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Antiretroviral drugs do not interfere with bryostatin-mediated HIV-1 latency reversal

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ABSTRACT

Although an effective combination of antiretroviral therapy (cART) controls HIV-1 viraemia in infected patients, viral latency established soon after infection hinders HIV-1 eradication. It has been shown that bryostatin-1 (BRY) inhibits HIV-infection *in vitro* and reactivates the latent virus through the protein kinase C-NF-κB pathway. We determined the *in vitro* potential effect of BRY in combination with currently used antiretroviral drugs. BRY alone or in combination with maraviroc (MVC)/Atripla (ATP) was tested for its capacity to reactivate latent virus and inhibit new infections. JLTRG-R5 cells and two latent HIV-1-infected cell lines, J89GFP and THP89GFP, were used as latency models. To quantify HIV infection, the reporter cell line TZM-bl was used. We found that BRY reactivates HIV-1 even in combination with MVC or ATP. Antiretroviral combinations with BRY do not interfere with BRY activity (i.e., the reactivation of latently infected cells) or with the antiviral activity of antiretroviral drugs. In addition, BRY-mediated down-modulation of surface CD4 and CXCR4 was not affected when it was used in combination with other antiretrovirals, and no hyperactivation or high-proliferation effects were observed in primary T cells. Moreover, the BRY treatment was able to reactivate HIV-1 in CD4+ T cells from HIV-1-infected patients under cART. Thus, we propose the use of BRY to purge the viral reservoir and recommend its combination with current antiretroviral treatments.

1. Introduction

An effective combination of antiretroviral therapy (cART) successfully controls HIV-1 viraemia in most HIV-1 infected patients. Although an undetectable viral load is achieved in most treated patients, HIV-1 establishes a long-term infection in a small pool of memory CD4+ T cells, which contains an integrated but transcriptionally silent provirus (Alexaki et al., 2008; Chun et al., 1997; Shen and Siliciano, 2008). Moreover, cells belonging to the monocyte/macrophage lineage represent one of the persistent major reservoirs of the virus because they maintain a low level of HIV-1 replication (Kumar et al., 2014). The origin and clinical implications of persistent low levels of viraemia are uncertain. Some studies postulate that it might be the result of the release of virus from latently infected cells (Joos et al., 2008; Kieffer et al., 2004), whereas other studies suggest that it could arise from ongoing viral replication (Chun et al., 2010, 2005; Havlir et al., 2003; Sharkey et al., 2000).

Abbreviations: AIDS, acquired immunodeficiency syndrome; ATP, Atripla®; BRY, bryostatin-1; cART, combination of antiretroviral therapy; DMSO, dimethyl sulfoxide; EFV, efavirenz; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FDA, food and drug administration; FTC, emtricitabine; GFP, green fluorescent protein; HDACi, histone deacetylase inhibitors; HIV-1, human immunodeficiency virus-1; IL-2, interleukin-2; iMFI, integrated mean fluorescence intensity; LRAs, latency reversal agents; LTR, long terminal repeat; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MVC, maraviroc; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PKC, protein kinase C; SEM, standard error of the mean; TFV DF, tenofovir disoproxil fumarate; TNF, tumor necrosis factor.

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Recently, several strategies have been developed to purge HIV-1 reservoirs, and the use of therapies with small molecules targeting HIV-1 reservoirs is one of the major challenges in the fight against AIDS. Clinical trials with cART intensification have failed to eliminate viral replication, which further supports the hypothesis that low levels of viraemia are produced by latently infected cells (Buzon et al., 2010; Gandhi et al., 2010; Gutierrez et al., 2011; McMahon et al., 2010; Vallejo et al., 2012). Therefore, it might be possible to reactivate the latent virus with a therapeutic approach known as "shock and kill" (Katlama et al., 2013).

A wide range of small molecules, such as disulfiram (Xing et al., 2011), HDAC inhibitors (HDACi); (Archin et al., 2012; Shirakawa et al., 2013; Siliciano et al., 2007) and protein kinase C (PKC) activators such as ingenols (Warrilow et al., 2006), prostratin (Kulkosky et al., 2001), 1,2-diacylglycerol analogs (Hamer et al., 2003), and bryostatin-1 (BRY); (del Real et al., 2004; Mehla et al., 2010), have been proposed as agents to reactivate HIV-1 and eradicate the pool of latently HIV-infected CD4+ T cells (Kulkosky and Bray, 2006). Several clinical trials are currently ongoing to evaluate the effectiveness of these compounds to hit HIV-1 reservoirs. The results of a clinical trial with disulfiram did not show a reduction in the size of the latent reservoir (Spivak et al., 2014). However, an *ex vivo* approach that evaluated the effectiveness of potential candidates, including BRY and HDACi, revealed that BRY was the best candidate to reactivate HIV-1 from latency (Bullen et al., 2014). Therefore, PKC activators, alone or in combination with HDACi, are of particular relevance for purging HIV-1 reservoirs in patients. For instance, the synergistic effect of BRY with HDACi to antagonize the HIV-1 latency has been shown *in vitro* (Perez et al., 2010).

Before clinical trials can be conducted on ART intensification with BRY to assess its impact on the size of the HIV-1 latent reservoirs, the potential effect of BRY in combination with antiretrovirals should be determined *in vitro*. In this study, maraviroc (MVC) as a monotherapy regimen and a fixed-dose combination of emtricitabine, tenofovir and efavirenz (Atripla®, ATP) as a full cART regimen were tested in combination with BRY at two levels. Using different *in vitro* models of latency and an *ex vivo* model, we demonstrated the viral reactivation from latent reservoirs and the inhibition of acute infection.

2. Materials and methods

2.1. Cell lines and culture

JLTRG-R5 (Division of AIDS, NIAID, NIH. Catalog number 11586; Dr. Olaf Kutsch), J89GFP and THP89GFP cells (kindly donated by Dr. David N Levy, NYU, USA) were maintained according to the protocol described by Kutsch et al. (2002). ACH-2 and J1.1 cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Thomas Folks (Clouse et al., 1989; Folks et al., 1989; Perez et al., 1991). Buffy coats from healthy subjects were obtained from the Madrid Transfusion Center. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by a ficoll-paque density gradient (Garcia-Merino et al., 2009) and maintained in complete RPMI supplemented with 30 U/ml IL-2 and, when indicated, activated for 3 days with 1 μg/ml phytohemagglutinin (PHA; Murex Biotech, England, UK). TZM-bl (Division of AIDS, NIAID, NIH, Catalog number 8129; Dr. John C. Kappes) and HEK 293T cells (ATCC number CRL-11268, Rockefeller University, USA) were cultured according to the manufacturers' instructions.

2.2. Reagents

Anti-human CD3-PC5, anti-human CD4-PC7, anti-human CXCR4-APC, anti-human CCR5-PE, anti-human CD38-FITC and anti-human HLA-DR-ECD were obtained from BD Biosciences Pharmingen (San Diego, CA, USA). BRY was obtained from Sigma–Aldrich (St. Louis, MO, USA), maraviroc (MVC) was obtained as a clinical formulation in a 150 mg tablet (Selzentry, Pfizer Labs, UK), and Atripla® (ATP) was obtained as a clinical formulation in a 1100-mg tablet (600 mg EFV, 200 mg FTC, 300 mg TFV DF; Gilead Sciences, CA, USA). MVC and ATP were freshly dissolved in distilled water with DMSO (Sigma–Aldrich, St. Louis, MO, USA) and sterile-filtered. ATP is not a chemical entity per se, so the theoretical molecular weight of 1198.44 g/mol was calculated based on the molecular weight of each component and the relative proportion of the single drugs. Hence, 1 mM of ATP corresponds to 2.07 mM of EFV, 0.87 mM of FTC, and 0.49 mM of TFV DF. The concentration of DMSO in cell cultures was less than 0.001%. TNF-α was purchased from R&D Systems (Minneapolis, Minn.).

2.3. Cell viability assays

The concentration range of each compound examined in this study is in agreement with previously published results (Arberas et al., 2013; Bousquet et al., 2009; Perez et al., 2010). The toxicity of compounds was measured by an MTT assay (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. 0.001% DMSO treated cells were included in each experiment as vehicle control (DMSO+); DMSO 10% (DMSO+++) was used as positive control of cytotoxicity.

2.4. Analysis of surface marker expression

Cells were stained for 1 h at 4 °C with the corresponding conjugated antibodies in a FACS staining buffer (phosphate-buffered saline, PBS, with 2% FBS) and analyzed in a Gallios flow-cytometer (Beckman-Coulter, CA, USA). At least 20,000 CD3+ cells, 50,000 JLTRG-R5 or 20,000 J89GFP and THP89GFP cells were collected for each sample and analyzed with the Kaluza software (Beckman-Coulter, CA, USA).

2.5. HIV-1 LTR reactivation

JLTRG-R5 cells were stimulated with the indicated compounds, and a GFP-fluorescence pattern was determined 24 h later. The percentage of GFP expressing cells was used as a measure of HIV-1 LTR activation.

2.6. Latent HIV-1 reactivation

To determine the viral reactivation, the EGFP-fluorescence pattern was measured by flow cytometry and HIVp24Gag release measured by ELISA (INNOTEST® HIV-Antigen mAb, Innogenetics, Belgium).

2.7. Latent HIV-1 reactivation in CD4+ T cells from infected individuals under cART

Fresh blood from HIV-1-infected individuals ($n = 3$) was obtained in accordance with protocols approved by the Hospital Ramón y Cajal Ethical Committee (Clinical characteristics depicted in Table S1). The participants signed an informed consent form. CD4+ T cells were isolated from PBMCs using the CD4+ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch-Gladbach, Germany) and cultured under different conditions. To measure the HIV-RNA levels, the supernatants were analyzed by a robotic COBAS AmpliPrep/TaqMan system (Roche Diagnostics, Indianapolis, IN, USA). The limit of detection in this assay was 50 copies/ml.