Dolutegravir Disrupts Mouse Blood-Brain Barrier by Inducing Endoplasmic Reticulum Stress

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Research Article

Keywords:

Posted Date: May 24th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4420818/v1

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Additional Declarations: No competing interests reported.
Abstract

Background

Dolutegravir (DTG) - based antiretroviral therapy is the contemporary first-line therapy to treat HIV infection. Despite its efficacy, mounting evidence has suggested a higher risk of neuropsychiatric adverse effect (NPAE) associated with DTG use with a limited understanding of the underlying mechanisms. Our laboratory has previously reported a toxic effect of DTG comparable to efavirenz in disrupting the blood-brain barrier (BBB) integrity in vitro and in vivo. The current study aimed to investigate, in vitro, the potential mechanisms involved in DTG toxicity.

Methods

Primary cultures of mouse brain microvascular endothelial cells were used as a robust rodent BBB cell model. The cells were treated with DTG at therapeutic relevant concentrations (2500, 3500, 5000 ng/ml) for 3–48 h with or without the presence of three endoplasmic reticulum (ER) sensor inhibitors (GSK2606414, 4µ8c, 4PBA). RNA-sequencing, qPCR, western blot analysis and cell stress assays (Ca$_2^+$ flux, H$_2$DCFDA, TMRE, MTT) were performed.

Results

Our initial Gene Ontology (GO) analysis of RNA-Sequencing data revealed an enriched transcriptome signature of ER stress pathway in DTG treated cells. We further demonstrated that therapeutic concentrations of DTG significantly activated the ER stress sensor proteins (PERK, IRE1, p-IRE1) and downstream ER stress markers (eIF2$\alpha$, p-eIF2$\alpha$, Hspa5, Atf4, Ddit3, Ppp1r15a, Xbp1, spliced-Xbp1). In addition, DTG treatment resulted in a transient Ca$_2^+$ flux, an aberrant mitochondrial membrane potential, and a significant increase in reactive oxygen species in primary cultures of mouse brain microvascular endothelial cells. Furthermore, we found that prior cell treatment with 4PBA (a broad-spectrum ER stress inhibitor) significantly rescued DTG-induced downregulation of tight junction proteins (Zo-1, OcI, Cldn5), whereas GSK2606414 (a PERK inhibitor) elicited the greatest protective effect on DTG-induced elevation of pro-inflammatory cytokines and chemokines (Il6, Il23a, Il12b, Cxcl1, Cxcl2).

Conclusions

The current study provides valuable insights into DTG toxicological cell mechanisms, which may serve as a potential explanation of DTG-associated NPAEs in the clinic.

Background
The integrase strand transfer inhibitor (INSTI) dolutegravir (DTG)-based antiretroviral regimen is the contemporary recommended first-line drug regimen for the treatment of human immunodeficiency virus (HIV) infection. Despite its great potency, high barrier to resistance and tolerability, DTG-based antiretroviral therapy (ART) is known to induce neuropsychiatric adverse effect (NPAEs) including insomnia, dizziness, anxiety and depression [1]. Moreover, some recent clinical studies raised a potential association between ART and a slow progressive degeneration of cognitive and motor functions [2]. Despite evidence revealing the toxic potential of DTG in the central nervous system (CNS), the molecular mechanisms responsible for this observed toxicity are not well understood. Considering the lifelong requirement of ART treatment, a comprehensive assessment of the potential toxicity of the first line antiretroviral drugs (ARVs) is urgently needed [3].

The blood-brain barrier (BBB), primarily composed of brain microvascular endothelial cells sealed by tight junction (TJ) proteins is the major physiological barrier separating the brain from the systemic circulation and plays a critical role in maintaining CNS homeostasis [4]. BBB dysfunction can lead to disruption of the brain microenvironment and is widely implicated in multiple neurological diseases including dementia, depression and schizophrenia [5]. Recent work in our laboratory has demonstrated the potential of DTG to disrupt the BBB by downregulating TJ proteins inducing pro-inflammatory cytokines and altering expression of efflux transporters in various human and rodent BBB models [6]. To further understand the cellular mechanisms related to DTG toxicity in brain microvascular endothelial cells, RNA-sequencing was performed and revealed the endoplasmic reticulum (ER) stress as a major dysregulated pathway.

The ER is a large membrane-enclosed cellular compartment that is primarily responsible for protein synthesis and folding [7]. Physiological or pathological challenges such as increased secretory protein load or the presence of mutated proteins overloading ER capacity can result in ER stress, activating protective strategies, collectively termed the “unfolded protein response” (UPR) [7, 8]. The UPR in mammalian cells is activated by three ER transmembrane receptors: type I transmembrane protein inositol requiring 1 (IRE1α); protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) and an activating transcription factor 6 (ATF6) [8]. The UPR operates in multiple ways as a homeostatic mechanism to prevent further accumulation of unfolded proteins in the cells, which includes: i) increased ER folding capacity by transcriptionally upregulating ER-chaperones; ii) attenuation of secretory protein transcription and translation; and iii) ER-associated degradation of misfolded protein [7]. In addition to protein folding, ER serves as the largest site of cellular free Ca\(^{2+}\) storage, which plays a key role in ER-mitochondria crosstalk [9]. The presence of ER stress can result in Ca\(^{2+}\) dysregulation, and UPR acts as a critical adaptive mechanism to cope with Ca\(^{2+}\) imbalance [10]. Severe or prolonged ER stress can cause oxidative stress and ultimately lead to cell death by inducing mitochondrial membrane permeabilization [11], cytochrome c release and caspase activation [12]. This process is believed to be primarily mediated by the activation of the c-Jun N-terminal kinase (JNK) pathway [12], which has been attributed to IRE1/TRAIR2/ASK1 pathway activation in response to UPR failure [13].
In the context of HIV treatment, several ARVs, particularly efavirenz (EFV) (a non-nucleoside reverse transcriptase inhibitor) have been reported to induce ER stress by activating PERK and IRE1α receptors and autophagy dysfunction in brain microvascular endothelial cells [14]. Protease inhibitors ritonavir and lopinavir have also been reported to induce ER stress, oxidative stress and inflammatory response in human and mouse macrophages and hepatocytes [15, 16]. Several other events including alteration of Ca²⁺ homeostasis, cellular respiratory metabolism, mitochondrial function, and DNA replication have also been documented with EFV and some HIV-protease inhibitors [17, 18]. In contrast, the toxicity mechanisms of the first-line ARV, DTG, is not well understood in any cell types. Our laboratory recently reported the toxic potential of DTG in disrupting BBB at comparable levels to EFV [6]. The goal of the current study was to investigate ER stress as an underlying mechanism of DTG-induced toxicity at the BBB. The current study revealed a significant upregulation of ER stress and UPR associated with DTG treatment using primary cultures of mouse brain microvascular endothelial cells as a robust rodent BBB in vitro cell model.

**Materials and Methods**

**RNA Sequencing (RNA-Seq) and analysis**

Total RNA was isolated from primary cultures of mouse brain microvascular endothelial cells using TRIzol reagent (Invitrogen) treated with DTG at 5000 ng/ml for 24 h. RNA quality was first assessed by Agilent Fragment Analyzer, then was subjected to library preparation using the Illumina TruSeq Stranded mRNA Library Preparation Kit (RS-122-2101) according to the manufacturer’s instructions. Libraries fragment size was checked using an Agilent Fragment Analyzer, then quantified with Qubit and qPCR using Collibri™ Library Quantification Kit (ThermoFisher, Cat#A38524500) on a BioRad CFX96 Touch Real-Time PCR Detection System. Quality checked libraries were loaded onto an Illumina NextSeq500 running SR 75 cycles. Real-time base call (.bcl) files were converted to FASTQ files using Illumina bcl2fastq2 conversion software. Qiagen CLC Genomic Workbench v23.0.4 with default parameters was used for the differential gene expression data analysis. Principal components analysis (PCA) was performed with DEseq on expression data to observe patterns with respect to experimental factors [19]. Volcano plot was generated using false discovery rate-adjusted P value cut-off of < 0.05 and log₂ fold change cut-offs of <-1 or > 1. Gene set enrichment analysis (GSEA) of differentially expressed gene sets was conducted using "fgsea" R package 1.20.0 (version 4.1.0) using M5: Gene Ontology (GO) subcollection (v2023.2) [20].

**Reagents/materials**

All cell culture reagents were of the highest purity and obtained from Invitrogen (Carlsbad, CA, USA), unless indicated otherwise. Real-time quantitative polymerase chain reaction (qPCR) reagents, including reverse transcription cDNA kits and qPCR TaqMan primers, were purchased from Applied Biosystems (Foster City, CA, USA) and Life Technologies (Carlsbad, CA, USA), respectively. ER stress inhibitors GSK2606414, 4µ8c and 4PBA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and
PBS buffer were purchased from Sigma-Aldrich. Primary rabbit polyclonal anti-TJP1 (ZO-1; 402300), anti-OCLN (OCLN; 711500), anti-CLDN5 (CLDN5; 341600) and anti-Phospho-IRE1α (PA5-85738) antibodies were purchased from Invitrogen (Burlington, ON, Canada). Primary rabbit polyclonal anti-PERK (ab229912), anti-eIF2α (ab169528), anti-IRE1α (ab37073), primary mouse polyclonal anti-cytochrome c (ab110325), anti-VDAC1 (ab14734) antibodies, Fluo-8 Calcium Flux Assay Kit (ab112129) and Mitochondria/Cytosol Fractionation Kit (ab65320) were purchased from Abcam (Boston, MA, USA). Primary rabbit polyclonal anti-Phospho-eIF2α antibody (SAB4504388) Hoechst 33342 (14533) and H₂DCFDA (D6883) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Mouse monoclonal anti-β-actin (sc-47778) antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies and DTG were purchased from Jackson ImmunoResearch Inc. (Baltimore, PA, USA) and MedChem Express (Deer Park, NJ, USA), respectively. µ-Slide 8 Well (80826) were purchased from Ibidi (Grafelfing, Germany).

**Cell cultures**

Primary cultures of mouse (C57BL/6) brain microvascular endothelial cells were cultured (passage 2–6) in complete Mouse Endothelial Cell Medium (M1168, Cell Biologics Inc, Chicago, Illinois, USA), supplemented with vascular endothelial growth factor, endothelia cell growth supplements, heparin, epidermal growth factor, hydrocortisone, L-glutamine, antibiotic-Antimycotic Solution, and 5% FBS, and grown on gelatin-coated tissue culture plates. Cell lines were maintained in a humidified incubator at 37°C with 5% CO₂, 95% air with fresh medium replaced every 2–3 days. Cells were split using 0.25% trypsin-EDTA upon reaching 95% confluence.

**Cell viability assay**

Cell viability of primary cultures of mouse brain microvascular endothelial cells in the presence of DTG (1000–10000 ng/ml) was assessed using the MTT assay as previously published [21]. Briefly following 48 h treatment, cells were incubated for 2 h at 37°C with 2.5 mg/mL MTT in PBS. The resulting formazan content in each well was dissolved using DMSO and quantified by UV absorbtion at 580 nm using a SpectraMax 384 microplate reader (Molecular Devices, Sunnyvale, CA). Cell viability was assessed by comparing the absorbance of cellular reduced MTT in DTG-treated cells to that of vehicle (DMSO)-treated cells. MTT assays revealed that the viability of primary cultures of mouse brain microvascular endothelial cells was not significantly affected by DTG in a wide range of concentrations (1000–10000 ng/ml) including therapeutic relevant concentrations after 48 h exposure (Supplemental Fig. 1).

**Supplemental Fig. 1.** Cell viability was assessed by MTT assay in primary cultures of mouse brain microvascular endothelial treated with DTG (1000–10000 ng/ml) for 48 h. Results are presented as mean relative cell viability ± SEM normalized to the DMSO control from n = 3 independent experiments. One-way ANOVA with Bonferroni’s post-hoc test analysis was applied.

**ARVs and ER stress inhibitors treatment**
Confluent monolayers of primary cultures of mouse brain microvascular endothelial cells were treated with either DMSO (vehicle control), DTG (2500, 3500, 5000 ng/ml) or tunicamycin (3000 ng/ml) for a period of 3, 6, 16, 24 or 48 h at 37°C. Doses of DTG were carefully chosen to correspond to human therapeutic plasma levels [22]. The naturally occurring nucleoside antibiotic tunicamycin, known to behave as a strong ER stressor by inhibiting the biosynthesis of N-linked glycans in the proteins, was used as a positive control [23]. For experiments involving ER stress inhibitors, cells were pre-treated by GSK2606414 (5 µM; PERK inhibitor), 4µ8c (10 µM; IRE1α inhibitor) or 4PBA (2 mM; a broad inhibitor) for 6 h, following the treatment of DTG (5000 ng/ml) with specific inhibitors at desired concentrations for 24 or 48 h. At the desired time interval, treated cells were harvested and processed for subsequent assays.

Gene expression analysis

The mRNA expression of specific genes of interest was quantified using qPCR. Total RNA was isolated from cell samples (primary mouse BBB cells) using TRIzol reagent (Invitrogen) and treated with DNase I to remove contaminating genomic DNA. RNA concentration (absorbance at 260 nm) and purity (absorbance ratio 260/280) was assessed using NanoDrop One Spectrophotometer (Thermo Scientific). A total amount of 2 µg of RNA was then reverse transcribed to cDNA using a high-capacity reverse transcription cDNA kit (Applied Biosystems) according to the manufacturer’s instructions. Specific mouse primer pairs for Tjp1 (Zo-1; Mm01320638_m1), Ocln (Ocln; Mm00500912_m1), Cldn5 (Cldn5; Mm00727012_s1), Il6 (Il6; Mm00446190_m1), Cxcl1 (Cxcl1;Mm04207460_m1), Cxcl2 (Cxcl2;Mm00436450), Il23a (Il23α;Mm00518984), Il12β (Il12β,Mm01288989) were designed and validated by Life Technologies for use with TaqMan qPCR chemistry. Specific mouse primer pairs for spliced Xbp1 were customized using the following sequence <forward> 5’GCTGAGTCCGCAGCAGGT3’ <reverse> 5’CAGGGTCCAACTTGTCCAGAAT3’ and validated by Life Technologies with TaqMan qPCR chemistry. All assays were performed in triplicates with the housekeeping gene for mouse cyclophilin B Ppib (Ppib; Mm00478295_m1) or glyceraldehyde-3-phosphate dehydrogenase Gapdh (Gapdh; Mm99999915_g1) as an internal control. For each gene of interest, the critical threshold cycle (CT) was normalized to Ppib or Gapdh using the comparative CT method. The difference in CT values (ΔCT) between the target gene and cyclophilin B was then normalized to the corresponding ΔCT of the vehicle control (ΔΔCT) and expressed as fold expression ($2^{-ΔΔCT}$) to assess the relative difference in mRNA expression.

Cytosolic and mitochondrial protein isolation

Cytosolic and mitochondrial protein fractions were extracted from the vehicle, DTG or tunicamycin-treated primary mouse brain microvascular endothelial cells using the Mitochondria/Cytosol Fractionation Kit (ab65320, Abcam) according to the manufacturer’s protocol. Briefly, 4×10^7 cells were harvested, washed, and centrifuged at 1000 × g for 10 min at 4°C. Cells were then resuspended in cytosol extraction buffer, incubated on ice for 10 min, and homogenized on ice using a Dounce tissue grinder. The homogenate was centrifuged at 1000 × g for 10 min at 4°C. The supernatant was collected and centrifuged at 10,000 × g for 30 min at 4°C. The supernatant was used as the cytosolic fraction. The
pellet was resuspended in mitochondrial extraction buffer, vortexed for 10 sec, and used as the mitochondrial fraction.

**Western blot analysis**

Western blots were performed in accordance with our published protocol with minor modifications [24]. Cell lysates were obtained using modified RIPA buffer [50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM sodium o-vanadate, 0.25% (v/v) sodium deoxycholic acid, 0.1% (v/v) sodium dodecyl sulfate (SDS), 1% (v/v) NP-40, 200 µM PMSF, 0.1% (v/v) protease inhibitor]. Protein concentrations of the lysates were quantified using Bradford's protein assay (Bio-Rad Laboratories) with BSA as the standard. Total protein (50 µg) for each sample was loaded, separated on 10%, 12% or 14% SDS-polyacrylamide gel, and electro-transferred onto a polyvinylidene fluoride membrane overnight at 4°C. The blots were blocked for 1 h at room temperature in 5% skim milk Tris-buffered saline solution containing 0.1% Tween 20 and incubated with primary rabbit polyclonal anti-TJP1 (1:250), anti-OCLN (1:250), anti-CLDN5 (1:250), anti-PERK (1:1000), anti-IRE1α (1:1000), anti-pIRE1α (1:1000), anti-eIF2α (1:1000), anti-peIF2α (1:1000) antibodies, primary mouse polyclonal anti-Cytochrome c (1:2000), anti-VDAC1 (1:1000) antibodies, and murine monoclonal anti-β-actin antibody (1:2000) overnight at 4°C. The blots were then incubated with corresponding horseradish peroxidase-conjugated anti-rabbit (1:5000) or anti-mouse (1:5000) secondary antibody for 1.5 h. Protein bands were detected using enhanced chemiluminescence SuperSignal West Pico System (Thermo Fisher Scientific).

**Calcium assay and imaging**

Cytosolic Ca\(^{2+}\) was quantified using Fluo-8 Calcium Flux Assay Kit (Cat# ab112129, Abcam) according to manufacturer’s protocol. Briefly, the primary mouse brain microvascular endothelial cells were cultured overnight on 96-black well plates (1 x 10\(^5\) cells/well) or µ-Slide 8 Well chambered coverslips (3 x 10\(^4\) cells/well) prior to the assay. Cells were first incubated with Fluo-8 for 30 min at 37°C and then incubated at room temperature for 30 min. Spontaneous calcium activity was recorded prior to DTG challenge (time 0). Baseline signal was measured at Ex/Em = 490/525 nm using multi-mode microplate reader (Biotek, Life Science, Inc., USA). DTG or DMSO (vehicle control) was then added to the wells, and the calcium flux was monitored and recorded by the fluorescence intensity at Ex/Em = 490/525 nm every minute for 5 min. The DTG-induced amplitude change in the 490/525 nm fluorescence ratio was calculated by subtracting the baseline ratio before DTG challenge. For calcium imaging, pre-stimulation images were taken to establish baseline calcium in cells (time 0) by fluorescence microscopy (Zeiss Axio Observer Apotome-2) using a 40× objective. Time lapse images were then taken every 20 s for one minute following DTG challenge.

**Measurement of intracellular ROS generation**

Reactive oxygen species (ROS) generation was quantified by fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H\(_2\)DCFDA) according to the following published protocol with minor modification [25]. Briefly, primary mouse brain microvascular endothelial cells were seeded overnight on 96-black well plates (1 x 10\(^5\) cells/well) prior to the assay. Cells were incubated with
H$_2$DCFDA (20 µM in pre-warmed cell medium) for 30 min at 37°C and the washed by pre-warmed PBS. Cells were then treated by DTG, tunicamycin or vehicle control for 1 or 6 h. ROS content was then measured by acquiring the fluorescence intensity at Ex/Em = 494/522 nm using multi-mode microplate reader (Biotek, Life Science, Inc., USA). The DTG-induced change in the 494/522 nm fluorescence was calculated by subtracting the background fluorescence and then normalized to the vehicle control group.

**Measurement of MMP with TMRE**

Changes in mitochondrial membrane potential (MMP) was assessed by tetramethyl rhodamine ethyl ester (TMRE)-MMP assay kit according to manufacturer’s protocol (Cat# ab113852, Abcam, UK). In summary, the primary mouse brain microvascular endothelial cells were seeded overnight on 96-well black plates (2 x 10$^5$ cells/well) or µ-Slide 8 Well chambered coverslips (3 x 10$^4$ cells/well) prior to the assay. After exposure to DTG for 10 min or 6 h, cells were incubated with TMRE (600 nM) for 25 min and washed twice with warm PBS/0.2% BSA. Fluorescence intensity was measured at Ex/Em = 549/575 nm using multi-mode microplate reader (Biotek, Life Science, Inc., USA). FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone), an ionophore uncoupler of oxidative phosphorylation was used as a positive control in each experiment [26]. Cells were treated by FCCP for 10 min prior to the TMRE incubation.

**Data Analysis**

All experiments were repeated 3–4 times using cells obtained from different passages. Results are presented as mean ± SEM. All statistical analyses were performed using Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance between two groups was assessed by two-tailed Student’s t test for unpaired experimental values. Multiple group comparisons were performed using one-way analysis of variance (ANOVA) with Bonferroni’s post-hoc test. p < 0.05 was considered statistically significant.

**Results**

**Bulk mRNA sequencing in primary cultures of mouse brain microvascular endothelial cells revealed activated ER stress as a result of DTG toxicity.**

To investigate the alteration of transcriptomic profile by DTG treatment in primary cultures of mouse brain microvascular endothelial cells, RNA-seq and subsequent gene set enrichment analysis was performed. PCA analysis revealed that the two sample groups (DMSO vs. DTG treated) cluster separately, indicating distinct transcriptomic signatures due to the treatment of DTG (Fig. 1A). A volcano plot was generated as determined by False Discovery Rate (FDR) based on differential gene expression analysis, revealing key genes ($Il6$, $Hspa5$, $Cxcl2$, $Atf4$, $Xbp1$) involved in inflammatory and ER stress response were significantly upregulated in DTG treatment group compared to control (Fig. 1B). The transcriptome signature in DTG treated cells was enriched for gene ontology (GO) molecular function...
DTG induces ER stress by activating PERK signaling pathway in primary cultures of mouse brain microvascular endothelial cells.

To examine whether DTG activates PERK signaling pathway in a dose-dependent and time-dependent manner, primary cultures of mouse brain microvascular cells were treated with DTG at 2500, 3500, 5000 ng/ml or tunicamycin at 3000 ng/ml for 3, 6, 16, 24 and 48 h. Compared to DMSO control, PERK protein expression was also significantly upregulated (~ 50%) by DTG (5000 ng/ml) following 24 or 48 h exposure (Fig. 2A). DTG (5000 ng/ml) treatment resulted in a time-dependent upregulation of p-eIF2α protein with greater elevation observed at 48 h (~ 50%) compared to 24 h (~ 25%) (Fig. 2A). Although DTG at 2500 ng/ml did not affect PERK or p-eIF2α protein expression, it significantly increased the gene expression of Hspa5 (BiP), Ddit3 (CHOP) and Ppp1r15a (GADD34) in a time-dependent manner. Consistent with this, stronger induction of Hspa5, Ddit3 Ppp1r15a gene expression was observed at 3500 ng/ml DTG together with a significant increase in Atf4 gene expression in a time-dependent manner (Fig. 2B). Finally, DTG at 5000 ng/ml resulted in a robust upregulation of Hspa5, Atf4, Ddit3 and Ppp1r15a gene expression at all three time points (3, 6 and 16 h) with a peak response observed at 6 h (Fig. 2B). The positive control tunicamycin robustly activated PERK pathway as expected (Fig. 2A, Fig. 2B).

DTG induces ER stress by activating IRE1 signaling pathway in primary cultures of mouse brain microvascular endothelial cells.

To examine whether DTG activates IRE1α signaling pathway in a dose and time-dependent manner, primary cultures of mouse brain microvascular cells were treated with DTG at 2500, 3500 and 5000 ng/ml or tunicamycin at 3000 ng/ml for 3, 6, 16, 24 and 48 h. A mild but significant increase (~ 30%) of p-IRE1α protein expression was observed following DTG treatment at 2500 ng/ml for 24 h. Consistent with this, p-IRE1α protein upregulation was more robust at DTG concentration of 5000 ng/ml (~ 2 fold) at 24 h; and returned to basal level after 48 h. As expected, the positive control tunicamycin robustly activated p-IRE1α protein expression after 24 and 48 h as expected (Fig. 3A). The gene expression of Xbp1 was significantly upregulated following DTG treatment at 2500, 3500 and 5000 ng/ml at all three time points (3, 6, 16 h) in a dose-dependent manner, with the highest response observed at 6 h. The gene expression of the activated form of Xbp1 (spliced-Xbp1) was mildly increased by DTG 2500 ng/ml after 16 h, and by DTG 3500 ng/ml at an earlier time point (6 h) (Fig. 3B). Notably, DTG 5000 ng/ml robustly increased the Xbp1 and s-Xbp1 gene expression at a comparable level as induced by the positive control tunicamycin (Fig. 3B).

ER stress inhibitors mitigated the DTG-induced downregulation of TJ proteins in primary cultures of mouse brain microvascular endothelial cells.
To further investigate the role of ER stress in DTG-induced downregulation of TJ proteins, primary cultures of mouse brain microvascular cells were pre-treated with 3 different ER stress inhibitors: GSK2606414 (PERK inhibitor); 4µ8c (IRE1α inhibitor) and 4PBA (a broad inhibitor) separately for 6 h following DTG treatment (5000 ng/ml) for 24 or 48 h. The expression of TJ protein was examined at both gene (24 h) and protein (48 h) levels. In agreement with our previous report, DTG 5000 ng/ml robustly downregulated the gene expression of \textit{Tjp1} (Zo-1), \textit{Ocln} (Ocln) and \textit{Cldn5} (Cldn5) by > 60% following 24 h of treatment (Fig. 4A). The observed downregulation of TJ protein expression was significantly mitigated by three inhibitors at the gene level, with the rescue effect being the most noticeable following 4PBA pre-treatment (Fig. 4A). In parallel with the gene expression data, 48 h DTG (5000 ng/ml) treatment significantly downregulated the protein expression of Zo-1 and Ocln (Fig. 4B). The downregulation of Zo-1 and Ocln protein was fully rescued by 4PBA pre-treatment. Though GSK and 4µ8c also appeared to mitigate the DTG-induced downregulation of Zo-1 and Ocln, this effect did not reach statistical significance (Fig. 4B). Unlike the gene expression data, DTG treatment (5000 ng/ml) did not significantly affect Cldn5 protein expression (Fig. 4B).

**ER stress inhibitors mitigated the DTG-induced upregulation of pro-inflammatory cytokines and chemokines at the gene level in primary cultures of mouse brain microvascular endothelial cells.**

To further investigate the role of ER stress in DTG-induced inflammatory responses, primary cultures of mouse brain microvascular cells were pre-treated with 3 different ER stress inhibitors: GSK2606414 (PERK inhibitor); 4µ8c (IRE1α inhibitor) and 4PBA (a broad inhibitor) separately for 6 h following DTG treatment (5000 ng/ml) for 24 h. The expression of pro-inflammatory cytokines and chemokines was examined at the gene level. Consistent with our previous data, DTG 5000 ng/ml robustly induced the gene expression of \textit{Il6} (~ 23 fold), \textit{Il23a} (~ 30 fold), \textit{Il12b} (~ 20 fold), \textit{Cxcl2} (~ 15 folds) and mildly induced the gene expression of \textit{Cxcl1} (~ 2 fold) after 24 h treatment (Fig. 5). The induction of \textit{Il6}, \textit{Il23a}, \textit{Il12b}, and \textit{Cxcl1} gene expression was mitigated by ~ 50% following ER stress inhibitors 4PBA and 4µ8c pre-treatment (Fig. 5). GSK pre-treatment, specifically, rescued the \textit{Il6}, \textit{Il23a}, \textit{Il12b}, \textit{Cxcl1} and \textit{Cxcl2} gene upregulation to a similar level as the control group (Fig. 5).

**DTG treatment induced Ca^{2+} release in primary cultures of mouse brain microvascular endothelial cells.**

Dysregulation of cytosolic Ca^{2+} level is known to be associated with ER stress. To further characterize the mechanisms and downstream effects of DTG-induced ER stress, cytosolic Ca^{2+} level was measured in primary cultures of mouse brain microvascular cells 1–5 min following DTG exposure (2500, 5000 ng/ml) using Fluo-8 Ca^{2+} assay kit. The data demonstrated that, the cytosolic Ca^{2+} level was transiently increased in the presence of DTG (2500, 5000 ng/ml) within the first minute post DTG challenge, quickly reaching a plateau and remaining stable after that (Fig. 6A, Fig. 6B). To better characterize the timing of this Ca^{2+} increase, samples were dynamically examined by immunofluorescence microscopy following the DTG challenge. Ca^{2+} induction was first observed as early as 20 s after DTG challenge, persisted at 40 s and gradually reached a plateau by 60 s. The effect was more pronounced with DTG at 5000 ng/ml compared to 2500 ng/ml (Fig. 6C).
DTG disrupted MMP, induced ROS generation but did not induce cytochrome-c release from mitochondria in primary cultures of mouse brain microvascular endothelial cells.

To further investigate whether DTG-induced ER stress results in the alteration of mitochondrial bioenergetics, MMP was assessed by TMRE assay kit using a microplate reader in primary mouse brain microvascular cells treated with DTG (2500, 5000 ng/ml). Acute DTG treatment for 10 min at 2500 or 5000 ng/ml did not demonstrate any significant change in relative MMP. By contrast, prolonged DTG treatment (6 h) at both 2500 and 5000 ng/ml resulted in a significant decrease in MMP compared to controls, as indicated by the dose-dependent reduction in TMRE fluorescence (Fig. 7A). FCCP, an ionophore uncoupler, was used as a positive control in this assay, and resulted in an aberrant MMP after 10 min-treatment as expected (Fig. 7A). To investigate whether the observed ER stress and elevated UPR led to oxidative stress, ROS was quantified by a microplate reader using H$_2$DCFDA assay. Despite the unaltered MMP, a significant increase (~ 30%) of ROS content was observed following DTG treatment at 2500 ng/ml for 1 h, with a greater ROS induction (~ 50%) observed at 5000 ng/ml. Also, the DTG-induced ROS generation was time-dependent, as reflected by a greater increase in ROS (~ 2 fold) after a 6 h at 5000 ng/ml (Fig. 7B). However, a 48 h-treatment of DTG at 5000 ng/ml did not induce the cytochrome-c translocation from the mitochondria to cytosol, whereas such effect was observed with tunicamycin as expected (Fig. 7C).

**Discussion**

Owing to the great efficacy and tolerability in suppressing viral replication, DTG - based ART is one of the recommended first-line regimens to treat HIV infected individuals worldwide, including pregnant women [27]. However multiple clinical reports have revealed a significantly higher incidence of NPAEs associated with DTG than other INSTIs [1, 28]. The underlying mechanisms of DTG-induced toxicity remain largely unknown. Recently our laboratory reported an unexpected toxic potential of DTG in disrupting the BBB using several human and mouse BBB models *in vitro*, *ex vivo* and *in vivo* [6]. In the present study, our initial RNA-sequencing data illustrates an enrichment of transcriptome signature related to ER stress. We have therefore aimed to further investigate ER stress as a potential underlying mechanism of DTG toxicity at the BBB.

ER stress has been reported with the use of multiple ARVs particularly EFV, some nucleoside reverse transcriptase inhibitors (abacavir, lamivudine) and protease inhibitors (lopinavir, ritonavir) in various CNS cell culture systems including human brain microvascular endothelial cells (hCMEC/D3) and primary cultures of human astrocytes [14, 29]. To the best of our knowledge, our current study demonstrates for the first time that clinically relevant concentrations of the first line INSTI, DTG induces ER and oxidative stress, inflammatory responses, cytosolic Ca$^{2+}$ imbalance and mitochondrial bioenergetic alteration in primary cultures of mouse brain microvascular endothelial cells, which together suggest the underlying mechanism by which DTG induces toxicity at the BBB.
PERK and IRE1α are the two major ER stress transducers of UPR response [12]. The PERK protein is activated by phosphorylation and dimerization in response to the accumulation of misfolded/unfolded protein, which leads to the phosphorylation of the eukaryotic Initiation Factor 2 alpha (eIF2α) and promotes the transcription/translation of the Activating Transcription Factor 4 (ATF4) [30]. Prolonged or excessive levels of ER stress stimulates the genes of CCAAT-enhancer-binding protein homologous protein (CHOP) by activating ATF4 which plays a key role in the initiation of apoptosis [31]. Similar to PERK, the IRE1α protein is activated by autophosphorylation in response to overloaded misfolded/unfolded protein, which will induce a splicing event of a transcription factor X-box binding protein 1 (XBP1) mRNA as an adaptive mechanism to cope with cell stress [12]. Alternatively in response to excessive ER stress and UPR failure, activated IRE1α can lead to cell death by triggering pro-apoptotic cascades through activation of the c-Jun N-terminal kinase (JNK) pathway [32].

In this study we showed that a therapeutic relevant concentration of DTG (5000 ng/ml) activates PERK by significantly upregulating the protein expression of PERK and phosphorylated-eIF2α at 24 and/or 48 h in primary cultures of mouse brain microvascular endothelial cells. Despite the absence of significant upregulation of PERK or p-eIF2α protein by DTG 2500 ng/ml, our gene expression data revealed a dose- and time-dependent induction of downstream chaperone, pro-apoptotic and transcription factor genes (Ddit3, Hspa5, Atf4, Ppp1r15a) by DTG at 2500, 3500 and 5000 ng/ml. Similarly, the IRE1α pathway was activated by both concentrations of DTG (2500, 5000 ng/ml), as reflected by significant upregulation of phosphorylated IRE1α protein expression at 24 h as well as a dose-dependent and time-dependent induction of Xbp1 and s-Xbp1 mRNA expression. Notably, the induction of pro-apoptotic genes as well as adaptive gene markers (Xbp1 and s-Xbp1) was modest with DTG treatment at 2500 ng/ml (median therapeutic plasma concentration) and 3500 ng/ml, but was more pronounced at the higher DTG concentration of 5000 ng/ml (Cmax), suggesting a potential adaptative UPR response at least below 3500 ng/ml.

Our recently published study demonstrated that DTG can result in BBB leakage by disrupting TJ proteins [6]. To investigate whether ER stress is responsible for the observed effects, we pre-treated the cells with three ER sensor inhibitors 6 h before DTG exposure. Inhibitor concentrations were carefully selected based on prior literature to ensure efficacy of inhibition, with effects of the inhibitors were assessed in inhibitor groups alone [14, 33]. Our gene expression data demonstrate that all three inhibitors elicited protective effects on DTG-induced Tjp1, Ocln and Cldn5 mRNA downregulation, with the most noticeable protective effects being observed with 4PBA (a broad inhibitor). We noticed the inhibitors alone upregulated the gene expression of TJ proteins, this was potentially due to the inhibition of intrinsic RNase activity of the ER sensor proteins, which has been previously well documented in the literature [14, 34]. In agreement with our gene expression data, western blotting further demonstrated that 4PBA pre-treatment successfully rescued the DTG-induced downregulation of Zo-1 and Ocln proteins. Even though some protective effects were also observed by the GSK and 4µ8c, these did not reach statistical significance. Overall, our data suggests that ER stress plays a key role in DTG-induced TJ proteins downregulation at the BBB.
Apart from structural damage, inflammatory response is another important aspect in the context of DTG toxicity at the BBB. To assess whether ER stress is also responsible for the DTG-induced inflammatory responses, the gene expression of several pro-inflammatory cytokines and chemokines was assessed in the presence or absence of the ER sensor inhibitors before the DTG exposure. Our data revealed that GSK, 4µ8c and 4PBA all significantly mitigated DTG-induced elevation of Il6, Il23a, Il12b, Cxcl1 and Cxcl2 gene expression. Interestingly in contrast to the TJ protein data where 4PBA elicited the most significant protective effect, the most noticeable rescue effect in the inflammatory response was observed in the presence of GSK (PERK inhibitor), suggesting PERK pathway being the major mediator in the context of DTG-associated inflammation, whereas the DTG-induced BBB structural damage related to TJ protein dysfunction appears to be resulted from general activation of ER sensors.

Cytosolic Ca\(^{2+}\) is one of the key regulators in ER stress and plays an important role in determining the cell fate [35]. A cytosolic Ca\(^{2+}\) imbalance can induce ER stress, and vice versa, the activated UPR response can aggravate the cytosolic Ca\(^{2+}\) dysregulation [35]. Our data demonstrated that DTG treatment (2500, 5000 ng/ml) resulted in a transient cytosolic Ca\(^{2+}\) surge in primary cultures of mouse brain microvascular endothelial cells. These results are in agreement with several published studies which have implicated ARV (EFV) with ER stress, evoking Ca\(^{2+}\) flux in various cell types including hepatocytes, neurons, and brain microvascular endothelial cells [14, 36, 37]. Interestingly DTG has also been reported to induce cytosolic Ca\(^{2+}\) in human erythrocytes and potentiate platelet activation [38]. Due to the important role of cytosolic Ca\(^{2+}\) in the crosstalk between ER and mitochondria, we next assessed DTG’s potential to induce mitochondrial dysfunction. Our data showed that a 6 h DTG (5000 ng/ml) treatment resulted in a rapid drop in MMP, but an acute treatment (10 min) of DTG did not produce any significant effect. These data suggest that DTG is unlikely to act as a direct mitochondrial uncoupler but attenuates mitochondrial bioenergetics as a result of cellular stress. This is in agreement with other studies reporting that some ARVs (EFV, 2′,3′-dideoxyinosine, tenofovir, ritonavir, lopinavir) treatment (4–24 h) resulted in a rapid drop in MMP in various cell types including human hepatoma cell line, primary cultures of human umbilical vein endothelial cells (HUVEC) and primary rat neuronal and glial cultures [37, 39–42]. While the underlying mechanism remains unknown, such changes are likely due to the ER-mitochondrial uncoupling resulting from ARVs-induced disruption of Ca\(^{2+}\) signal, which can subsequentially lead to impaired mitochondrial metabolism [43]. Interestingly, DTG has been reported as a potential Ca\(^{2+}\) chelator [38]. Whether the DTG’s chelating potential may affect Ca\(^{2+}\)-regulated ER-mitochondrial coupling which may lead to a differential toxicological mechanism from other ARVs [11, 38] is an interesting aspect that needs further investigation.

An aberrant MMP is closely associated with mitochondrial dysfunction and oxidative stress [10]. To further investigate the DTG-mediated oxidative stress, we next sought to quantify the DTG-induced ROS generation with cytosolic cytochrome c level. Tunicamycin was used as a positive control due to its widely documented potential to induce ROS formation and cytochrome c release [44]. Our data demonstrate ROS was significantly elevated by DTG treatment at both 2500 and 5000 ng/ml doses 1 h and 6h post-treatment. Notably, similar to our mRNA and protein data, the ROS content was significantly
higher at $C_{\text{max}}$ DTG (5000 ng/ml), with a modest effect at 2500 ng/ml (median therapeutic plasma concentration), suggesting an adaptative UPR below 2500 ng/ml. Our results agree with others showing that several ARVs (EFV, ritonavir, lopinavir) could induce ROS production and impair mitochondrial function in multiple CNS cell types including neurons and oligodendrocytes in rodents [45, 46]. Importantly some clinical studies documented a higher oxidant level in the serum of HIV + individuals on ARVs compared to treatment naïve HIV + individuals and uninfected controls [47, 48], suggesting that the ARVs-induced oxidative stress is translatable to clinical conditions. It is worth noting that as a signalling molecule, ROS activation can serve as an adaptive purpose in response to acute UPR or promote cell death after excessive or prolonged ER stress [44]. Here we demonstrated ROS generation shortly after DTG exposure. To further investigate whether DTG-induced ROS activation potentially leads to cell death, we then assessed the level of cytochrome c translocation, which acts as a key initiator in caspase activation [12]. Our data showed that 48 h DTG (5000 ng/ml) treatment did not result in a significant increase in cytosolic cytochrome c level when compared to tunicamycin, which is known to provoke cytochrome c cytoplasmic release to initiate apoptosis [49]. Similarly, the MTT assay did not reveal any significant changes in cell viability following any DTG treatment (up to 10000 ng/ml) after 48 h. Together, these data suggest that despite the high level of cell stress and inflammatory response, DTG at $C_{\text{max}}$ (5000 ng/ml) did not lead to subsequent endothelial cell death.

**Conclusion**

In conclusion, this study demonstrates that DTG disrupts the BBB via induction of ER and oxidative stress, inflammatory response, and an alteration of mitochondrial bioenergetics. The current work provides insight into the underlying mechanisms responsible for DTG-induced toxicity in the CNS, which serve as a potential explanation for the high incidence of NPAEs associated with DTG use in the clinic; and further reveals that elevated DTG plasma concentrations could present a potential risk factor for DTG-induced toxicity in the CNS. In the clinic, a dose adjustment during ongoing DTG - based ART treatment may be beneficial to alleviate DTG-induced NPAEs.

**Abbreviations**

- **ART**
  antiretroviral therapy
- **ARV**
  antiretroviral drugs
- **ATF6**
  activating transcription factor 6
- **BBB**
  blood-brain barrier
- **CNS**
  central nervous system
Declarations
Availability of data and materials

The data that support the findings of this study are available in the Materials and Methods, Results, and/or Supplemental Material of this article or from the corresponding author on reasonable request.

Ethics declarations

Ethics approval and consent to participate

Not applicable. The current study does not involve any human or animal subjects.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported, in part, by the Canadian Institutes of Health Research (CIHR Grant# 511794) and the Ontario HIV Treatment Network (OHTN Grant# 506657) awarded to Dr. Reina Bendayan.

Authors’ Contributions

CH, QRQ, MTH performed the experimental work, RB conceived the study and directed the research. CH, MTH, and RB drafted the manuscript. All authors have read and approved the final version of the manuscript.

Acknowledgements

The authors thank Network Biology Collaborative Center (NBCC), Mount Sinai Hospital (University of Toronto, Canada) and Yiyan Wu (University of Toronto, Canada) for the help in RNA-Sequencing and data analysis. We acknowledge Dr. Jeffery Henderson (University of Toronto, Canada) for his help in experimental design and manuscript review.

References


**Figures**
Figure 1

Bulk mRNA sequencing primary cultures of mouse brain microvascular endothelial cells treated by DMSO or DTG (5000 ng/ml) for 24 h. (A) Principal component plot of the first and second components from PCA analysis in these samples (n = 3). (B) Volcano plot illustrates differential gene expression between control and DTG-treated groups defining significantly upregulated or downregulated genes as those with \( \log_{2} FC > 2 \) and \( \log_{10} FDR > 1 \). (C) Dot plot: gene ontology (GO) analysis to identify key molecular function (MF), biological process (BP) and cellular component (CC). Dot size: number of genes in data attributed to each GO term; dot color: red indicates upregulated gene sets by DTG-treatment; blue indicates downregulated gene sets by DTG-treatment.
Figure 2

Protein and gene expression of ER stress markers was assessed by immunoblotting and qPCR in primary cultures of mouse brain microvascular endothelial cells treated with DTG (2500, 3500, 5000 ng/ml) or tunicamycin (Tuni) (3000 ng/ml; positive control) for 24 or 48 h, respectively. (A) Representative immunoblots and densitometric analysis of PERK and p-eIF2α protein expression. Western blot analysis was applied using specific antibodies to detect the protein expression of PERK, p-eIF2α and eIF2α; b-actin was used as a loading control. (B) The mRNA expression of Hspa5, Atf4, Ddit3 and Ppp1r15a genes were assessed by qPCR normalized to the housekeeping mouse Gapdh gene. Results are presented as mean relative mRNA or protein expression ± SEM normalized to the DMSO control from n=3-4 independent experiments. One-way ANOVA with Bonferroni’s post-hoc test analysis was applied, * p < 0.05; ** p < 0.01, *** p < 0.001.
Figure 3

Protein and gene expression of ER stress markers was assessed by immunoblotting and qPCR in primary cultures of mouse brain microvascular endothelial cells treated with DTG (2500, 3500, 5000 ng/ml) or tunicamycin (Tuni) (3000 ng/ml; positive control) for 24 or 48 h, respectively. (A) Representative blots and densitometric analysis performed on p-IRE1α protein expression level. Western blot analysis was applied using specific antibodies to detect the protein expressions of p-IREα and IREα; β-actin was used as a loading control. (B) The mRNA expression of Xbp1 and sXbp1 genes was assessed by qPCR normalized to the housekeeping mouse Gapdh gene. Results are presented as mean relative mRNA or protein expression ± SEM normalized to the DMSO control from n=3-4 independent experiments. One-way ANOVA with Bonferroni’s post-hoc test analysis was applied, * p < 0.05; ** p < 0.01.
ER sensor inhibitors (GSK; 4PBA; 4μ8c) mitigated the DTG-induced downregulation of TJ proteins. **(A)** Gene expression of TJ proteins was assessed by qPCR in primary cultures of mouse brain microvascular endothelial cells treated with DTG (5000ng/ml) for 24 h in the presence or absence of the ER sensor inhibitors. The mRNA expression is normalized to the housekeeping mouse *Gapdh* gene. **(B)** Representative immunoblots and densitometric analysis performed on Zo-1, Ocln and Cldn5 protein expression levels. Western blot analysis was applied using specific antibodies to detect the protein expression of Zo-1, Ocln and Cldn5; β-actin was used as a loading control. Results are presented as mean relative mRNA or protein expression ± SEM normalized to the DMSO control from n=3-4 independent experiments. One-way ANOVA with Bonferroni’s post-hoc test analysis was applied, * *p* < 0.05; ** *p* < 0.01. ****, *p* < 0.0001.
ER sensor inhibitors (GSK; 4PBA; 4μ8c) mitigated the DTG-induced proinflammatory cytokines and chemokines elevation. Gene expression of *Il6, Il23a, Il12b, Cxcl1, Cxcl2* was assessed by qPCR in primary cultures of mouse brain microvascular endothelial treated with DTG (5000ng/ml) for 24 h in the presence or absence of the ER sensor inhibitors. The mRNA expression is normalized to the housekeeping mouse Gapdh gene. Results are presented as mean relative mRNA expression ± SEM normalized to the DMSO control from n=4 independent experiments. One-way ANOVA with Bonferroni’s post-hoc test analysis was applied, * p < 0.05; ** p < 0.01; ***, p < 0.001. **** p < 0.0001.
Figure 6

Cytosolic Ca\(^{2+}\) level was assessed by fluo-8 calcium flux assay in primary cultures of mouse brain microvascular endothelial cells treated with DTG (2500, 5000 ng/ml) at various time points. (A) Cytosolic Ca\(^{2+}\) level was monitored by measuring the fluo-8 fluorescence within the first 1-5 min of post DTG challenge by a microplate reader. (B) Statistical analysis was performed by subtracting basal fluorescence and then normalized to untreated control. (C) Cytosolic Ca\(^{2+}\) level was recorded every 20 s within the first minute post DTG challenge by fluorescence microscopy. The arrows indicate the cytosolic Ca\(^{2+}\) flux. Nuclei were stained by Hoechst. Results are presented as mean fluorescence ± SEM normalized to the DMSO control from n=3 independent experiments. One-way ANOVA with Bonferroni's post-hoc test analysis was applied, * \(p < 0.05\); ** \(p < 0.01\).
Figure 7

MMP, ROS content and cytochrome c translocation were assessed in primary cultures of mouse brain microvascular endothelial treated with DTG at various time points. (A) MMP was assessed by TMRE assay kit in cells treated with DTG (2500, 5000ng/ml) or FCCP (20 μM; positive control) for 10 min and 6 h. Fluorescence intensity was measured by microplate reader, normalized, and compared to DMSO control, n = 4 independent experiments. (B) ROS content was measured by H2DCFDA assay in cells treated with DTG (2500, 5000 ng/ml) or tunicamycin (Tuni) (3000 ng/ml; positive control) for 1 and 6 h. Fluorescence intensity was measured by microplate reader, normalized, and compared to DMSO control, n = 3 independent experiments. (C) Cytochrome c expression in the cytosolic and mitochondrial cell fractions was assessed by western blot analysis in primary cultures of mouse brain microvascular endothelial treated with DTG (5000ng/ml) or tunicamycin (Tuni) (3000 ng/ml; positive control) for 48 h. Representative blot and densitometric analysis are shown, n= 3 independent experiments. One-way ANOVA with Bonferroni’s post-hoc test analysis was applied, * p < 0.05; ** p < 0.01. ****, p < 0.0001.

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