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HIV-1 Latency-Reversing Agents Prostratin and Bryostatin-1 Induce Blood–Brain Barrier Disruption/Inflammation and Modulate Leukocyte Adhesion/Transmigration

Clélia Dental,* Alizé Proust,* Michel Ouellet,* Corinne Barat,* and Michel J. Tremblay*†

A shock-and-kill approach involving the simultaneous treatment of HIV-1–infected patients with latency-reversing agents (LRAs) and combination antiretroviral therapy was proposed as a means to eradicate viral reservoirs. Currently available LRAs cannot discriminate between HIV-1–infected and uninfected cells. Therefore, the risks and benefits of using broad-spectrum LRAs need to be carefully evaluated, particularly in the CNS, where inflammation and leukocyte transmigration must be tightly regulated. We used a real-time impedance-sensing system to dynamically record the impact of different classes of LRAs on the integrity of tight monolayers of the immortalized human cerebral microvascular endothelial cell line hCMEC/D3. Results show that prostratin and bryostatin-1 can significantly damage the integrity of an endothelial monolayer. Moreover, prostratin and bryostatin-1 induce secretion of some proinflammatory cytokines and an increase of ICAM-1 expression. Additional studies demonstrated that prostratin and bryostatin-1 also affect adhesion and transmigration of CD4+ and CD8+ T cells as well as monocytes in an in vitro human blood–brain barrier (BBB) model. Prostratin and bryostatin-1 could thus be considered as potent regulators of BBB permeability and inflammation that influence leukocyte transport across the BBB. Altogether, these findings contribute to a better understanding of the potential risks and benefits of using a shock-and-kill approach with LRAs on the normal physiological functions of the BBB. The Journal of Immunology, 2017, 198: 1229–1241.

Despite great advances this past decade, current combination antiretroviral therapy (cART) regimens fail to completely eliminate HIV-1 in anatomical sanctuaries where drug concentrations are not fully suppressive and in latently infected cells where expression of the integrated provirus is limited. The difficulty of efficient delivery of cART to the CNS contributes to HIV-1 persistence in actively producing and long-lived latently infected cells, such as perivascular macrophages, microglia, and astrocytes. In the long term, even in patients in whom cART successfully reduces plasma viremia to less than detectable levels, cumulative injuries to the brain lead to HIV-1–associated neurocognitive disorders in up to 50% of patients and, in the more severe cases, to the development of HIV-1–associated dementia. Various strategies were recently developed and tested to enhance the delivery of antiretroviral drugs through the highly restrictive blood–brain barrier (BBB). These include a new delivery system of antiretroviral therapies (1–3), produgs with improved compartment distribution properties (4), and inhibitors of influx and efflux transporters (5).

Although these strategies aim to increase CNS drug concentrations and further limit active replication in the brain, these approaches do not target integrated silent provirus. To solve this serious limitation of cART, a shock-and-kill approach was used (6). This therapeutic strategy involves the use of transcriptional activators that turn on the expression of HIV-1 and, therefore, reactivate viral production in latently infected cells (shock). Cells expressing viral proteins could then be recognized and cleared by the immune system, whereas further infections by de novo virions would be inhibited by cART (kill). Many HIV-1 latency-reversing agents (LRAs) were tested for their capacity to reverse latency in vitro such as 2-(hexahydro-4-methyl-1H-1,4-diazepin-1-yl)-6,7-dimethoxy-N-[1-phenylmethyl]-4-piperidinyl]-4-quinazolinamine trihydrochloride hydrate (BIX-01294) (7, 8), 5-aza-2’-deoxycytidine (5-aza-dC) (9), suberanilohydroxamic acid (SAHA; also called vorinostat) (10, 11), 4-hydroxy-3-methoxybenzoic acid (hexamethylene bisacetamide [HMBA]) (12), prostratin (13), and bryostatin-1 (14). Furthermore, certain combinations of LRAs show synergistic effects in some experimental cell models (15–19).

None of these drugs, however, can specifically target latently HIV-1–infected cells, and their mode of action could potentially activate transcription of many host genes, including proinflammatory factors, in uninfected bystander cells. Their use must therefore be carefully evaluated, particularly in the CNS, where inflammation must be tightly regulated because it is now recognized as a dominant contributor to diverse CNS disorders.

In this study, a real-time electric impedance-sensing system was used to dynamically record the possible impacts of six LRAs from different classes on the hCMEC/D3 endothelial monolayer resistance (as a measure of the integrity and permeability of the monolayer). This well-characterized brain microvascular endothelial cell line of human origin recapitulates in vitro most properties of the BBB (20). To assess the effect of LRAs on transmigration of leukocytes through the BBB, we used a model of indirect coculture of hCMEC/D3 monolayers and human fetal astrocytes (HFA)s on cell culture inserts. Results indicate that, apart from 5-aza-dC, all tested LRAs induced a dose-dependent breakdown of the endothelial integrity. Interestingly, prostratin and bryostatin-1 further showed a strong induction of BBB breakdown and inflammation because they promote secretion of some proinflammatory cytokines, ICAM-1 surface expression, and an opening of the endothelial barrier while being noncytotoxic. Prostratin and bryostatin-1 also influence attachment of both CD4+ and CD8+ T cells, as well as monocytes, to the endothelial barrier, an essential step leading to their eventual transmigration. Taken together, these data suggest that prostratin and bryostatin-1 exert modulatory effects on BBB integrity and leukocyte transmigration, which might magnify neurological complications seen in the context of HIV-1 infection despite cART.

Materials and Methods

Study approval

This study was approved by the Bioethics Committee at the Centre Hospitalier Universitaire de Québec-Université Laval (Pavillon du Centre Hospitalier de l’Université Laval). Human peripheral blood mononuclear cells (PBMCs) were obtained from anonymous healthy volunteer donors who were specifically solicited for the donation of these samples. Written informed consent was obtained from each participant before inclusion in this study.

Reagents

5-Aza-dC (2′-deoxy-5-azacytidine), BIX-01294 (2-[hexahydro-4-methyl-1H-1,4-diazepin-1-yl]-6,7-dimethoxy-N-[1-(phenylmethyl)-4-piperidinyl]-4-quinazolinamine trihydrochloride hydrate), suberoyanilide hydroxamic acid, N,N′-hexamethylene bis(acetamide), bryostatin-1, and DMSO were purchased from Sigma-Aldrich (Oakville, ON). Prostratin was obtained from LC Laboratories (Woburn, MA).

Cell culture

The hCMEC/D3 cell line was a generous gift from Dr. Couraud (Institut Cochin, Paris, France) under the license from the INSERM (Paris, France). This immortalized human cerebral microvascular endothelial cell line had been shown to constitutively express many endothelial and/or BBB markers with the appropriate subcellular localization pattern and was maintained in culture as described previously (21, 22). In brief, hCMEC/D3 cells were seeded in 96-well plates coated with collagen I and allowed to form a confluent monolayer for 5 d. Cells were treated for 1 or 3 d with different concentrations of LRAs or VC. PMS solution was added at a 1:20 ratio to the MTS solution at room temperature, and 20 μl of PMS/MTS reagent was added to each well containing cells and 100 μl of cell culture medium. Cells were incubated for 2 h at 37°C. The conversion of MTS into formazan by mitochondrial-reducing enzymes is directly proportional to the amount of 490 nm absorbance. OD was measured at 490 nm using an ELX808 microplate reader (BioTek Instruments, Winooski, VT). Each value represented in percentage was normalized against the corresponding vehicle-treated control in triplicate.

GenePlex

To verify the relative expression of the IL-6, IL-8, CCL2, GM-CSF, OCLN, and CLDN5 genes, we performed the Quantigene Plex Assay according to the manufacturer’s protocol (Panomics, Fremont, CA). The fluorescence intensity in the hybridized complex was measured using a Bio-Plex 200 system (Bio-Rad, Mississauga, ON).

Detection of soluble factors

hCMEC/D3 cells were cultured in 96-well plates coated with collagen I at a concentration of 1 × 10^5 cells per well. At day 5, cells were treated with increasing doses of LRAs for 24 h. The levels of IL-6, IL-8, CCL2, TNF-α, and GM-CSF in collected supernatants were quantified in triplicates using ELISA assays following the manufacturer’s instructions (BioLegend, San Diego, CA). Optical absorbance was measured at 450 nm using an ELX808 microplate reader (BioTek Instruments). Each value represented in ratio was normalized against the corresponding vehicle-treated control in triplicate.

On-cell Western assay

hCMEC/D3 cells were cultured in 96-well plates coated with collagen I at a concentration of 1 × 10^5 cells per well. At day 5, cells were treated with increasing doses of LRAs for 24 h. Before staining, cells were incubated with 20% pooled human sera to block nonspecific binding sites and washed once with PBS supplemented with 0.5% BSA (Fitzgerald Industries International, Acton, MA). Cell surface expression of ICAM-1 (CD54) was monitored with an anti-human CD54 biotin (eBioscience, Germany) at a final concentration of 6–8 × 10^7 cells per flask in MEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.3 mm/ml l-glutamine, 1 mM of sodium pyruvate, MEM nonessential amino acids, 0.5 μg/ml amphotericin B (all from Life Technologies), and 0.1% dextrose (Sigma-Aldrich). HFA were grown in a humidified incubator maintained at 37°C under a 5% CO₂ atmosphere and left untouched for 2 wk before being passaged once a week. To ensure cell purity, we conducted all experiments on the third or fourth passage.

"xCELLigence assay

The xCELLigence Real Time Cell Analysis Dual Purpose system (Roche Applied Science) allow the continuous and noninvasive recording of electrophoresis impedance integrated into the bottom of 96-well E plates. The number, morphology, degree of adhesion of cells, and strength of their tight junctions all affect electrode impedance that represents the cellular responses in a time- and dose-dependent manner. The dimensionless cell index (CI) value is directly proportional to the relative change in electrical impedance normalized by the background value. Recording of CI values and analysis were performed using the Real Time Cell Analysis software version 1.2.1. In brief, hCMEC/D3 cells were evenly distributed in 96-well E plates precoated with Collagen-I in three to five replicates for each condition. To prevent edge effect, we allowed the cells to attach to the 96-well E plates at room temperature for 30 min before being inserted into the cell incubator (37°C and 5% CO₂) for continuous impedance recording. The hCMEC/D3 reached their confluent phase on day 3. LRAs were diluted in complete culture medium. The corresponding vehicle control (VC) (i.e., DMSO or ddH₂O) was diluted the same way as each tested LRA. On day 0, the medium was replaced by LRAs or VC-containing medium. On day 3 or 4, the cells were washed three times with PBS and incubated 4 more days with fresh cell culture medium. The normalized CI (nCI) at a certain time point was acquired by dividing the CI value by the value at the reference time point, day 0. We used for each condition the corresponding VC curve as baseline to obtain the BaseLine nCI.

MTS assay

Cellular viability was assessed using CellTiter 96 AQueous Nonradioactive Cell Proliferation Assay Kits (MTS/PS assay; Promega, Madison, WI). In brief, hCMEC/D3 cells were seeded in 96-well plates coated with collagen I and allowed to form a confluent monolayer for 5 d. Cells were treated for 1 or 3 d with different concentrations of LRAs or VC. PMS solution was added at a 1:20 ratio to the MTS solution at room temperature, and 20 μl of PMS/MTS reagent was added to each well containing cells and 100 μl of cell culture medium. Cells were incubated for 2 h at 37°C. The conversion of MTS into formazan by mitochondrial-reducing enzymes is directly proportional to the amount of 490 nm absorbance. OD was measured at 490 nm using an ELX808 microplate reader (BioTek Instruments, Winooski, VT). Each value represented in percentage was normalized against the corresponding vehicle-treated control in triplicate.
San Diego, CA) and streptavidin-PE solution (BioLegend). After three extensive washes with a buffer made of PBS (pH 7.4), 1 mM of EDTA, and 0.5% BSA, cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Fluorescence was measured at 575 nm using Variskan flash multimode reader (Thermo Fisher Scientific, Waltham, MA). Each value represented in ratio was normalized against the corresponding VC treated in triplicate.

Immunofluorescence staining and microscopy analyses

hCMEC/D3 cells were cultured on μ-Slide eight-well ibiTreat (ibidi, Martinsried, Germany) coated with collagen I at a concentration of 1 × 10^5 cells per well. At day 5, cells were either left untreated or treated for 8 h with prostratin (1 μM) or bryostatin-1 (1 nM). Cells were then fixed in 2% paraformaldehyde for 30 min at 4°C. After fixation, cells were permeabilized with 0.1% Triton X-100, and non-specific binding sites were blocked with a buffer containing PBS (pH 7.4), 1% BSA (Fitzgerald Industries International), 20% normal goat serum (Jackson ImmunoResearch, West Grove, PA), and 10% human AB serum (Corning) for 30 min. Subsequently, hCMEC/D3 cells were incubated with 10 μg/ml mouse anti–ZO-1 (ZO1; BD Transduction Laboratories, San Jose, CA) or anti–JAM-A (J3F.1; Santa Cruz Biotechnology, Dallas, TX) at 1:500, and DAPI (1:500; Thermo Fisher Scientific, Mississauga, ON, Canada) and Alexa Fluor 488 (1:1000; Sigma-Aldrich, St. Louis, MO) for 45 min at room temperature. Stained cells were washed extensively before addition of ibidi mounting medium (ibidi, Madison, WI). Visual observations with a 63× oil immersion objective under a fully automated inverted Leica DMi6000 B microscope (Leica Microsystems, Mississauga, ON, Canada) were then performed. Image acquisition and analysis were done using Velocity Software Version 5.4.0 (PerkinElmer, Waltham, MA).

In vitro human BBB model system

The cocultivation model included hCMEC/D3 cells and HFAs seeded on each side of a porous insert allowing cell-cell contacts as reported previously (24, 25). In brief, cell culture inserts for 24-well plates with 3.0-μm pore in translucent polyethylene terephthalate membrane (Corning Life Sciences) were coated with collagen I (Sigma-Aldrich) at 150 μg/ml. Next, HFAs were seeded on the basal side of the membrane. Stained cells were washed extensively before addition of ibidi mounting medium (ibidi, Madison, WI). Visual observations with a 63× oil immersion objective under a fully automated inverted Leica DMi6000 B microscope (Leica Microsystems, Mississauga, ON, Canada) were then performed. Image acquisition and analysis were done using Velocity Software Version 5.4.0 (PerkinElmer, Waltham, MA).

BBB permeability assays with dextran-rhodamine

The effect of LRAs on BBB integrity was assessed by measuring permeability to dextran-rhodamine. After 24 h of treatment with either prostratin (2.5 μM), bryostatin-1 (1 nM), or VC (i.e., DMSO at a 1:1000 dilution), the culture medium in the upper chamber was changed to complete culture medium supplemented with 1 mg/ml 70-kDa dextran-rhodamine (Life Technologies, Carlsbad, CA). The fluorescence intensity in collectors was measured using an ELX808 microplate reader (BioTek Instruments).

Transmigration assays as monitored by flow cytometry

BBB-containing inserts were placed into 24-well Ultra-Low Attachment Plates to maintain cells in a suspended, unattached state in collectors (Corning Life Sciences). Thereafter, prostratin at 1 μM, bryostatin-1 at 1 nM, or a similar volume of VC was added to the upper chamber. After 16 h, the medium in the upper chamber was rinsed and some prostratin-treated inserts were placed into new ultra-low attachment–containing medium (rProstratin [rP] and rinsed bryostatin-1 [rBryo], respectively). Finally, PBMCs or monocytes (10^5 each) were added to the upper chamber for 24 h in 50% RPMI 1640–50% complete hCMEC/D3 culture medium. PBMCs or monocytes were collected in the lower chamber, in the collector, and on both sides of BBB-containing inserts using Accutase-Enzyme Cell Detachment Medium (eBioscience). Before staining, cells were incubated with 20% pooled human serum to block nonspecific binding sites and washed once with PBS supplemented with 0.5% BSA. Cell surface expression of CD3, CD4, CD8, CD14, and CD16 was monitored by flow cytometric analyses. In brief, cells were incubated for 15 min with fluorochrome-labeled Abs (all from BioLegend) and then washed twice with a buffer made of PBS (pH 7.4), 1 mM of EDTA, and 0.5% BSA. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature. After acquisition on a FACS Canto flow cytometer (BD Biosciences, Mississauga, ON), data were analyzed with FlowJo software (FlowJo, Ashland, OR).

Statistics

Means of raw data were compared using either Student t test or one-way ANOVA. The p values <0.05 were deemed statistically significant. Calculations were performed with the GraphPad PRISM 6 for Windows (GraphPad Software, La Jolla, CA).

Results

A dynamic real-time method for monitoring endothelial barrier integrity in vitro

In the first series of investigations, an impedance-based xCELLigence Real Time Cell Analysis Dual Purpose system was used to dynamically monitor cerebral microvascular endothelial barrier integrity (see Materials and Methods). This technology was previously shown to efficiently monitor tight junction integrity in human brain endothelial cells (26, 27). Preliminary experiments were first performed to determine the optimal number of cells per well to allow growth of hCMEC/D3 cells until reaching an impedance plateau, expressed as a CI value, indicative of a highly confluent monolayer (Supplemental Fig. 1). CI values approaching 10 were previously described as indicative of tight monolayers of hCMEC/D3 cells (26). Interestingly, a strong effect of hCMEC/D3 passage number was observed on the maximal impedance of the endothelial barrier. At passage 28, the maximal CI value plateaued at 6–9, whereas it would reach 8–10 at passage 33 (Supplemental Fig. 1A). Experiments in this study were thus performed using 8 × 10^4 hCMEC/D3 cells per well at passages 28–33. These experimental conditions allowed cells to reach maximal confluence after 3 d (dark blue lines). On day 4, the endothelial layer was treated with different LRAs of various classes (i.e., BIX-01294, 5-aza-dC, SAHA, HMBA, prostratin, and bryostatin-1; see later for more details). A large range of LRA concentrations was tested, and three representative concentrations were selected for each LRA.

BIX-01294 transiently affects the endothelial barrier resistance and viability, whereas 5-aza-dC has no effect on confluent endothelial barrier integrity

BIX-01294 and 5-aza-dC modulate epigenetic signals by inhibiting enzymes involved in histone and DNA methylation. BIX-01294 is a small-molecule compound targeting euchromatic histone-lysine N-methyltransferase (EHMT) 2 (28) and EHMT1 (29). The heteromorphic complex EHMT1/2 catalyzes the monomethylated and dimethylated state of histone H3 at lysine 9 (H3K9me2) (30). By inhibiting EHMT1/2, BIX-01294 is also able to block their nonhistone substrates, including DNA methyltransferase 1. Inhibition of EHMT1/2 activity by BIX-01294 favors acetylation of H3K9, which is associated with transcriptional activation of surrounding promoters. Integrity of the endothelial barrier after BIX-01294 treatment was evaluated by recording the hCMEC/D3 BaseLine nCI every 15 min during 4 d of treatment with different doses of BIX-01294 ranging from 0.1 to 10 μM. At day 4, cell culture medium was changed for fresh medium without drug, and the BaseLine nCI was recorded during 2 more days. At 10 μM, a weak and transient decrease of BaseLine nCI was observed, with a maximal response at 14 h posttreatment (Fig. 1A). After 3 d of treatment, the BaseLine nCI approached zero, meaning that there was no observable difference with VC-treated cells. Removal of BIX-01294 from the cell culture medium had no significant incidence on BaseLine nCI. Under 10 μM, no significant and reproducible effect of BIX-01294 on BaseLine nCI was observed. Finally, MTS assays were performed on confluent hCMEC/D3 monolayers after 24 and 72 h in the presence of increasing concentrations of BIX-01294 (Fig. 1B). After 24 h, BIX-01294 at 10 μM produced a mild cytotoxicity (83 ± 1.5% of metabolically active cells in treated samples compared with untreated controls) that was not observed after 72 h (106 ± 2.9%). In contrast, during 4 d of treatment, 5-aza-dC had no measurable effect on confluent...
MTS assays revealed that the slight decrease of BaseLine nCI capable of reconstituting endothelial barrier integrity (data not shown). hCMEC/D3 BaseLine nCI at the tested concentrations and showed no cytotoxicity after 24 and 72 h of treatment (Fig. 1C, 1D).

Positive elongation factor b activators, SAHA and HMBA, induce a dose-dependent breakdown of endothelial barrier resistance that is partially explained by their cytotoxicity

SAHA is a strong inhibitor of human class I and II histone deacetylases (31), which leads to acetylation of the Nuc1-repressive nucleosome overlapping the HIV-1 transcription start site (19, 32) and dissociation of histone deacetylase 1 from the viral promoter (19). However, its major activity required for reactivation of latent HIV-1 lies in its ability to release positive elongation factor b (P-TEFb) from the 7SK ribonucleoprotein complex, thereby increasing its availability for viral transcription (11, 33). As shown in Fig. 2A, although SAHA at 0.1 μM did not seem to affect the endothelial barrier integrity, we observed a decrease of BaseLine nCI at 2.5 μM and a drastic decline at 10 μM that started as early as 24 h after treatment (minimum BaseLine nCI plateaued at −0.22 and −0.86, respectively). Removal of the drug at day 4 allowed a restoration of the monolayer toward Baseline nCI only in the 2.5-μM condition. Beyond the threshold concentration of 5 μM of SAHA, hCMEC/D3 cells were no longer capable of reconstituting endothelial barrier integrity (data not shown). MTS assays revealed that the slight decrease of BaseLine nCI observed at 2.5 μM and beyond was not associated with cytotoxicity the first day of treatment (Fig. 2B). Thus, during the first 24 h, SAHA triggered modifications in hCMEC/D3 morphology and cell–cell junctions that did not affect the ratio of metabolically active hCMEC/D3. However, after 72 h of treatment, SAHA induced a dose-dependent cytotoxicity that is reflected by the drop of BaseLine nCI observed between days 1 and 4 posttreatment. The high cytotoxicity of SAHA after 72 h at 10 μM (22.5 ± 6% of metabolically active treated cells compared with VC) confirmed that the number of viable hCMEC/D3 cells was not sufficient to restore a confluent monolayer even after the drug was removed from the medium.

HMBA is known to transiently activate the PI3K-Akt pathway, leading to phosphorylation of HEXIM1 and then to the subsequent release of active P-TEFb. Availability of P-TEFb is crucial for Tat-mediated HIV-1 transcription and reactivation of latent HIV-1 (12). A dose-dependent decrease of BaseLine nCI was observed in the presence of HMBA that reaches and maintains a plateau after 14 h of treatment (Fig. 3A). The integrity of the endothelial barrier resistance was totally restored 2 d after HMBA removal from the medium. Although HMBA did not affect cell viability in the first 24 h of treatment, a low mortality was observed after 3 d in the presence of HMBA at 5 and 10 mM (percentage of
metabolic activity in treated cells compared with VC of 83.3 ± 0.3 and 82.2 ± 1.1%, respectively) (Fig. 3B). This delayed mortality was not sufficient to explain the decrease of hCMEC/D3 resistance at 14 h posttreatment.

Proestratin and bryostatin-1 cause a cytotoxic-independent breakdown of hCMEC/D3 resistance

Proestratin and bryostatin-1 are two protein kinase C (PKC)–activating compounds that upregulate latent HIV-1 provirus expression. Previous studies have described an important role of PKC in the regulation of BBB functions (34–38). After exposure to proestratin, a dose-dependent decrease of BaseLine nCI was observed, which reached a plateau after 16 h (Fig. 4A). Removal of the drug triggered a quick return of BaseLine nCI toward zero in 24 h. Although proestratin causes a dose-dependent breakdown of endothelial barrier resistance, it does not affect cell viability at 24 h regardless of the concentration used (Fig. 4B). Only the highest dose of proestratin tested could induce a slight decrease of the percentage of metabolic activity at 72 h compared with VC (89.5 ± 5.7%). Proestratin has been shown to activate T cells without leading to their proliferation (39). Reflecting this, although the hCMEC/D3 metabolism did rise after 24 h of treatment, proestratin had no effect on their proliferation per se (data not shown).

Bryostatin-1 shows a PKC-binding affinity in the nanomolar range with a higher affinity toward PKCδ compared with proestratin. A large range of bryostatin-1 concentrations was tested showing that this drug affects BaseLine nCI at nanomolar concentrations (data not shown). Fig. 4C illustrates its effect on the hCMEC/D3 monolayer at final concentrations of 0.04, 0.15, and 2.5 nM. Like proestratin, bryostatin-1 causes a breakdown of endothelial monolayer resistance that reaches a plateau at 16 h. Regardless of the concentration used, the BaseLine nCI returned back to the initial value after a few days. Interestingly, a significant delay to return to the initial Baseline nCI after drug removal could be observed after bryostatin-1 exposure. Five to seven days were required to restore the Baseline nCI after washing the drug. Considering that no significant effect of bryostatin-1 on hCMEC/D3 metabolism was observed at any time points tested, this phenomenon seems independent of any drug cytotoxicity (Fig. 4D).

LRA combinations differently potentiate the breakdown of hCMEC/D3 resistance

Combining multiple LRAs, because they synergistically activate HIV-1 production in several in vitro studies (15–19), represents a strong interest for future investigations. Five combinations of two...
LRAs were thus tested including either prostratin or bryostatin-1 associated with compounds releasing active P-TEFb (i.e., SAHA and HMBA) or the two PKC agonists together. No synergistic effect was observed on hCMEC/D3 resistance when prostratin was associated with bryostatin-1 (Fig. 5). However, a clear additive effect was observed between prostratin and HMBA on endothelial barrier breakdown. Although this additive effect could also be observed with SAHA, it was more moderate and resistance restoration was significantly slower after drug removal. Finally, bryostatin-1 did not show any additive nor synergistic effect with either of the P-TEFb activators.

Prostratin and bryostatin-1 induce secretion of proinflammatory cytokines by hCMEC/D3 cells and modulate transcription of occludin and claudin 5 genes

The effect of LRAs on secretion of some soluble factors by brain microvascular endothelial cells was next studied by treating confluent hCMEC/D3 monolayers with three concentrations of each LRA for 24 h. Secretion of IL-6, IL-8, TNF-α, CCL2 (also known as monocyte chemotactic protein 1), and GM-CSF in the cell culture supernatants was then quantified by commercial ELISA tests. Results show that, although none of the tested LRAs triggered production of a quantifiable amount of TNF-α, prostratin...
and, to a lesser extent, bryostatin-1 could provoke a significant dose-dependent secretion of IL-6, IL-8, CCL2, and GM-CSF (Fig. 6). 5-Aza-dC also enhanced the secretion of IL-6 but had no effect on IL-8, TNF-α, GM-CSF, and CCL2. BIX-01294, SAHA, and HMBA did not induce the secretion of any of the soluble factors tested. On the contrary, higher concentrations of SAHA and HMBA could significantly reduce the basal secretion of CCL2 by hCMEC/D3 cells.

Given the strong secretion of proinflammatory cytokines and chemokines induced by both PKC activators, we tested their effect on hCMEC/D3 gene expression profiles of GM-CSF, IL-6, IL-8, and CCL2, as well as two tight junction protein-coding genes (i.e., occludin/OCLN and claudin 5/CLDN5). Fig. 7 shows a sequential gene induction triggered by prostratin and bryostatin-1 in the endothelial cell line. Both PKC activators, but more particularly prostratin, induced a robust expression of GM-CSF, IL-6, IL-8, and CCL2 mRNAs in the first 4 h, which returned nearer to baseline values thereafter. Modulation of the expression of tight junction protein coding genes by prostratin and bryostatin-1 occurred only at later time points. A decrease of OCLN gene expression could be observed 8 h posttreatment with prostratin and bryostatin-1. Transcription of CLDN5, in contrast, was induced by both PKC activators, albeit only after 24 h of treatment.

**Prostratin and bryostatin-1 disrupt ZO-1 localization and reduce expression of JAM-A in hCMEC/D3**

We carried out immunofluorescence microscopy analyses to validate the model used in this study and further investigated the

![FIGURE 7. Prostratin and bryostatin-1 affect OCLN and CLDN5 gene expression. Gene expression profiles of hCMEC/D3 were analyzed using QuantiGene 2.0 Multiplex assay. In brief, hCMEC/D3 monolayers were treated with 1 μM of prostratin (A) and 1 nM of bryostatin-1 (B) for the indicated time periods. Expression variation for each gene is presented in fold change compared with VC for each time point. Data are expressed as means ± SD of five samples. Asterisks denote statistically significant data as defined by the Student t test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).](http://www.jimmunol.org/)
FIGURE 8. Prostratin and bryostatin disrupt ZO-1 localization and reduce expression of JAM-A in hCMEC/D3. Immunofluorescence microscopic analyses of ZO-1 (green) and JAM-A (green) expression were performed in untreated hCMEC/D3 cells and prostratin- or bryostatin-treated hCMEC/D3 cells. Cells were first stained with an mAb specific for ZO-1 or JAM-A before adding an Alexa488-conjugated secondary Ab. Stained cells were visualized by an inverted fluorescence microscope. Original magnification ×630.

Prostratin and bryostatin-1 increase ICAM-1 expression in hCMEC/D3 monolayers

ICAM-1 is a cell surface molecule expressed by several cell types including leukocytes and endothelial cells; it is involved in the transendothelial migration of leukocytes to sites of inflammation. ICAM-1 interacts with its integrin counterparts LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) to support leukocyte–endothelial interactions and facilitate rolling and adhesion of leukocytes on the endothelial wall. Considering the essential role of ICAM-1 when expressed on endothelial cells in increased infiltration of immune cells across the brain vasculature, we assessed the effect of LRAs on ICAM-1 expression at the apical surface of hCMEC/D3 cells. This goal was reached by performing on-cell Western assays after 24 h of treatment with increasing concentrations of LRAs and VCs. Fig. 10 shows a dose-dependent increase of ICAM-1 expression on hCMEC/D3 cells after exposure to prostratin or bryostatin-1 compared with VC. BIX-01294 and SAHA had no effect on ICAM-1 expression, whereas HMBA and 5-aza-dC led to a reduction of basal cell surface ICAM-1 expression. However, an increase in ICAM-1 expression was seen when using the highest concentration of 5-aza-dC (i.e., 10 μM).

Prostratin and bryostatin-1 increase BBB permeability and affect leukocyte transmigration

The inflammatory response and the drastic reduction of the resistance of the hCMEC/D3 endothelial monolayer induced by prostratin and bryostatin-1 led us to investigate the impact of these two compounds on an in vitro human BBB experimental model system. To this end, confluent monolayers of hCMEC/D3 human brain endothelial cells and HFAs were cocultured on each side of cell culture inserts (to permit cell–cell contacts) to test the effect of PKC-activating LRAs on BBB permeability and leukocyte transmigration potential. First, the impact of prostratin and bryostatin-1 on BBB permeability was assessed by measuring the diffusion of fluorescent rhodamine (70 kDa) from the upper chamber to the collector after 24 h of treatment with prostratin, bryostatin-1, and VC at 37°C. An empty control (EC) insert was used to assess passive diffusion of the fluorescent marker. Consistent with the breakdown of hCMEC/D3 monolayer resistance, BBB permeability was also affected by prostratin and bryostatin-1 because they both induced a significant increase in dextran-rhodamine concentrations in the collector compared with the VC condition (Fig. 11A).

Transmigration of lymphocytes and monocytes across the BBB is critical to the neuropathogenesis of HIV-1, because this process promotes inflammation and facilitates infection of the CNS. PKC activators, and especially prostratin, can induce the secretion of soluble factors involved in the recruitment, differentiation, or activation of various leukocytes while also increasing the expression of CCL2, a chemokine in the prostratin-induced hCMEC/D3 resistance breakdown. Prostratin-induced CCL2 is involved in hCMEC/D3 monolayer resistance breakdown

CCL2 was previously shown to disrupt the integrity of cultured brain endothelial cell monolayers while inducing a redistribution and reduction in the expression of tight junction proteins (40–42). Given the potent induction of CCL2 mRNA and protein expression after prostratin treatment, we monitored the involvement of this chemokine in the prostratin-induced hCMEC/D3 resistance breakdown. Addition of a purified blocking Ab against human CCL2 along with prostratin led to a partial restoration of endothelial barrier resistance (Fig. 9). Although restoration of barrier integrity is far from complete, these results suggest that CCL2 might be involved in the breakdown of the endothelial barrier after prostratin treatment.

endothelial barrier integrity. Although Occludin and Claudin 5 were not consistently detected at the cell-to-cell contacts of hCMEC/D3 cells (data not shown), our immunodetection studies indicated a clear junctional expression of the submembranous tight junction-associated protein ZO-1 and the integral membrane protein JAM-A (Fig. 8). Although ZO-1 and JAM-A were clearly observed at the cell–cell borders in untreated hCMEC/D3 cells, treatment with prostratin or bryostatin-1 resulted in a significant relocation of ZO-1 to cytoplasmic and perinuclear compartments, whereas JAM-A expression was considerably reduced.

FIGURE 9. CCL2 is involved in the prostratin-mediated diminution in endothelial barrier integrity. The BaseLine nC1 calculated from the measure of an hCMEC/D3 monolayer electrical impedance, was recorded in real-time during 3 d of treatment with 2.5 μM of prostratin either used alone (red line) or in combination with an anti-human CCL2 mAb (5 μg/ml, light brown line). The corresponding VC associated with the anti-human CCL2 mAb was used as baseline (black line). Results are the mean of five replicates ± SD.
adhesion molecules such as ICAM-1 on the luminal surface of endothelial cell monolayers. As such, this LRA could significantly facilitate transmigration of leukocytes across the BBB. When compounded with a significant increase of the endothelial cell monolayer permeability, this effect could potentially lead to unwanted neuroinvasion, neuroinflammation, and/or cerebral edema. Using the previously described in vitro human BBB model, we developed a transmigration assay using PBMCs. PBMCs were isolated from fresh whole blood of healthy donors and then added to the upper chamber of the in vitro human BBB model. After 24 h, the absolute cell numbers were counted in the luminal (upper chamber), BBB adherent (insert membrane), and basal (collector) compartments by flow cytometry. Prostratin significantly enhances adhesion and transmigration of leukocytes compared with the VC condition (Fig. 11B). Bryostratin-1 promoted PBMC adhesion to the BBB, but its effect on transmigration was muted compared with prostratin, both at their optimal concentrations of 1 nM and 1 μM, respectively.

Various experimental conditions were tested to study the effect of PKC-activating LRAs on leukocyte transmigration through the model BBB. One strategy involved washing the drug 24 h after its addition to observe persisting effects of drug treatment on the BBB. In these experimental conditions, termed rBryo and rPro, the two sides of each insert were carefully washed with PBS and put back in collectors containing fresh culture medium before adding PBMCs in the upper chamber. Whereas rinsing prostratin restored PBMCs adhesion and transmigration to basal levels, rinsing bryostatin-1 did not yield similar results. Indeed, rBryo and bryostatin-1 conditions did not differ significantly, and even rinsing the drug still resulted in a significant increase in the absolute number of cells found in the BBB adherent compartment. These results are consistent with the...
rapid restoration of hCMEC/D3 monolayer resistance observed in Fig. 4 after removal of prostratin from the medium, whereas this restoration was significantly delayed after bryostatin-1 removal.

Prostratin and bryostatin-1 differentially regulate CD8+ T cell adhesion and transmigration across the in vitro human BBB model

The transmigration assay described earlier was also used to determine the relative ratio of adherent and transmigrated CD8+ and CD4+ T cells by flow cytometry. Exposure of the in vitro human BBB model with prostratin or bryostatin-1 for 24 h strongly increased both adhesion and transmigration of CD4+ T cells (Fig. 12). In sharp contrast with prostratin, which also augments adhesion and transmigration of CD8+ T cells, bryostatin-1 instead severely reduced their number in the BBB adherent and basal compartments compared with the VC condition. These results suggest that distinct mechanisms of regulation are involved in the transmigration of CD8+ T cells induced by prostratin compared with bryostatin-1. In these assays, rinsing and changing the collectors of pretreated inserts (rPro and rBryo) totally abolished their effect on adhesion and transmigration of CD4+ and CD8+ T cells.

Prostratin and bryostatin-1 increase adhesion of monocytes but limit their transmigration through the in vitro human BBB model

In addition to CD8+ and CD4+ T cells, we also studied the transmigration of different subpopulations of monocytes, namely, CD14+, CD14high/CD16dim, CD14dim/CD16high, and CD16+. Depending on the monocyte population, we noted a 3- to 20-fold increase in adhesion to the BBB upon treatment with prostratin without washes (Fig. 13). This increase was highest for the CD14+ subpopulation (relative ratio to VC-treated conditions of 24.11 ± 5.24). Comparatively, prostratin treatment of the BBB led to a significant reduction in the number of monocytes that transmigrated to the basal compartment. Similar observations were made with bryostatin-1, but the modulatory effects were less dramatic.

Analyzing the relative proportions of each subpopulation of monocytes in each compartment provides another perspective

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**FIGURE 12.** Prostratin (Pro) and bryostatin-1 (Bryo) differentially regulate CD8+ T cell adhesion and transmigration across an in vitro human BBB model. Inserts containing both hCMEC/D3 cells and HFAs were treated for 16 h either with 1 μM of Pro, 1 nM of Bryo, or the corresponding VC. Next, the medium of the upper chambers was rinsed and some Pro-treated and Bryo-treated inserts were placed into new medium-containing collectors (rPro and rBryo, respectively). PBMCs were added to the inner/upper chamber for 24 h. The results are expressed in ratio of absolute adherent cell number (A) and absolute basal cell number (B) measured by flow cytometry and compared with the corresponding VC. Data are expressed as means ± SD of five donors.

**FIGURE 13.** Prostratin (Pro) and bryostatin-1 (Bryo) increase adhesion of monocytes to an in vitro human BBB model and limit their transmigration.

Inserts containing both hCMEC/D3 cells and HFAs were treated for 16 h with either 1 μM of Pro, 1 nM of Bryo, or the corresponding VC. Next, the medium of the upper chambers was rinsed, and some Pro- and Bryo-treated inserts were placed into new medium-containing collectors (rPro and rBryo, respectively). Monocytes were added to the inner/upper chamber for 24 h. The results are expressed in ratio of absolute adherent cell number (A) and absolute basal cell number (B) measured by flow cytometry and compared with the corresponding VC. Data are expressed as means ± SD of five donors. Asterisks denote statistically significant data as defined by the Student t test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).
about the effect of LRAs on the transmigration of leukocytes across the BBB. Fig. 14 shows that prostratin and bryostatin-1 caused an increase of the relative proportion of CD14\(^+\) cells in all three compartments compared with VC. Furthermore, in the BBB adherent compartment, we noted an increase of the relative proportion of CD14\(^{high}/CD16\(^{dim}\) monocytes versus CD14\(^{dim}/CD16\(^{high}\) and CD16\(^+\) subpopulations. Interestingly, removal of the drugs by rinsing the inserts abolished their effects and showed that their action was reversible.

**Discussion**

The maintenance of a highly restrictive BBB is a multifactorial process that plays a critical role in preserving CNS homeostasis (43). Our study shows that LRAs such as BIX-01294, SAHA, HMBA, prostratin, and bryostatin-1 clearly affect the endothelial barrier resistance. The majority of studies about LRAs focus on their role in reactivation of HIV-1 from transcriptional latency. To our knowledge, no research has yet been carried out to date on the effect of LRAs on the BBB function. Interestingly, a recent study demonstrated that certain LRAs including panobinostat, romidepsin, chaetocin, disulfiram, SAHA, HMBA, and IQ-1 are well tolerated by primary fetal astrocytes, monocyte-derived macrophages (as a model of perivascular macrophages), and the SH-SY5Y neuroblastoma cell line (44). The mean 50% cytotoxic concentrations of SAHA and HMBA on the SVG fetal astrocyte cell line reported in this study were 2.6 and 2.1 \(\mu\)M, respectively, after 24 h of treatment. Unfortunately, the potential alteration, by each studied LRA, of the cellular functions of all cell types tested in this work were not evaluated. In our hands HMBA showed no cytotoxicity toward hCMEC/D3 cells even at millimolar concentrations (Fig. 3B), but SAHA induced a 51.4% mortality rate after 72 h of treatment at a final concentration of 2.5 \(\mu\)M (Fig. 2B). These results are consistent with previous experiments showing that SAHA triggers growth arrest and/or apoptosis of a wide variety of transformed cells (45). Results from Fig. 1C indicate that 5-aza-dC has no effect on confluent endothelial barrier integrity. These results were not surprising because the DNA methyltransferase 1 trapping and degradation by 5-aza-dC agent is dependent on cell cycle progression and DNA replication (46, 47).

The PKC family of protein kinases includes 10 isoforms that are classified into three distinct subtypes, that are conventional (\(\alpha, \beta I, \beta II,\) and \(\gamma\), novel (\(\delta, \theta, \eta, \) and \(\epsilon\)), and atypical (\(\upsilon\lambda\) and \(\zeta\)). Activation of most of PKC isoforms results in endothelial barrier breakdown (34–38, 48). As an exception, however, activation of PKCe leads to an upregulation of CLDN5, which is an integral membrane protein and component of tight junction strands and protects the BBB from TNF-\(\alpha\)-induced disruption (49). As expected, treatment with prostratin and bryostatin-1, as strong activators of PKC, led to a significant decrease of BBB permeability that was not linked with any observed cytotoxicity (Fig. 4). Furthermore, association of a PKC activator such as prostratin with the P-TEFb activator HMBA enhanced endothelial barrier breakdown (Fig. 5). These results raise the possibility that prostratin and bryostatin-1 might improve the access of cART to the CNS anatomical sanctuary during a shock-and-kill strategy. However, a compromised BBB is commonly observed in HIV-1–infected patients and is associated with disease progression (50). The ultimate outcome of PKC activators on BBB permeability, access of cART to the CNS, viral replication in the brain, and disease progression are thus important issues that should be investigated before such LRAs are actually considered as therapeutic strategies. Data from Fig. 7 suggest that both PKC activators induce a significant decrease of OCLN gene expression at 8 h posttreatment but cause a late increase in CLDN5 gene expression after 24 h of treatment. One possible reason for these important variations in tight junction protein expression could come from the activation of multiple PKC isoforms by prostratin and bryostatin-1, including PKC\(\epsilon\) (14, 51, 52). Such modulation of tight junction proteins could thus explain the alteration of BBB permeability by these LRAs. Prostratin and bryostatin-1 also altered the localization of ZO-1 and reduced JAM-A expression, two other important components of the tight junctions (Fig. 8). This concordant destabilization of

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**FIGURE 14.** Prostratin (Pro) and bryostatin-1 (Bryo) increase the proportion of CD14\(^+\) monocytes in all BBB compartments. Inserts containing both hCMEC/D3 cells and HFA were treated for 16 h with either 1 \(\mu\)M of Pro, 1 nM of Bryo, or the corresponding VC. Next, the medium of the upper chambers was rinsed, and some Pro- and Bryo-treated inserts were placed into new medium-containing collectors (rPro and rBryo, respectively). Monocytes were added to the inner/upper chamber for 24 h. The results are expressed in parts of all luminal (top row), adherent (middle row), and basal (bottom row) monocyte subtypes measured by flow cytofluorometry. Data are expressed as means of five donors.
the tight junctions after 8 h of treatment is consistent with the breakdown of endothelial barrier resistance induced by these two distinct LRAs.

Prostratin and bryostatin-1, unlike BIX-01294, SAHA, and HMBA, not only trigger BBB opening, but also cause inflammation by inducing IL-6, IL-8, GM-CSF, and CCL2 secretion by brain microvascular endothelial cells (Fig. 6). Upregulation of proinflammatory cytokines in the CSF and brain tissues of patients with HIV-1–associated neurocognitive disorders was associated with the severity of HIV-1 disease progression (53–56). In particular, CCL2 was shown to increase vascular endothelial cell monolayer permeability in vitro (40–42) and to enhance transmigration of HIV-1–infected monocytes (57). Interestingly, results from Fig. 9 suggest that the prostratin-induced CCL2 secretion is involved in hCMEC/D3 monolayer resistance breakdown. It has been reported that IL-6 also modulates expression of tight junction proteins and their distribution, and increases endothelial permeability by inducing PKC-mediated signaling events (58–60). Thus, it is possible that the induction of IL-6 secretion by prostratin and bryostatin-1 can potentiate the loss of permeability of the BBB via a retroactive loop involving PKC activation. Additional studies are needed to solve this issue.

Finally, prostratin and bryostatin-1 increased ICAM-1 surface expression on hCMEC/D3 cells and promoted adhesion of CD4+ T cells and monocytes on monolayers of this endothelial cell line (Figs. 10–14). ICAM-1 expression is essential for the firm adhesion and crawling of leukocytes on the BBB before diapedesis (61, 62) but is not sufficient per se for complete cell transmigration. The high secretion of chemokines by hCMEC/D3 after treatment with prostratin or bryostatin-1 is probably involved in the attraction of leukocytes to the BBB. However, it is possible that the lack of a gradient of chemokines in the collector or the lack of another secondary signal would limit an efficient transmigration of monocytes across the BBB. In addition, distinct affinities of prostratin and bryostatin-1 to the diverse family of PKC isozymes might explain why a differential response is observed for their ability to induce adhesion and transmigration of CD8+ T cells across the BBB (Fig. 12). Finally, these results raise the possibility that CD4+ and CD8+ T cells could transmigrate with a high degree of specificity depending on the proinflammatory signal to which the endothelial layer and either of the T cell subtypes are exposed.

Several studies have suggested that bryostatin-1 could be a beneficial adjunct to the treatment of HIV-1–mediated CNS damage. Notably, bryostatin-1 was shown to reactivate latent viral infection in normal human astrocytes and the U87 astrocyoma cell line (63). In vitro, combination of bryostatin-1 with the CCR5 inhibitor maraviroc (Selzentry) or the efavirenz/emtricitabine/tenofovir disoproxil fumarate combination antiretroviral (Atripla) (65). The concentration of the single dose of bryostatin-1 evaluated the effects of bryostatin-1 on HIV-1–infected patients on cART (65). The concentration of the single dose of bryostatin-1 was grade 1 headache and myalgia. Further clinical studies are needed to shed light on this important issue and to define the risks and benefits of using a shock-and-kill strategy for the treatment of HIV-1–infected individuals.

Disclosures
The authors have no financial conflicts of interest.

References
1. Mahajan, H. S., M. S. Mahajan, P. P. Nerkar, and A. Agrawal. 2014. Neurotoxicity and/or exacerbate neurocognitive disorders already seen in the context of HIV-1 infection. Additional studies are thus needed to shed light on this important issue and to define the risks and benefits of using a shock-and-kill strategy for the treatment of HIV-1–infected individuals.

References
1. Mahajan, H. S., M. S. Mahajan, P. P. Nerkar, and A. Agrawal. 2014. Neurotoxicity and/or exacerbate neurocognitive disorders already seen in the context of HIV-1 infection. Additional studies are thus needed to shed light on this important issue and to define the risks and benefits of using a shock-and-kill strategy for the treatment of HIV-1–infected individuals.

References
1. Mahajan, H. S., M. S. Mahajan, P. P. Nerkar, and A. Agrawal. 2014. Neurotoxicity and/or exacerbate neurocognitive disorders already seen in the context of HIV-1 infection. Additional studies are thus needed to shed light on this important issue and to define the risks and benefits of using a shock-and-kill strategy for the treatment of HIV-1–infected individuals.

References
1. Mahajan, H. S., M. S. Mahajan, P. P. Nerkar, and A. Agrawal. 2014. Neurotoxicity and/or exacerbate neurocognitive disorders already seen in the context of HIV-1 infection. Additional studies are thus needed to shed light on this important issue and to define the risks and benefits of using a shock-and-kill strategy for the treatment of HIV-1–infected individuals.


