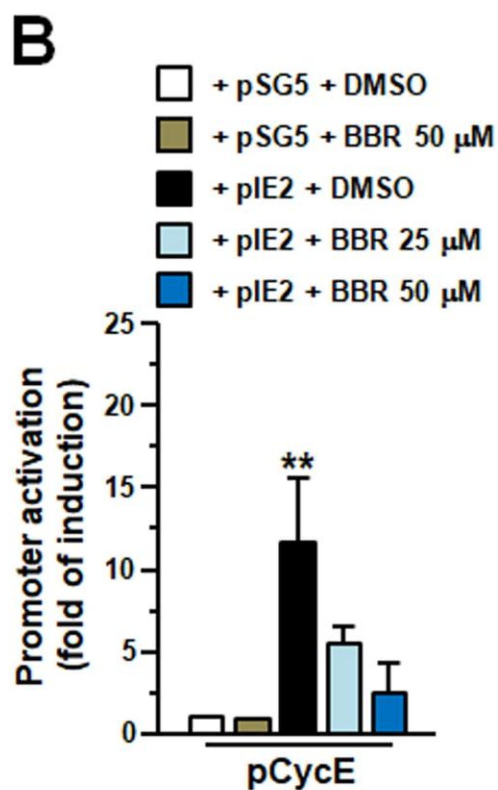
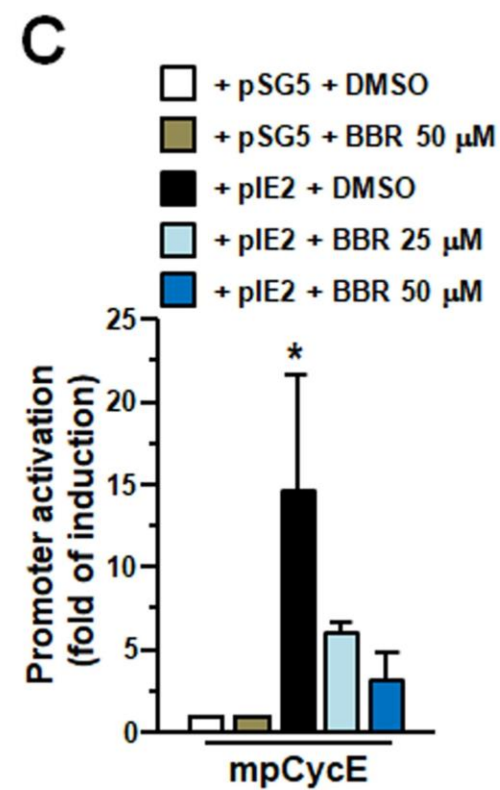
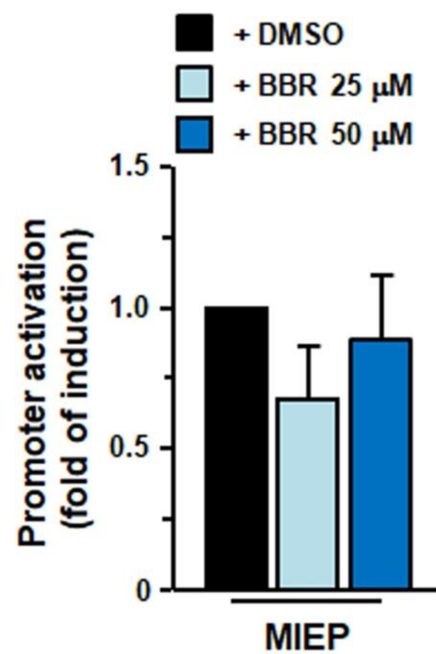
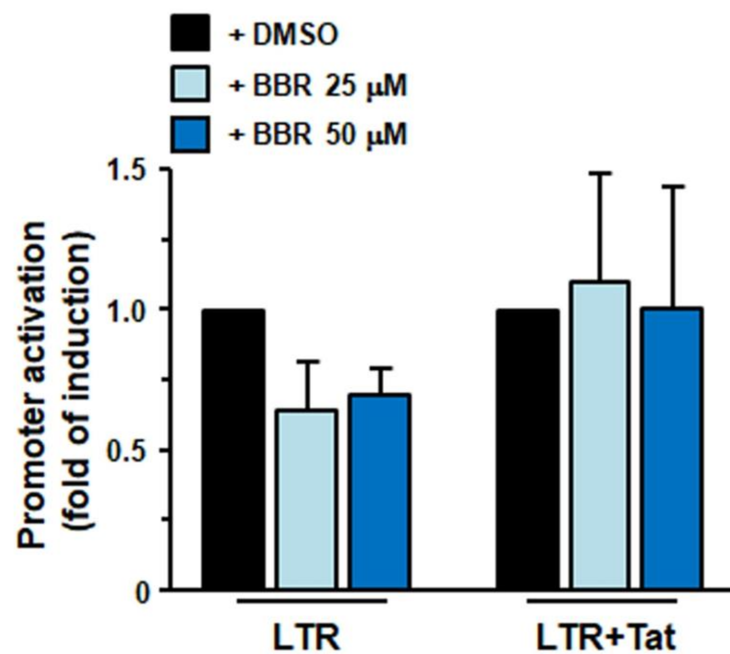
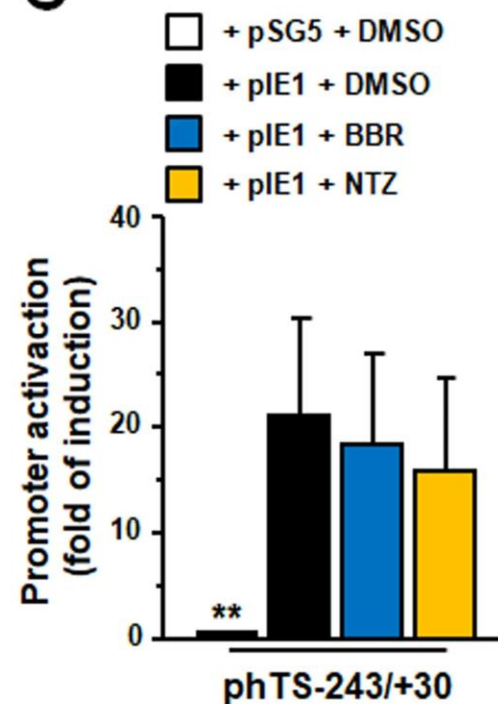
pUL54-0.4
pUL54-0.15

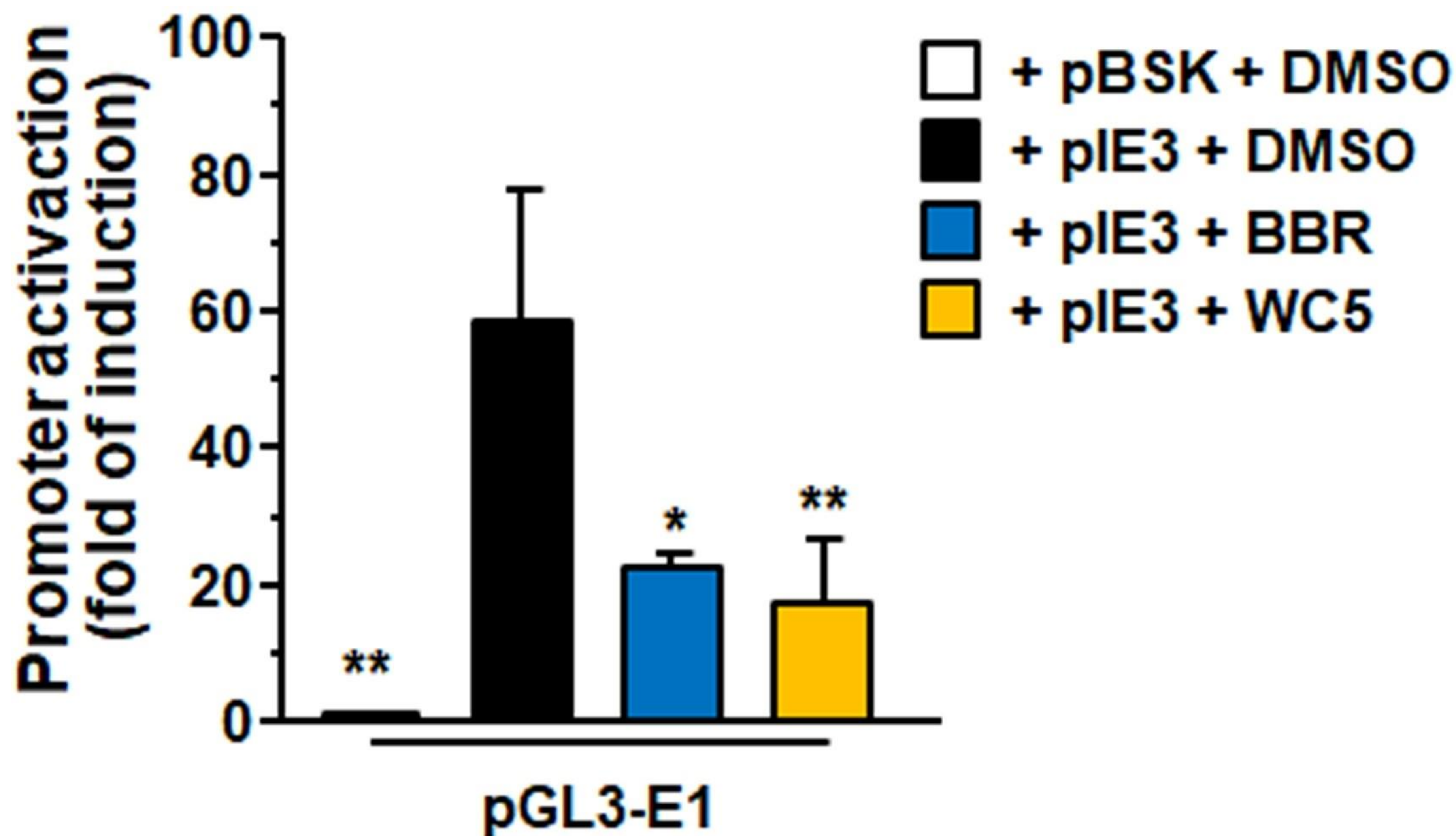

B
+ pSG5 + DMSO
+ pSG5 + BBR 50 μM
+ pIE2 + DMSO
+ pIE2 + BBR 25 μM
+ pIE2 + BBR 50 μM
Promoter activation (fold of induction)
25
20
15
10
5
0
**
pCycE


C
+ pSG5 + DMSO
+ pSG5 + BBR 50 μM
+ pIE2 + DMSO
+ pIE2 + BBR 25 μM
+ pIE2 + BBR 50 μM
Promoter activation (fold of induction)
25
20
15
10
5
0
*
mpCycE


A
+ DMSO
+ BBR 25 µM
+ BBR 50 µM
Promoter activation (fold of induction)
1.5
1.0
0.5
0
MIEP


B
+ DMSO
+ BBR 25 µM
+ BBR 50 µM
Promoter activation (fold of induction)
1.5
1.0
0.5
0
LTR
LTR+Tat


C
+ pSG5 + DMSO
+ pIE1 + DMSO
+ pIE1 + BBR
+ pIE1 + NTZ
Promoter activaction (fold of induction)
40
30
20
10
0
**
phTS-243/+30


Promoter activaction (fold of induction)
100
80
60
40
20
0
**
*
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pGL3-E1
+ pBSK + DMSO
+ pIE3 + DMSO
+ pIE3 + BBR
+ pIE3 + WC5