EFdA, a Reverse Transcriptase Inhibitor, Potently Blocks HIV-1 *Ex Vivo* Infection of Langerhans Cells within Epithelium

Journal of Investigative Dermatology (2014) **134,** 1158–1161; doi:10.1038/jid.2013.467; published online 2 January 2014

TO THE EDITOR

Despite increasing access to antiretroviral drugs, sexual transmission of HIV-1 remains a significant public health threat. A recent clinical trial, CAPRISA 004, of a vaginally administered microbicide using a nucleoside reverse transcriptase inhibitor (NRTI), tenofovir (TDF), has demonstrated that 1% TDF gel reduced HIV-1 acquisition by an estimated 39% overall (Abdool Karim *et al.*, 2010), indicating a potential utility of NRTI-based microbicides. In the VOICE study, however, a once-daily dosing regimen with TDF gel failed to demonstrate protective effects in at-risk women. These studies demonstrate the need to develop additional more potent microbicide candidates to potentially increase the activity to protect women from HIV-1 transmission.

We previously reported that a series of 4′-substituted NRTIs have excellent antiviral properties (Ohrui, 2006), and through optimization of such 4′-substituted NRTIs, 4′-ethynyl-2-fluoro-2′-deoxyadenosine (EFdA) was found to exert extremely potent activity against a wide spectrum of HIV-1 strains including highly multidrug-resistant clinical HIV-1 isolates, with favorable *in vitro* cell toxicities (Nakata *et al.*, 2007; Ohrui *et al.*, 2007). EFdA inhibited HIV-1 replication in activated peripheral blood mononuclear cells with an EC_{50} of 0.05 nM, a potency several orders of magnitude greater than any of the current clinically available NRTIs (Michailidis *et al.*, 2009). As the prevalence of new infections with drug-resistant HIV-1 variants could increase in the coming years (Nichols *et al.*, 2011), EFdA may be useful as a topical microbicide.

Langerhans cells (LCs) are dendritic cells located, among other sites, within genital skin and mucosal epithelium (Lederman *et al.*, 2006). In female rhesus macaques exposed intravaginally to simian immunodeficiency virus, up to 90% of initially infected target cells were LCs (Hu *et al.*, 2000). *Ex vivo* experiments with human foreskin explants show that epidermal LCs in inner foreskin are primary target cells for HIV-1 infection, providing a plausible explanation for why circumcision greatly reduces the probability of acquiring HIV-1 (Ganor *et al.*, 2010; Zhou *et al.*, 2011). LCs also express CD4 and CCR5, but not CXCR4, and demonstrate the distinctive characteristics of emigrating from tissue to draining lymph nodes in order to interact with T cells following contact with pathogens (Lederman *et al.*, 2006). Indeed, epidermal LCs are readily infected *ex vivo* with R5-HIV-1, but not with X4-HIV-1, and initiate and promote high levels of infection upon interactions with cocultured $CD4^+$ T cells (Kawamura *et al.*, 2000; Ogawa *et al.*, 2009, 2013), consistent with previous epidemiologic observations that the majority of HIV-1 strains isolated from newly infected patients are R5-HIV-1 strains (Zhu *et al.*, 1993). Thus, LCs likely have an important role in disseminating HIV-1 soon after exposure to the virus.

To understand how HIV-1 traverses skin and genital mucosa, an *ex vivo* model was developed in which resident LCs within epithelial tissue explants obtained from suction blisters are exposed to HIV-1 and then allowed to emigrate from the tissue, thus mimicking conditions that occur following mucosal exposure to HIV (Kawamura *et al.*, 2000; Ogawa *et al.*, 2009, 2013). In this model, although relatively few productively infected LCs are identified, these cells induce high levels of HIV-1 infection when cocultured with resting autologous $CD4^+$ T cells (Kawamura *et al.*, 2000; Ogawa *et al.*, 2013). As expected, when epidermal tissue explants were pretreated with various concentrations of TDF, EFdA, and CCR5 inhibitor, maraviroc (MVC), prior to R5-tropic $HIV\text{-}1_{Ba\text{-}L}$ exposure, HIV-1 infection of resident LCs within epidermis as well as subsequent virus transmission from emigrated LCs to cocultured $CD4^+$ T cells was decreased in a dose-dependent manner (Figure 1a and c; for detailed methods, see Supplementary Material). The blocking was confirmed by repeated experiments using skin explants from three additional randomly selected individuals (Figure 1b and d). Strikingly, although the blocking efficiency of TDF or MVC even at 5,000 nM was partial, EFdA demonstrated complete blocking of R5-HIV-1 replication in LCs as well as subsequent virus transmission from emigrated LCs to $CD4^+$ T cells at doses of 100–5,000 nM (Figure 1a–d). Furthermore, EFdA blocked *ex vivo* virus infection of LCs as well as subsequent virus transmission when two strains of R5-HIV-1, $HIV\text{-}1_{JR\text{-}FL}$ and $HIV\text{-}1_{AD8}$, were utilized in experiments ($n=3$, Supplementary Figure S1 online).

Similar to the results in epidermal LCs, preincubation of monocyte-derived LCs (mLCs) with 100–5,000 nM of EFdA completely blocked HIV-1 replication in mLCs as well as subsequent virus transmission from mLCs to cocultured $CD4^+$ T cells, whereas both TDF and MVC at the same doses only partially inhibited the transmission (Figure 2a and b; for detailed methods, see Supplementary Material online).

Abbreviations: EFdA, 4′-ethynyl-2-fluoro-2′-deoxyadenosine; LC, Langerhans cell; mLC, monocyte-derived LC; MVC, maraviroc; NRTI, nucleoside reverse transcriptase inhibitor; TDF, tenofovir

Accepted article preview online 11 November 2013; published online 2 January 2014

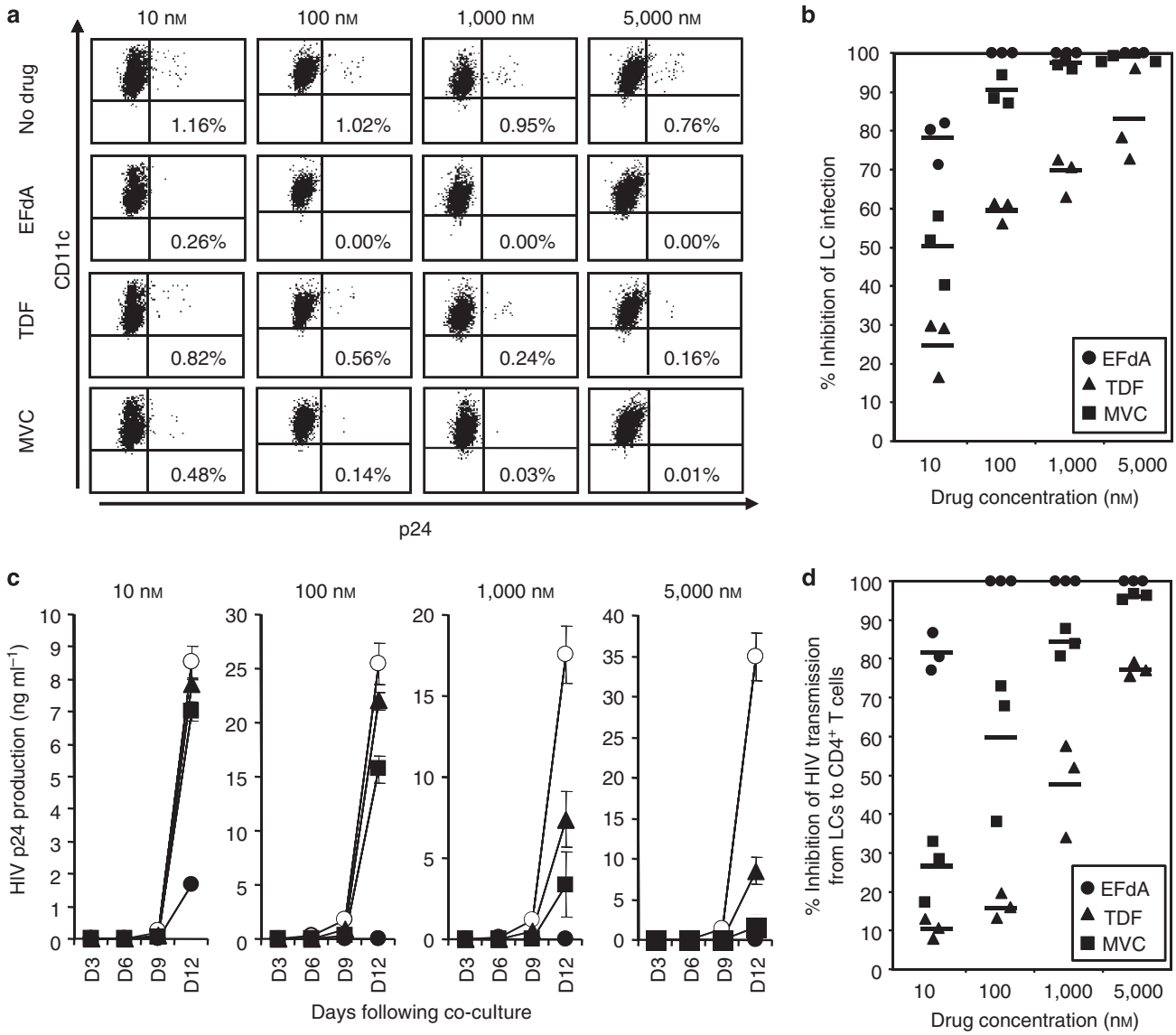

Figure 1. Preincubation of skin explants with EFdA blocks R5-HIV-1 infection in LCs and subsequent virus transmission to cocultured CD4$^+$ T cells. LCs within skin explants were preincubated with no drug (○) or the indicated concentrations of EFdA (●), TDF (▲), and MVC (■) for 30 minutes, exposed to HIV-1_{Ba-L} for 2 hours, and then floated on culture medium to allow migration of LCs from the explants. Emigrating cells from the epidermal sheets were collected 3 days following HIV-1 exposure. HIV-1-infected LCs were assessed by HIV-1 p24 intracellular staining in langerin$^+$ CD11c$^+$ LCs (**a**, **b**), or further cocultured with autologous CD4$^+$ T cells and culture supernatants were assessed for p24 content by ELISA on the indicated days (**c**, **d**). Summary of percent inhibition of LC infection (**b**) and virus transmission to CD4$^+$ T cells (**d**) of 12 experiments using skin explants from 12 individuals with the indicated each concentration of EFdA (●), TDF (▲), and MVC (■) are shown. Mean values obtained from different donors are shown as horizontal marks (**b**, **d**). EFdA, 4′-ethynyl-2-fluoro-2′-deoxyadenosine; LCs, Langerhans cells; MVC, maraviroc; TDF, tenofovir.

Intriguingly, even in 1–3 days following the removal of EFdA (1,000 nM), EFdA completely blocked HIV-1 infection of mLCs as well as subsequent virus transmission from mLCs to cocultured CD4$^+$ T cells, whereas TDF and MVC rapidly lost their anti-HIV-1 activity within days (Figure 2c–f). No cellular toxicity was noted for any of these drugs at the doses used in these experiments (Supplementary Figure S2 online). When similar experiments were conducted using peripheral blood mononuclear cell as target cells, virtually identical favorable persistency of EFdA in antiviral activity compared with that of TDF was observed (data not shown).

In the present work, we demonstrated that EFdA exerted extremely more potent anti-HIV-1 activity in LCs than did TDF and MVC, and the potent anti-HIV-1 activity of EFdA persisted for at least 3 days. Of note, the efficacy of TDF gel in CAPRISA 004 has been linked to its long intracellular half-life (Abdool Karim *et al.*, 2010; Rohan *et al.*, 2010). Our data strongly indicate that EFdA may serve as a promising microbicide to block sexual transmission of HIV-1 because of its potent anti-HIV-1 activity, low cytotoxicity, and superior

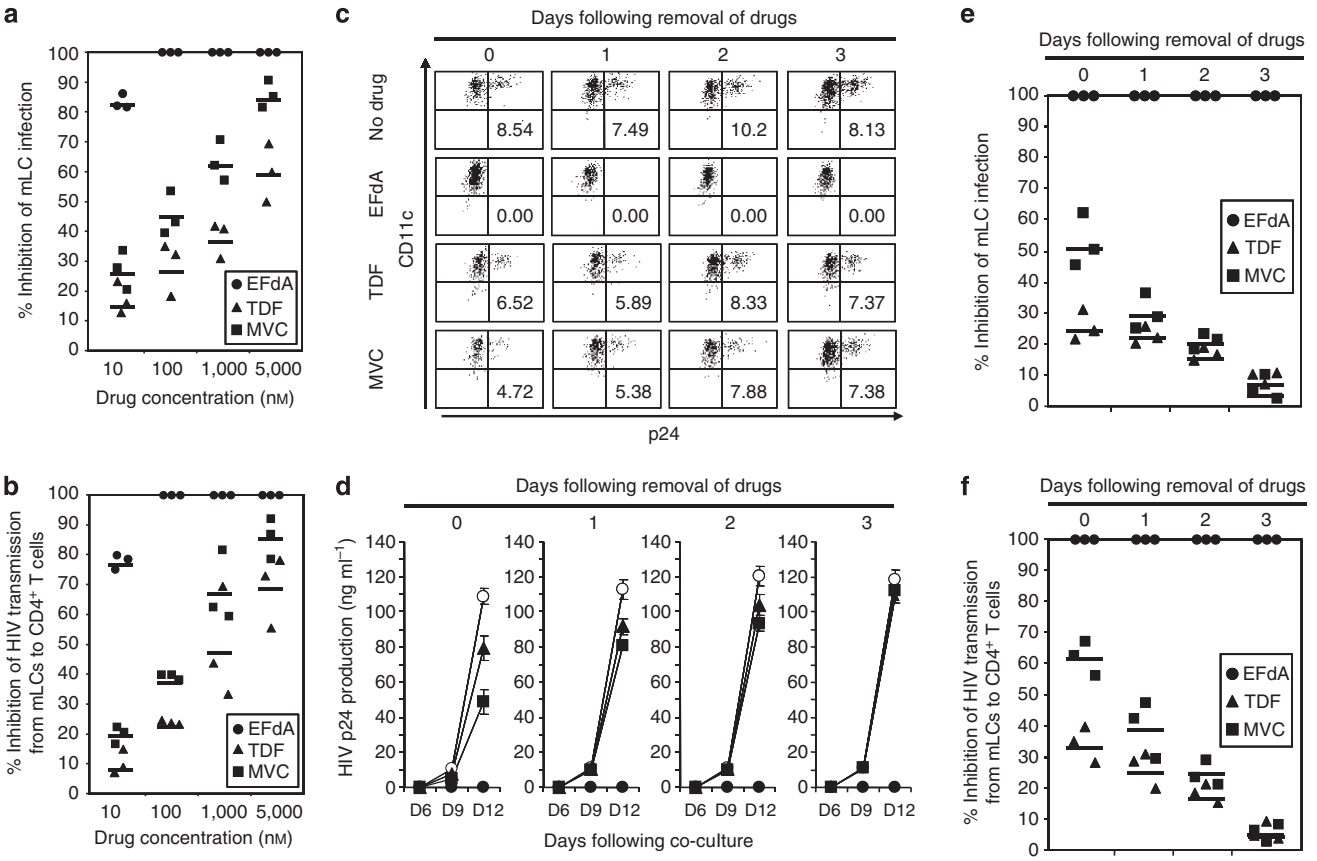

Figure 2. Preincubation of skin explants with EFdA blocks subsequent R5-HIV-1 infection in LC in a dose-dependent manner. mLCs were preincubated with no drug (○) or the indicated concentrations of EFdA (●), TDF (▲) and MVC (■) for 30 minutes, and then immediately exposed to HIV-1Ba-L for 2 hours (**a**, **b**), or thoroughly washed to remove the extracellular drug and further cultured for 1, 2, or 3 days prior to exposure to HIV-1Ba-L for 2 hours (**c**–**f**). After 7 days of HIV-1 exposure, HIV-1-infected mLCs were assessed by HIV-1 p24 intracellular staining in langerin$^+$ CD11c$^+$ mLCs (**a**, **c**, **e**), or further cocultured with autologous CD4$^+$ T cells and culture supernatants were assessed for p24 content by ELISA on the indicated days (**b**, **d**, **f**). Summary of percent inhibition of mLC infection (**a**, **e**) and virus transmission to CD4$^+$ T cells (**b**, **f**) of three independent experiments are shown. Mean values are shown as horizontal marks (**a**, **b**, **e**, **f**). EFdA, 4′-ethynyl-2-fluoro-2′-deoxyadenosine; LCs, Langerhans cells; mLCs, monocyte-derived LCs; MVC, maraviroc; TDF, tenofovir.

persistence of antiviral activity against HIV-1 in LCs.

CONFLICT OF INTEREST

HM is among coinventors on a patent for EFdA; all rights, title, and interest to the patent have been assigned to Yamasa Corporation, Chiba, Japan. The other authors state no conflict of interest.

ACKNOWLEDGMENTS

Financial support was provided by the Ministry of Health Science of the Japanese Government (201029002), the Intramural Research Program of Center for Cancer Research, National Cancer Institute, National Institutes of Health, and a grant for global education and research center aiming at the control of AIDS (Global Center of Excellence supported by Monbu-Kagakusho).

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Suppression of miR135b Increases the Proliferative Potential of Normal Human Keratinocytes

Journal of Investigative Dermatology (2014) **134,** 1161–1164; doi:10.1038/jid.2013.427; published online 14 November 2013

TO THE EDITOR

Cell fate is regulated by the activation and repression of specific genes (Sonkoly *et al.*, 2007), and microRNAs (miRNAs) are a class of posttranslational regulators of gene expression.

Psoriasis is a hyperproliferative skin disorder (Schneider, 2012) and it has been reported that the stratified epidermis expresses miR135b (Joyce *et al.*, 2011), which suggests that miR135b is closely related to epidermal keratinization. Previously, we isolated three populations of epidermal cells that differed according to their ability to adhere to type IV collagen (Kim *et al.*, 2004). Rapidly adhering (RA) cells, which are considered to be epidermal stem cells, express high levels of α_6 integrin and low levels of CD71. In contrast, slowly adhering (SA) cells express low levels of α_6 integrin and high levels of CD71 (Kim *et al.*, 2004). Type IV collagen-coated dishes were prepared (CellmatrixType IV, Nitta Gelatin, Osaka, Japan) and a subpopulation of cells able to adhere within 10 minutes at 37 °C was selected (RA cells). Thereafter, non-adherent cells were incubated for another 24 hours, and, among them, slowly adhering cells were selected (SA cells). Total RNAs were prepared and real-time reverse-transcriptase–PCR (RT–PCR) analysis was performed. Levels of miR135b were significantly higher in SA cells (three different cell lines from three volunteers, Supplementary Figure S1 online). To test the effects of miR135b, cultured keratinocytes were transfected with miR135b mimic (30 nM, AM17100, PM13044, Ambion, Austin, TX) or mock (as negative controls, 30 nM, AM17110, Ambion). Results showed that miR135b mimic – transfected cells showed abnormal changes compared with mock-transfected cells (Supplementary Figure S2 online). These findings suggested that miR135b initially induced early differentiation of keratinocytes. Therefore, we suppressed miR135b to clarify whether inhibition of miR135b might target and delay differentiation of keratinocytes. The transfection was performed with an anti-miRNA inhibitor (ib-miR135b, AM17000, AM13044, Ambion) designed for hsa-miR135b or a negative control (AM17010, Ambion) at a final concentration of 30 nM according to the manufacturer's instruction. To check transfection efficiency, an FAM-labeled miRNA was transfected and the results showed successful transfection (Supplementary Figure S3 online). Thereafter, the transfected FAM-labeled miRNA was not observed at day 7 after transfection (Supplementary Figure S3 online), which means that the transfected miRNA persisted only for a few days. At every passage, transfection was repeatedly performed, and portions of cells were collected for cell counting, RNA extraction, and protein extraction. RT–PCR analysis showed that transfection of ib-miR135b effectively suppresses miR135b (Figure 1a). Cumulative cell numbers showed a large difference (Figure 1b). A colony assay showed that ib-miR135b-transfected cells showed a higher colony-forming ability than mock-transfected cells (Supplementary Figure S4 online). At the 9th passage, ib-miR135b-transfected cells reached $\sim 5 \times 10^{10}$ cells (Figure 1b). However, mock-transfected cells increased to only 1.6×10^{10} cells (Figure 1b). In addition, mock-transfected cells showed large vacuoles earlier than ib-miR135b-transfected cells (Figure 1b, arrow). Large vacuoles frequently appeared in the cytoplasm, especially in late passages, during the culture of keratinocytes (personal observation). However, these findings are not described in detail in the literature. The vacuolar alteration has

Abbreviations: miRNA, microRNA; RA, rapidly adhering; SA, slowly adhering

Accepted article preview online 15 October 2013; published online 14 November 2013