Original Contribution

Comparative analysis of ER stress response into HIV protease inhibitors: Lopinavir but not darunavir induces potent ER stress response via ROS/JNK pathway

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Abstract

HIV protease inhibitor (PI)-induced ER stress has been associated with adverse effects. Although it is a serious clinical problem for HIV/AIDS patients, comparative analyses of ER stress induction by clinically used PIs have rarely been done. Especially, there is no report on the differential ER stress response between lopinavir (LPV) and darunavir (DRV), although these PIs are the most clinically used PIs. We show here that LPV induces the most potent CHOP expression, ER stress marker, among the 9 Food and Drug Administration (FDA)-approved PIs in human peripheral blood mononuclear cells, several human epithelial cells, and mouse embryonic fibroblasts. LPV induced the most potent ROS production and JNK activation in 9 PIs. A comparison among the most clinically used PIs, ritonavir (RTV), LPV, and DRV, revealed that LPV potently and RTV moderately but not DRV induced ER stress via ROS-dependent JNK activation rather than proteasome inhibition. Finally, we analyzed ER stress induction in tissues of mice intraperitoneally injected with RTV, LPV, and DRV. RTV and LPV but not DRV showed ER stress induction in several mice tissues. In conclusion, we first identify LPV as the most potent ER stress inducing PI among 9 FDA-approved PIs in human cells, and although clinical verification is necessary, we show here that DRV has the advantage of less ROS and ER stress induction potential compared with LPV in vitro and in vivo.

Introduction

The combination antiretroviral therapy (cART) effectively improves HIV/AIDS patients’ life prognosis [1]. The HIV-1 protease inhibitor (PI) is a component of this combination therapy based on its suppressive effect on HIV-1 protease and viral maturation to block viral proliferation [2]. Various PIs have been developed in recent years. Ritonavir (RTV)-boosted lopinavir (LPV) and darunavir (DRV) are the most clinically used PIs for cART because these drugs have high binding affinity to HIV-1 protease and are highly active to drug-resistant HIV-1 [3-5]. But despite rigorous drug development, PIs do not completely cure HIV/AIDS, and HIV/AIDS patients are required to maintain long-term PI treatment, which causes serious side effects such as hyperlipidemia, diabetes, diarrhea, and atherosclerosis [6].

ER stress is a common molecular mechanisms for PI-induced side effects. Several PIs disrupt lipid metabolism in hepatocytes [7], induce apoptosis in pancreatic β cells to inhibit insulin secretion [8], activate the expression of inflammatory cytokines in macrophage [9], and disrupt the barrier integrity in intestinal epithelial cells [10] via ER stress induction as a common molecular mechanism. Although ER stress is thought to be important for the induction of several side effects by PIs, exhaustive comparison of clinically used PIs in terms of their potential to induce ER stress has not been done. Additionally, there is no report comparing the ER stress-inducing effects of LPV and DRV despite some clinical reports indicating the less adverse effects of DRV compared to those of LPV [11-15].

The accumulation of unfolded proteins, calcium disruption, and ROS production are well known triggers of ER stress [16]. Physiologically, ER stress correlates to several diseases such as diabetes,
hyperlipidemia, neurodegenerative disorder, autoimmune disease, and cancer [17]. Mammalian cells have a homeostasis response against ER stress, called ER stress response or unfolded protein response. ER stress activates several signaling molecules, PERK and IRE1, to activate the transcription factors XBP1, ATF4, and ATF6. These transcription factors regulate cellular transcription and translation to decide cellular responses including protein synthesis, cell cycle, and apoptosis regulation [16–19]. It was previously reported that several PIs induce ER stress via proteasome inhibition and or ROS production [20–22]. Therefore examining ER stress induced by PI is important for clarifying the mechanisms of PI-induced side effects.

In this study, first we screened the ER stress induction potential of 9 Food and Drug Administration (FDA)-approved PIs (SQV, saquinavir; RTV, ritonavir; IDV, indinavir; NVP, nelfinavir; APV, amprenavir; LPV, lopinavir; TPF, tipenavir; ATV, atazanavir; DRV, darunavir), and identified that LPV has the most potent ER stress induction potential among these PIs in human peripheral blood mononuclear cells, several human cell lines, and mouse embryonic fibroblasts. By a comparison between LPV and DRV, we clearly indicated that DRV does not induce ER stress and apoptosis. On the other hand, LPV induced ER stress and apoptosis, not by proteasome inhibition but by ROS-dependent JNK activation. Collectively, these results indicated that the most clinically used PIs, LPV and DRV, show clear differences in terms of ER stress and cytotoxicity induction potential.

Materials and methods

Reagents, plasmids, and antibodies

The reagents used are as follows: Nine PIs were obtained as previously described [23]. DCFH-DA was from Sekisui Medical (Tokyo, Japan). Ampex Red was from Invitrogen Japan (Tokyo, Japan). SP600125 and SB203580 were from Wako (Osaka, Japan). Ubiquitinated protein antibody was from Abcam (Cambridge, UK). Antibodies used are as follows: Antibodies for XBP1, ATF4, ATF6, CHOP, eIF2α, eIF2α phosphatase, caspase, JNK, JNK1/2, JNK, p38, phosphorylated-p38, ERK, phosphorylated-ERK, cleaved caspase-3, caspase-9, and HRP-conjugated anti-mouse or anti-rabbit IgG were from Cell Signaling Technology (Danvers, MA). HRP-conjugated anti-rat IgG was from DakoCytomation (Glostrup, Denmark).

Cell culture, treatment, and transfection

Human embryonic kidney cells, HEK293, human hepatoma cells, HepG2, lung adenocarcinoma cells, A549, human colorectal cells, HCT116, and mouse embryonic fibroblasts, MEF, were maintained as previously described [25]. All cell lines were cultured at 37 °C in a humidified atmosphere of 5% CO2. Treatment of cells with indicated doses of PIs was carried out for indicated times. For inhibition of caspase, JNK, p38, and eIF2α phosphatase, cells were pretreated with Q-VD-OPh, SP600125, SB203580, and salubrinal for 1 h before PI treatment, respectively. Transient transfections of plasmids were performed using Hilymax (Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's instruction. Stably transfected HEK293 cells were established by 500 μg/ml G418 treatment. Human peripheral mononuclear cells (PBMC) were collected from adult male donors after informed consent was obtained in accordance with the Declaration of Helsinki and based on a protocol approved by the Institutional Review Board of the Faculty of Medical and Pharmaceutical Sciences, Kumamoto University. PBMC was maintained in RPMI-1640 containing 10% FBS and antibiotics. Small interfering RNA (siRNA) for JNK1/2 (si-JNK1/2) was transfected into HEK293 cells using Trans-IT TKO (Mirus, Madison, WI) according to the manufacturer's instructions. A 50 nM JNK1/2 siRNA duplex was transfected into 70% confluent cells to knock down JNK1/2. GL2-luciferase (luc) siRNA duplex was used as a control. The cells were treated with HIV-PIs and harvested 48 h after transfection. The siRNA oligonucleotide sequences are as shown below. JNK1 siRNA sense, 5′-GACAUUUCAGAAUCAGACUU-3′; JNK1 siRNA antisense, 5′-AUGUCUGAUUGAAUGGGUC-3′; JNK2 siRNA sense, 5′-GAUGCUAACUAUGACGGUU-3′; JNK2 siRNA antisense, 5′-AACCUGAAUAUGACGAC-3′.

RT-PCR analysis

To analyze the XBP-1 splicing, semi-RT-PCR analyses for XBP1, XBP1s, and internal controls 18S ribosomal RNA (18S rRNA) were carried out as previously described [25]. The normalized gene expression values were expressed as the relative quantity of CHOP gene-specific messenger RNA (mRNA). The oligonucleotide primers used in quantitative RT-PCR are as shown below. Human CHOP-Fw, 5′-ATGGCGGAGATGCCATCTGCTTC3′; human CHOP-Rv, 5′-AGAAGCGGCTCAAGTGTTGAA-3′; human XBP1s-Fw, CCGCAGGCGTGAGG; human XBP1s-Rv, GAGTCATAA CCGCAGAATCCA; human 18S-Fw, 5′-CGGCTACCATCAGAAAGGAA-3′; human 18S-Rv, 5′-GTCGGTACCTACGCGGCCT-3′; mouse CHOP-Fw, 5′-CATTACCACACACAGGAG-3′; mouse CHOP-Rv, 5′-CGTTCGTATCTCGAG-3′; mouse 18S-Fw, 5′-GTAACCGGGTGACACACCTT-3′; mouse 18S-Rv, 5′-CCTACCCATGCTGAGG-3′.

Western blotting

For Western blotting analysis of XBP1s, ATF4, ATF5, and γ-tubulin, HEK293 cells were treated with PIs. Nuclear proteins were obtained as described previously [27]. For detection of XBP1s, a short form of the XBP1 band was separately detected from the full length of the XBP1 band by molecular size. For expression analysis of CHOP, cleaved caspase-3/4/9/12, Hsc70, or JNK, p38, ERK, and phosphorylated form, whole proteins were recovered as described previously [28]. For examining CHOP and Hsc70 in mice tissues, tissue proteins were lysed in glycerol buffer, described previously [29]. Protein lysates were subjected to SDS-PAGE and Western blotting. Blots were probed with the indicated antibodies, and visualized using Chemi-Lumi One Super (Nakarai Tesk, Kyoto, Japan).
Lactate dehydrogenase (LDH) assay

Cells were assayed for LDH release according to the protocol as described previously [30]. Lactate dehydrogenase release was expressed as percentage of LDH in the medium over the total LDH (medium and lysate). Values are means ± SE of triplicate testing for a representative experiment. At least two independent experiments were performed.

Fluorescence microscopy and flow cytometry

For detection of ER stress or ubiquitinated protein accumulation, we utilized pCAX-F-XBP1ΔDBD-venus plasmid or proteasome sensor vector. pCAX-F-XBP1ΔDBD-venus expresses green fluorescence protein on induction of ER stress via trans-activation by XBP-1 [31]. Proteasome sensor vector encodes ZsGreen conjugated with C-term of mouse-derived ornithine decarboxylase. This ZsGreen-conjugated protein half-life is 30 min and specifically degraded by proteasome immediately. Proteasome sensor vector expresses green fluorescence protein on induction of proteasome inhibition [32]. HEK293 cells seeded onto 12-well plates were transfected with 0.5 μg of pCAX-F-XBP1ΔDBD-venus plasmid or proteasome sensor vector. Twenty-four hours after transfection, cells were treated with Pls for 24 h. Cells were fixed, and venus- or ZsGreen-positive cells were detected by fluorescence microscopy using BIOREVO BZ-9000 (Keyence, Osaka, Japan).

For analysis of ROS production, HEK293 cells seeded onto 24-well plates were pretreated with DCFH-DA or Amplex Red for 1 h before PI treatment. After PI treatment for 30 min, DCFH-DA or Amplex Red-positive cells were detected. For quantification of DCFH-DA or Amplex Red fluorescence intensity, cells were recovered and analyzed by an LSR2 flow cytometer (BD Biosciences, SanJose, CA).

For Annexin V assay, apoptosis was measured by dual labeling with the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences Pharmingen). Briefly, after treatment with Pls, cells were harvested, washed, and then incubated with Annexin V-FITC and propidium iodide (PRI) for 20 min in the dark, before being analyzed on an LSR2 flow cytometer.

Luciferase reporter assay and protease activity assay

HEK293 cells seeded onto 12-well plates were transfected with 0.2 μg of pCAX-HA-2xXBP1ΔDBD (anATG)-LUC-F, pGL4-ATF6-reporter, and pGL4-ARE-reporter together with Renilla luciferase plasmid (pRL-TK; Promega). Renilla luciferase was used as control. For ATF4 reporter assay, HEK293 cells seeded onto 12-well plates were transfected with 0.2 μg of pCAX-hATF4(1-285)-hRL-HA together with Firefly luciferase plasmid (pGL3b; Promega) Twenty-four hours after transfection, cells were treated with Pls for another 24 h and luciferase activity was determined using a dual luciferase reporter assay system (Promega) as described previously [27]. For analysis of proteasome activity, HEK293 cells seeded onto 6-well plates were treated with Pls or bortezomib for 4 h. After washing with PBS solution, the cell pellet was lysed on ice for 15 min in 200 μl lysis buffer (25 mM Heps, 10 mM Na2HPO4-1H2O, 100 mM NaF, 5 mM EDTA, 2 mM Na3VO4, and 1% Triton X-100). After clearing cell debris by centrifugation at 4°C, the extract (25 μg) was subjected to proteasomal activity using the Proteasome-Glo cell-based assay (Promega) according to the manufacturer’s protocol and a previous report [33]. Briefly, each sample was incubated with three luminesogenic proteasome substrates such as Suc-LLVY-aminoluciferin (chymotrypsin-like), Z-LRR-aminoluciferin (trypsin-like), and Z-nLpLD-aminoluciferin (caspase-like) for 30 min. Following cleavage by the proteasome, each substrate for luciferase (aminoluciferin) is released, allowing the luciferase reaction to proceed and produce light. Luminescence was measured on a luminometer Centro XS 3LB960 (Berthold Japan KK, Tokyo, Japan).

Animals

Bablic male mice were housed in a vivarium in accordance with the guidelines of the animal facility center of Kumamoto University. The animals were fed with chow ad libitum. All experiments were performed according to the protocols approved by the Animal Welfare Committee of Kumamoto University (No. B24-140).

Statistical analysis

Data are presented as mean ± SE. For statistical analysis, the data were analyzed by one-way ANOVA with the Tukey–Kramer multiple comparison test or Student’s t test (JMP software, SAS Institute, NC, USA) as indicated in each figure legend. The differences were considered statistically significant when the P value was less than 0.05.

Results

Comparison of ER stress response and apoptosis induction by 9 Pls

Previous studies have indicated that several Pls activate the ER stress response, which correlates with Pls’ side effects [7–10]. However, comprehensive analysis of ER stress induction by clinically used Pls has not been done yet. Additionally there is no report investigating the effect of Pls on ER stress in peripheral blood mononuclear cells, although the target of Pls is PBMC which is a main reservoir of HIV-1. To address this issue, we first analyzed the CHOP expression, which is an ER stress marker, induced by 9 Pls (SQV, saquinavir; RTV ritonavir; IDV, indinavir; NFV, nelfinavir; APV, amprenavir; LPV, lopinavir; TPV, tipranavir; ATV, atazanavir; DRV, darunavir) in human PBMC derived from healthy donors (Fig. 1A), several human cell lines (Fig. 1B, C, D, and E), and MEF cells (Fig. 1F). Although all cell types showed different CHOP expressions against 9 Pls, respectively, LPV commonly showed the most potent ER stress induction in all cell types. On the other hand, IDV, TPV, and DRV have no effect in these cells (Fig. 1A, B, C, D, E, and F). Consistent with the protein level, LPV showed the highest CHOP mRNA expression (Fig. 1G). CHOP is an apoptosis inducer during ER stress [17]. Therefore we next analyzed the cell death induction by 9 Pls. LPV, SQV, and RTV showed significant cell death (Fig. 1H). Consistent with cell death induction, expression of apoptosis marker, cleaved-caspase-3, was detected in LPV-, SQV-, and RTV-treated cells (Fig. 1I). These results indicated that LPV has the most potent ER stress and apoptosis induction potential among 9 FDA-approved Pls in human and mouse cells.

RTV and LPV but not DRV induce apoptosis via ER stress

RTV, LPV, and DRV are the most clinically used Pls as the standard regimens (RTV-boosted LPV and DRV) because of their highly active antiviral effect for HIV-1 [11–13]. Based on our results, RTV and LPV but not DRV showed clear ER stress induction and cell death (Fig. 1). Thus we selected RTV, LPV, and DRV as representative Pls to investigate the extent of ER stress induction by Pls. To analyze ER stress and cytotoxicity induction by three Pls, we utilized HEK293 cell derived from normal human kidney cells which explained similar ER stress responses against 9 Pls to human PBMC (Fig. 1A and B). Our data showed that RTV and LPV induced CHOP expression and cytotoxic effects in a dose- and
time-dependent manner (Fig. 2A, B, D, and E). Consistent with cytotoxic effects by PIs, LPV potently and RTV moderately increased the expression of cleaved-caspase-3 (Fig. 2C and F). To confirm the early apoptosis cell population (AnnexinV+/PI−) and the late apoptosis/necrosis cell population (AnnexinV+/PI+) in RTV-, LPV-, and DRV-treated HEK293 cells, cells were stained with AnnexinV/PI. Consistent with LDH assay, LPV potently and RTV moderately but not DRV induced early and late apoptosis (Fig. 2G). Additionally, its cytotoxicity was inhibited by pan-caspase inhibitor treatment (Fig. 2H). On the other hand, DRV had no ER stress and cytotoxicity induction despite a high dose and lengthy treatment compared with RTV and LPV (Fig. 2A, B, C, D, E, and F). These results indicated that RTV and LPV but not DRV induced apoptotic cell death consistent with its ER stress induction. Next, we confirmed the contribution of ER stress on PI-induced cytotoxicity by using salubrinal, an ER stress-induced apoptosis inhibitor [34]. Interestingly, salubrinal inhibited the LPV-induced cytotoxicity in a dose-dependent manner (Fig. 2I) and also suppressed RTV-induced cytotoxicity (Fig. 2J). These results indicated that RTV and LPV but not DRV induced ER stress-dependent apoptosis.

**RTV and LPV but not DRV induce ER stress signaling**

Several PIs induce an ER stress response by activating ER stress signaling [10]. Therefore, we next aimed to clarify the differences of the mechanisms of ER stress signal induction by 3 PIs. First, we compared the effect of 3 PIs on XBP1s activation, which is the transcription factor activated by ER stress to induce an ER stress response such as CHOP induction [16]. The fluorescence microscopy indicated that RTV and LPV activated the XBP1s (Fig. 3A). To quantify the activation of ER stress signaling by PI treatment, we examined the luciferase activity using the ER stress-associated transcription factors XBP1s, ATF4, and ATF6 reporter plasmids. LPV potently and RTV moderately increased the XBP1s and ATF4 reporter activity (Fig. 3B and C), but did not affect ATF6 reporter activity (Fig. 3D). On the other hand, DRV had no effect on these transcriptional activities (Fig. 3A, B, C, and D). Next, we checked the expression of ER stress signaling molecules PERK, IRE1, and eIF2α, which are upstream molecules of XBP1s and ATF6, expressions were increased by RTV and LPV but not by DRV (Fig. 3E). Consistent with the reporter assay, XBP1s and ATF4 but not ATF6 were increased by RTV and LPV but not by DRV (Fig. 3F). Additionally we analyzed the XBP1 activation by examining the expression of the spliced form of XBP1 mRNA by semi-RT-PCR and Q-PCR. Consistent with the protein expression of XBP1s was induced by RTV and LPV but not by DRV (Fig. 3G and H). These results indicated that RTV and LPV but not DRV activate ER stress signaling to induce ER stress response via transcription factors XBP1s and ATF4.

**RTV and LPV but not DRV induce ROS production rather than proteasome inhibition**

It was previously reported that some PIs induce ER stress response by proteasome inhibition and/or ROS production [20–22].
Therefore, we first examined the proteasome inhibition activity of RTV, LPV, and DRV by using proteasome sensor vector which is the ZsGreen-conjugated substrate specifically degraded by proteasome immediately as noted under Materials and methods \[32\]. Proteasome-inhibited cells exhibited green fluorescence as shown in cells treated with 10 nM bortezomib, a clinically used proteasome inhibitor (Fig. 4A). However, green-fluorescent cells were not observed in cells treated with the PIs, suggesting that the PIs did not promote proteasome inhibition (Fig. 4A). Consistent with Fig. 4A, the PIs did not induce the accumulation of ubiquitinated proteins compared with bortezomib, as determined by immunoblotting (Fig. 4B). PIs did not suppress proteasomal protease activities (trypsin-like, chymotrypsin-like, caspase-like activity), although bortezomib clearly suppressed protease activities except for trypsin-like activity as previously reported \[33\] (Fig. 4C). These results indicated that proteasome inhibition is not involved in the activation of PI-induced ER stress. Therefore we next checked the expression of ROS production by DCFH-DA and Amplex Red staining which is the fluorescent ROS indicator \[35,36\]. Interestingly, the fluorescent microscopy and flow cytometry analysis indicated that RTV and LPV but not DRV induced the ROS production (Fig. 4D and E). Additionally, we examined the PI-induced oxidative stress by...
antioxidant response (ARE) reporter assay which monitors the oxidative stress response by transcription factor Nrf2. Consistent with ROS production, LPV potently and RTV moderately but not DRV induced oxidative stress (Fig. 4F). Comparison among 9 PIs on ROS production by DCFH-DA staining indicated that RTV and LPV clearly induced ROS production in 9 PIs (Fig. 4G). Amplex Red staining indicated that hydroxy peroxide production is induced by RTV and LPV but not by DRV (Fig. 4H). These results indicated that ROS production might be involved in the PI-induced ER stress rather than proteasome inhibition.

ER stress induced by LPV is dependent on ROS-induced JNK activation

To examine the effect of ROS production on ER stress response, we investigated the signaling cascade after LPV and DRV treatment (Fig. 5A). Interestingly, the activation of JNK/p38 was earlier than CHOP induction in LPV treatment. Especially, considering the activation of caspase pathway (caspase-3/9) contributing to apoptosis induction, JNK activation is relevant to the induction of apoptosis rather than p38 activation. Actually, the inhibition of JNK suppressed LPV-induced JNK activation and cell death in dose-dependent manner (Fig. 5B and C) but p38 inhibition did not protect the LPV-induced cell death (Supplementary Figure). Additionally, JNK inhibition suppressed CHOP induction by LPV (Fig. 5D). CHOP mRNA induction by RTV and LPV was also attenuated by JNK inhibition (Fig. 5E). To confirm the implication of JNK activation in CHOP induction by LPV, we also examined the effect of si-RNA for JNK1/2. JNK1/2 knock-down attenuated the CHOP induction consistent with JNK inhibition by NAC treatment (Fig. 5F and G). The suppression of ROS production by NAC (Fig. 5I) inhibited ER stress marker expression (CHOP and BIP), JNK and caspase-3 activation (Fig. 5I). On the other hand, DRV had no effect on these signaling cascade as expected (Fig. 5A). Collectively, these results indicated that the ROS-induced JNK activation is a clear correlator with LPV-induced ER stress and apoptosis.
To partially extend our in vitro observations to the in vivo system, we compared the CHOP protein expression in some tissues of RTV, LPV, and DRV intraperitoneally injected mice. Similar to results in vitro, the CHOP expression was increased in liver, kidney, and small intestine of LPV-injected mice in comparison with control (Fig. 6A, B, and C). RTV showed a slight increase of CHOP expression (Fig. 6A, B, and C). DRV did not up-regulate CHOP expression (Fig. 6A, B, and C). Additionally, we analyzed ER stress marker expression (CHOP and BIP), JNK, and caspase-3 activation in liver of mice treated with LPV by intraperitoneal injection for 3, 6, and 12 h. JNK and eIF2α activation and CHOP and BIP expression were induced by LPV in a time-dependent manner except for caspase-3 (Fig. 6D). Consistent with the protein level, LPV induced CHOP mRNA expression in liver of mice treated with LPV (Fig. 6E). These data suggested that LPV potently induces ER stress response and that ER stress induction is a critical difference between LPV and DRV in mouse tissue consistent with in vitro analysis.

Discussion

Previously, several basic studies indicated that PIs have adverse effects associated with ER stress in some tissues of mouse and cell types [7–10]. Clinical studies also indicated that the PI-induced side effects in long-term administrated patients are known as ER stress-associated diseases such as metabolic syndrome [11–13,15,37]. Because all previous studies used a limited selection of PIs and did not compare LPV and DRV, although these are the most clinically used PIs, we focused on 9 FDA-approved PIs for screening to identify
Fig. 5. HIV-PI-induced ER stress is dependent on ROS-induced JNK activation. (A) MAPK (p-JNK, JNK, p-ERK, ERK, p-p38, and p38), caspase-3/4/9/12, CHOP, and Hsc70 expressions were examined in 40 μM LPV or DRV treated for the indicated times in HEK293 cells. (B, C) HEK293 cells were untreated or treated with 40 μM LPV in the absence or presence of SP600125 (1, 5, 10, and 50 μM) for 6 h (B) or 24 h (C). p-JNK and JNK expressions were analyzed by Western blotting (B). Hsc70 was used as internal control. % LDH release was determined (C). Values are expressed as mean ± SE from triplicate tests. ***P < 0.001; **P < 0.01; *P < 0.05 versus LPV-treated sample, determined by ANOVA with Tukey–Kramer. (D) CHOP protein induction was examined in HEK293 treated with 40 μM LPV for 24 h in the absence or presence of 50 μM SP600125. (E) CHOP mRNA induction was examined in HEK293 treated with 40 μM RTV, LPV, and DRV in the absence or presence of 50 μM SP600125. (F) CHOP protein was examined in HEK293 cells transfected with 50 nM si-JNK1/2 or si-GL2 and untreated or treated with LPV for 24 h. Hsc70 was used as a loading control. (G) CHOP mRNA was examined in HEK293 cells transfected with 50 nM si-JNK1/2 or si-GL2 and untreated or treated with RTV, LPV, and DRV for 6 h. ***P < 0.001 vs the control group, assessed by Student's t test. (H) p-JNK, JNK, CHOP, BIP, and cleaved-caspase-3 expressions were examined in HEK293 treated with 40 μM LPV in the absence or presence of 1, 5, and 25 mM NAC. (I) ROS production was examined by 10 μM DCFH-DA activation in HEK293 treated with 40 μM LPV for 30 min in the absence or presence of 5 mM NAC by using flow cytometry. (J) p-JNK, JNK, p-p38, and p38 expressions were examined in HEK293 treated with 40 μM of 9 PIs for 6 h.
potent and non-ER stress-inducing PIs. The principal and novel finding of this research is that LPV shows the most potent ER stress induction among 9 FDA-approved PIs in different types of cells including human PBMC.

ER stress is one of the key pathogenic factors for a wide variety of disorders [17]. It has been previously associated with the development of metabolic disease [37], neurodegenerative disease [38], and autoimmune disease [39], via ER stress response by both translational and transcriptional mechanisms. Some clinical drugs induce ER stress as the mechanism of side effects such as nonsteroidal anti-inflammatory drugs [40], disease-modifying antirheumatic drugs [41], and anticancer drugs [42]. In this study, we identified that LPV is the most potent PI for ER stress induction among 9 FDA-approved PIs in several human cell lines and PBMC (Fig. 1). Except for LPV, some PI-induced ER stress was cell type dependent (Fig. 1). We also showed that the strength of RTV- and LPV-induced ER stress is dependent on tissues (Fig. 6). These results indicated that PI might induce different ER stress responses in cell types or tissues, and also explain other molecular mechanisms that correlate with induction of PI-induced side effects. It was previously reported that HIV-1 infection and HIV-1 accessory proteins Tat and Nef also induce ER stress [43,44]. Therefore, ER stress induction by HIV-1 infection might be accelerated by PIs and might be correlated with the development of metabolic syndrome in HIV/AIDS patients.

PIs induce ER stress via proteasome inhibition and or ROS production [20–22]. We showed here that LPV-induced ER stress mainly depends on ROS production (Fig. 4D, E, F, and 5G). On the other hand, DRV did not show any effects. Furthermore, the ROS-dependent JNK activation might be crucial for PI-induced ER stress and cytotoxicity (Fig. 5B, C, D, and 5E). Consistent with these results, a comparison among 9 PIs also indicated that ER-stress-inducing PIs showed clear ROS production and JNK activation (Figs. 4G and 5H). However, we could not clarify the mechanism of ROS production. Considering that ROS production is the key trigger of HIV-PI-induced ER stress, the investigation of the source of ROS and clarification of the ROS production mechanism are critical for HIV-PI research. Actually, several researchers focused on this subject. It was previously reported that HIV-PI-induced ROS production was suppressed by rotenone but not by NADPH oxidase blockage [45]. They concluded that mitochondrial ROS is the crucial source of ROS production by HIV-PIs rather than NADPH oxidase. Therefore, it is necessary to clarify the mechanism of mitochondrial ROS production by PIs for a clear understanding of PI-induced ER stress.

The therapeutic concentration of each PI is quite different among 9 PIs in a clinical setting. In this study we used the same concentration of PI to clearly show the ER stress induction potential of 9 PIs. Additionally, we utilized higher concentrations of PIs compared with therapeutic concentrations of PIs with the same strategy as previous reports to examine the molecular mechanisms [7–10]. To verify the contribution of ER stress induction to PI-induced side effects, it is necessary to examine lower or physiological concentrations of PIs as the blood concentration in HIV/AIDS patients. In this study, we are first to show that PIs could induced ER stress in PBMC (Fig. 1A). There is no report using human primary cells to analyze the effect of PIs on ER stress induction despite that the main target of PIs is PBMC including cells such as CD4 T cells and monocytes/macrophages which are HIV-1 main reservoirs. Therefore, by analyzing primary samples of HIV/AIDS patients such as PBMC, it is possible to monitoring the ER stress in treated patients. Now, we plan to do a comparative analysis using PBMC from PI-administrated patients as a clinical study.

In the case of PIs, its specificity for HIV-1 protease might be a key for deciding the frequency of side effects induction. DRV, which is a recently developed PI based on structure-based drug design strategy [46], showed the strongest binding affinity and inhibition efficiency to HIV-1 protease among 9 PIs including LPV [3]. Our present study indicated that DRV does not induce ER stress and cytotoxicity among 9 PIs (Fig. 1), which might explain the higher safety in comparison with LPV (Fig. 2). Clinical study also indicated that DRV has a higher inhibition efficacy for HIV-1 and shows a more favorable safety profile compared with LPV.
Combining our findings and previous studies, these clinical superiorities of DRV might be provided by the non-ROS and -ER stress induction potential of DRV based on its highly binding affinity to HIV-1 protease. PIs are crucial drugs for HIV/AIDS patients. Rapid drug development and accelerated approval are required for immediate stress induction. Therefore, it is not enough for clinical study of PIs compared with other therapeutic drugs. Thus the technical strategy for estimating or monitoring side effects will be useful for successful drug development of CART. Therefore, our ER stress monitoring system could be one of the estimating tools for drug development before human trials.

The present study is the first report indicating that LPV has the most potent PI for ER stress induction among 9 FDA-approved PIs, and that there is a critical difference between LPV and DRV on ER stress induction in vitro and in vivo. Considering the optimal cocktail for HIV/AIDS patients, the analysis of ER stress by PI could be an attractive strategy for estimating the safety of developing PI before human trials.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2013.08.161.

References


