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; NFV, nelfinavir; APV, amprenavir; LPV, lopinavir; TPV, tiprenavir; 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). Amplex Red was from Invitrogen Japan (Tokyo, Japan). SP600125 and SB203580 were from Wako (Osaka, Japan). Q-VD-OPh was from R&D Systems (Minneapolis, MN, USA). Salubrinal (Sal) was from Calbiochem (San Diego, CA). Bortezomib (Bor) was from Selleck Chemicals LLC (Houston, TX). *N*-Acetyl-L-cysteine (NAC) was from Sigma-Aldrich Co. (St. Louis, MO). Plasmids used are as follows: pCAX-F-XBP1ΔDBD-venus, pCAX-HA-2xXBP1ΔDBD (anATG)-LUC-F, and pCAX-hATF4(1-285)-hRL-HA. The pCAX plasmids were described previously [24]. pGL4-ARE-reporter (pGL4.37) and pGL4-ATF6-reporter (pGL4.39) plasmids were from Promega (Madison, WI). Proteasome sensor vector was from Clontech (Palo Alto, CA). Antibodies used are as follows: Antibodies for XBP1, ATF4, ATF6, CHOP, γ-tubulin, and HRP-conjugated anti-goat IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Caspase-12 and caspase-4 antibodies were from Millipore (Bedford, MA) and MBL (Nagoya, Japan), respectively. Ubiquitinated protein antibody was from AffinitiBioreagents (Golden, CO). eIF2α antibody was from Sigma-Aldrich Co. (St. Louis, MO). Antibodies for CHOP, PERK, IRE1, phoshorylated-eIF2α, JNK, phosphorylated-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% CO_2. 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′-GACCAUUUCAGAAUCAGACUU-3′; JNK1 siRNA antisense, 5′-AAGUCUGAUUCUGAAAUGGUC-3′; JNK2 siRNA sense, 5′-GAUGCUAACUUAUGUCAGGUU-3′; JNK2 siRNA antisense, 5′-AACCUGACAUAAGUUAGCAUC-3′. The negative control siRNA (MISSION siRNA Universal Negative Control; Sigma-Aldrich, Tokyo, Japan) was also used (con-si).

RT-PCR analysis

Total RNA isolation and quantitative RT-PCR (Q-PCR) analyses for CHOP, XBP1s, and internal controls 18S ribosomal RNA (18SrRNA) 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′-ATGGCAGCTGAGTCATTGCCTTTC-3′; human CHOP-Rv,5′-AGAAGCAGGGTCAAGAGTGGTGAA-3′; human XBP1s-Fw, CCGCAGCAGGTGCAGG; human XBP1s-Rv, GAGTCAATA-CCGCCAGAATCCA; human 18s-Fw,5′-CGGCTACCACATCCAAGGAA-3′; human 18s-Rv,5′-GCTGGAATTACCGCGGCT-3′; mouse CHOP-Fw, 5′-CATACACCACCACACCTGAAAG-3′; mouse CHOP-Rv, 5′-CCGTTTCCTA-GTTCTTCCTTGC-3′; mouse 18s-Fw,5′-GTAACCCGTTGAACCCCATT-3′; mouse 18s-Rv, 5′-CCATCCAATCGGTAGTAGCG-3′.

To analyze the XBP-1 splicing, semi-RT-PCR analyses for the human spliced form and the unspliced form of XBP-1 mRNA and internal control human GAPDH were carried out as previously described [26]. The oligonucleotide primers used in quantitative RT-PCR are as shown below. XBP1-Fw,5′-TTACGAGAGAAAACTCATGGCC-3′; XBP1-Rv,5′-GGGTCCAAGTTGTCCAGAATGC-3′; GAPDH-Fw, 5′-CGGGAAGCTT GTGATCAATGG-3′; GAPDH-Rv,5′-GGCAGTGATGGCATGGACTG-3′.

Western blotting

For Western blotting analysis of XBP1s, ATF4, ATF6, 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 their 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-XBP1DBD-venus expresses green fluorescence protein on induction of ER stress via transactivation 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 PIs 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 PIs, 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 (phRG-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 PIs 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 PIs 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 Hepes, 10 mM $Na_4P_2O_7 \cdot 10H_2O$, 100 mM NaF, 5 mM EDTA, 2 mM Na_3VO_4, 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 luminogenic proteasome substrates such as Suc-LLVY-aminoluciferin (chymotrypsin-like), Z-LRR-aminoluciferin (trypsin-like), and Z-nLPnLD-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

Balb/c 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 PIs

Previous studies have indicated that several PIs activate the ER stress response, which correlates with PIs' side effects [7–10]. However, comprehensive analysis of ER stress induction by clinically used PIs has not been done yet. Additionally there is no report investigating the effect of PIs on ER stress in peripheral blood mononuclear cells, although the target of PIs 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 PIs (SQV, saquinavir; RTV ritonavir; IDV, indinavir; NFV, nelfinavir; APV, amprenavir; LPV, lopinavir; TPV, tiprenavir; 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 PIs, 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 PIs. 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 PIs 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 PIs 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 PIs to investigate the extent of ER stress induction by PIs. To analyze ER stress and cytotoxicity induction by three PIs, we utilized HEK293 cell derived from normal human kidney cells which explained similar ER stress responses against 9 PIs to human PBMC (Fig. 1A and B). Our data showed that RTV and LPV induced CHOP expression and cytotoxic effects in a dose- and

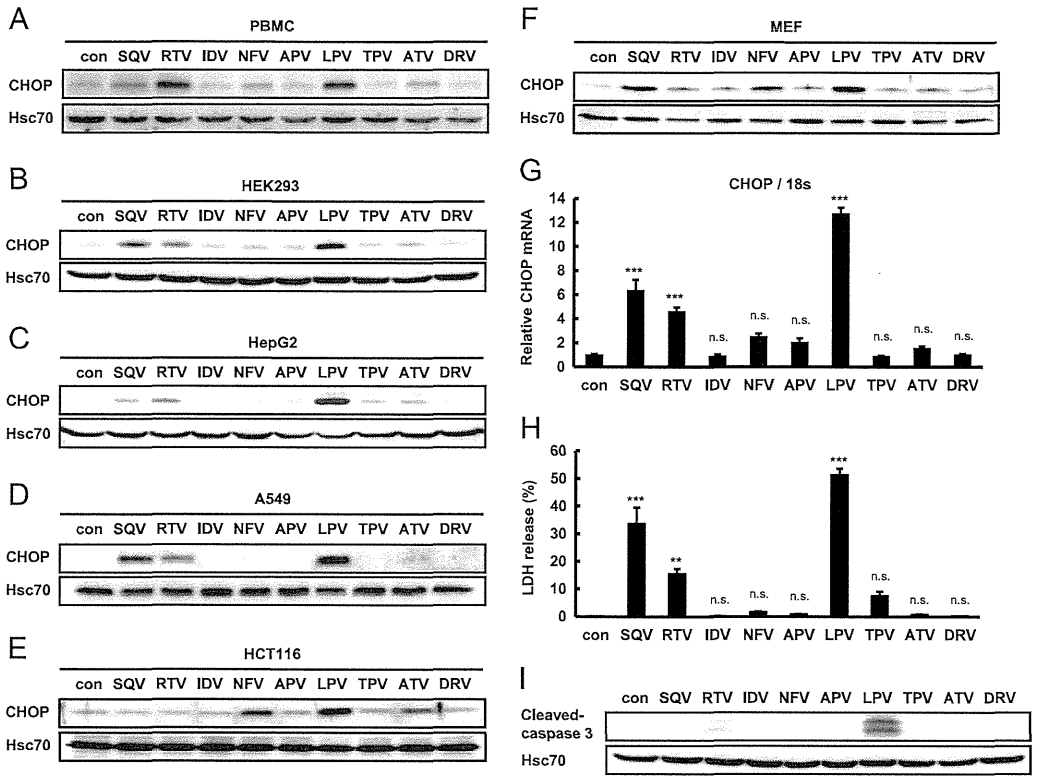

Fig. 1. Comparisons between ER stress response and apoptosis induction by 9 PIs.(A, B, C, D, E, F) CHOP protein expression was examined in PBMC (A), HEK293 (B), HepG2 (C), A549 (D), HCT116 (E), and MEF (F) cells treated with 40 μM of 9 HIV-PIs for 24 h. Hsc70 was used as internal control. (G) HEK293 cells were treated with 40 μM of 9 HIV-PIs for 6 h and total RNA from these cells was isolated for analysis of CHOP mRNA by Q-PCR. mRNA expression was normalized to human 18S RNA. Values are expressed as mean ± SE from triplicate tests. $^{***}P < 0.001$ versus control, determined by ANOVA with Tukey–Kramer. (H) HEK293 cells were treated with 40 μM of 9 HIV-PIs for 24 h. Cell death was measured by LDH assay, and % LDH release was determined. Values are expressed as mean ± SE from triplicate tests. $^{***}P < 0.001$; $^{**}P < 0.01$ versus control, determined by ANOVA with Tukey–Kramer. (I) Cleaved caspase-3 was examined in HEK293 cells treated with 40 μM of 9 HIV-PIs for 6h. Hsc70 was used as internal control.

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+/PRI−) and the late apoptosis/necrosis cell population (AnnexinV+/PRI+) in RTV-, LPV-, and DRV-treated HEK293 cells, cells were stained with AnnexinV/PRI. 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. PERK, IRE1, and phosphorylated-eIF2α, which are upstream molecules of XBP1s and ATF6, expressions were increased by RTV and LPV but not by DRV (Fig. 3E). Consist with the reporter assay, XBP1s and ATF4 but not ATF4 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].

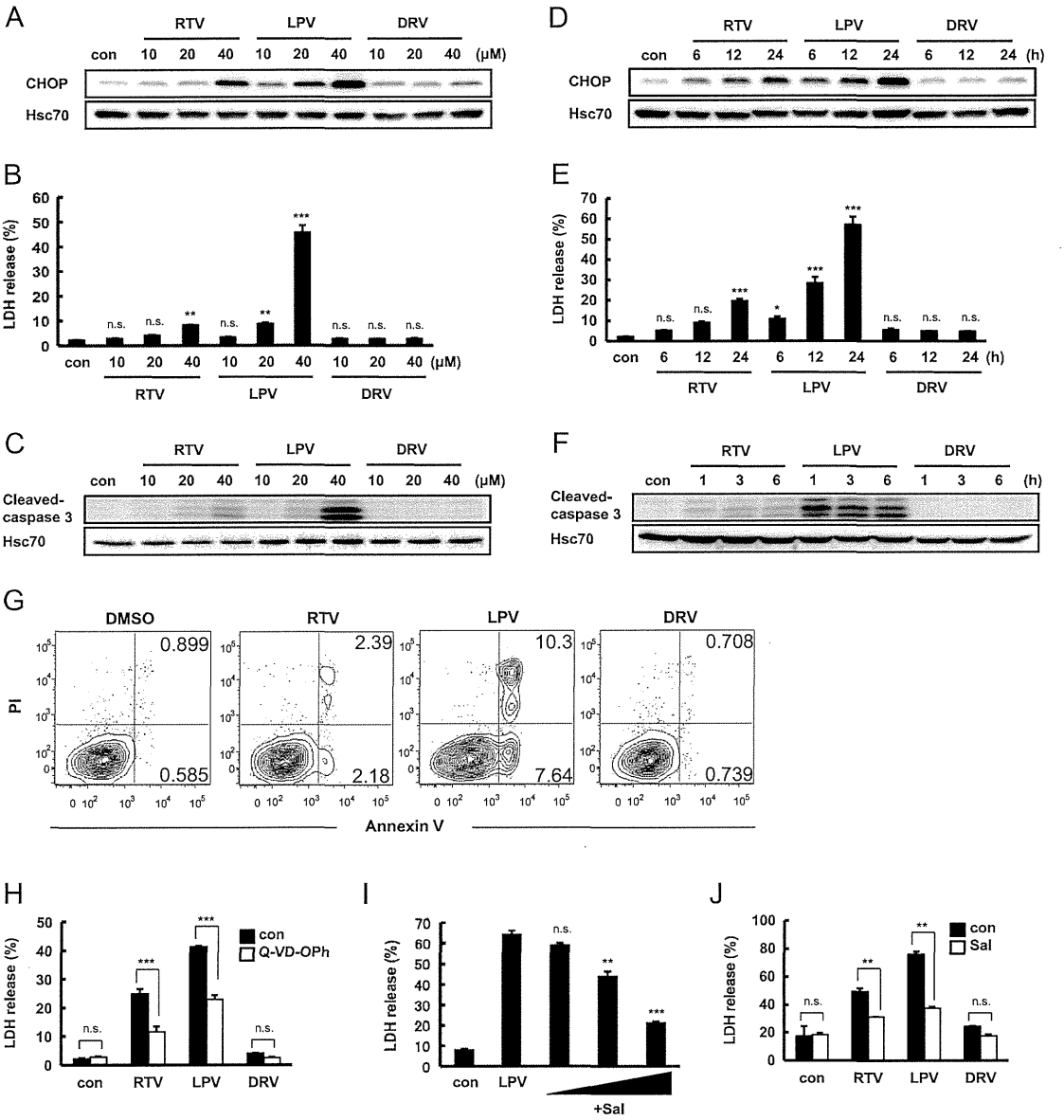

Fig. 2. RTV and LPV but not DRV induce apoptosis via ER stress. (A, B) HEK293 cells were treated with 10, 20, and 40 μM RTV, LPV, and DRV for 24 h. (D, E) HEK293 cells were treated with 40 μM RTV, LPV, and DRV for 6, 12, and 24 h. CHOP expression was analyzed by Western blotting (A, D). Hsc70 was used as internal control. % LDH release was determined (B, E). Values are expressed as mean ± SE from triplicate tests. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ versus control, determined by ANOVA with Tukey–Kramer. (C) Cleaved caspase-3 was examined in HEK293 cells treated with 10, 20, and 40 μM RTV, LPV, and DRV for 6 h. (F) Cleaved caspase-3 was examined in HEK293 cells treated with 40 μM RTV, LPV, and DRV for 1, 3, and 6h. Hsc70 was used as internal control. (G) AnnexinV/PI staining was examined in HEK293 cells treated with 40 μM RTV, LPV, and DRV for 12 h.(H, J) HEK293 cells were untreated or treated with 40 μM RTV, LPV, and DRV for 24 h in the absence or presence of 10 μM caspase inhibitor (Q-VD-Oph) (H) or 10 nM salubrinal (J). % LDH release was determined. $^{***}P < 0.001$; $^{**}P < 0.01$ vs the control group, assessed by Student's *t* test. (I) HEK293 cells were untreated or treated with 40 μM LPV for 24 h in the absence or presence of 0.1, 1, and 10 nM salubrinal. % LDH release was determined. $^{***}P < 0.001$; $^{**}P < 0.01$ versus control, determined by ANOVA with Tukey–Kramer.

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

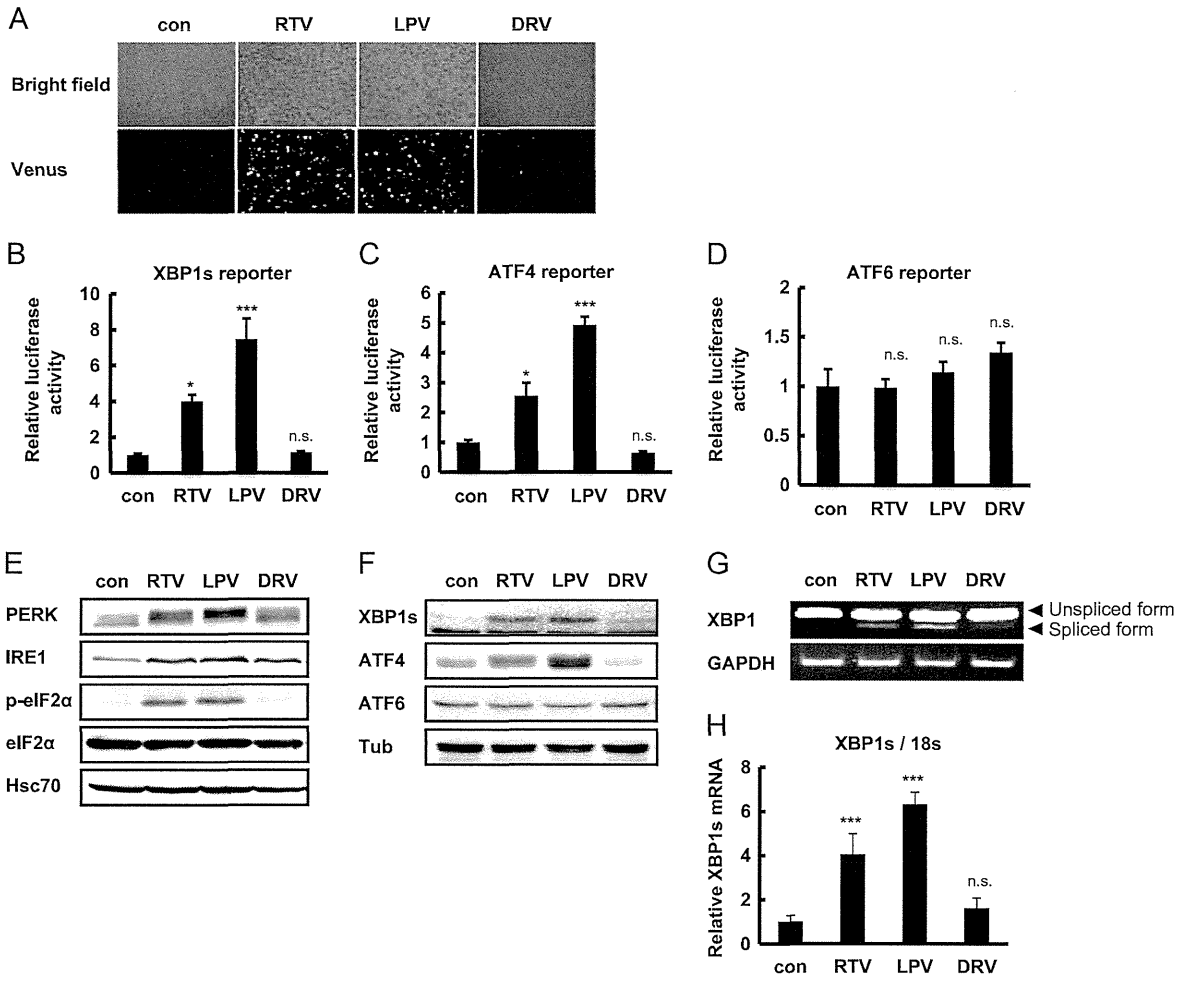

Fig. 3. RTV and LPV but not DRV induce ER stress signaling. (A) HEK293 cells were transfected with XBP1-venus reporter plasmid (pCAX-F-XBP1ΔDBD-venus) and treated with 40 μM RTV, LPV, and DRV for 24 h. Cells were fixed and imaged 24 h after treatment by using fluorescence microscopy. (B, C, D) HEK293 cells were transfected with XBP1 (B), ATF4 (C), ATF6 (D) reporter plasmids and treated with 40 μM RTV, LPV, and DRV for 24 h. Luciferase activity was determined 24 h after treatment and is expressed as fold activation over the DMSO treatment as control. Values are mean ± SE of triplicate platings. $^{***}P < 0.001$; $^{*}P < 0.05$ versus control, determined by ANOVA with Tukey–Kramer. (E, F) Cytoplasmic lysates (E) or nuclear extracts (F) from HEK293 cells untreated or treated with 40 μM RTV, LPV, and DRV for 6 h were analyzed by Western blotting for PERK, IRE1, p-eIF2α, eIF2α, XBP1, ATF4, and ATF6. Hsc70 was used as loading control for cytoplasmic lysates. γ-Tubulin was used as loading control for nuclear extracts. (G, H) mRNA from HEK293 cells untreated or treated with 40 μM LPV for 6 h were analyzed by semi-RT-PCR (G) and Q-PCR (H) for the spliced form of XBP1. 18S and GAPDH were used as internal control. For Q-PCR analysis of CHOP mRNA expression was normalized to human 18S RNA. Values are expressed as mean ± SE from triplicate tests. $^{***}P < 0.001$ versus control, determined by ANOVA with Tukey–Kramer.

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. 5H). 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 correlates with LPV-induced ER stress and apoptosis. To clarify the correlation between JNK activation and PI-induced ER stress, we examined this effect of 9 PIs. ER stress-inducing PIs, SQV, RTV, and LPV, showed clear activation of JNK (Fig. 5J). These results indicated that the ROS-induced JNK activation is a clear

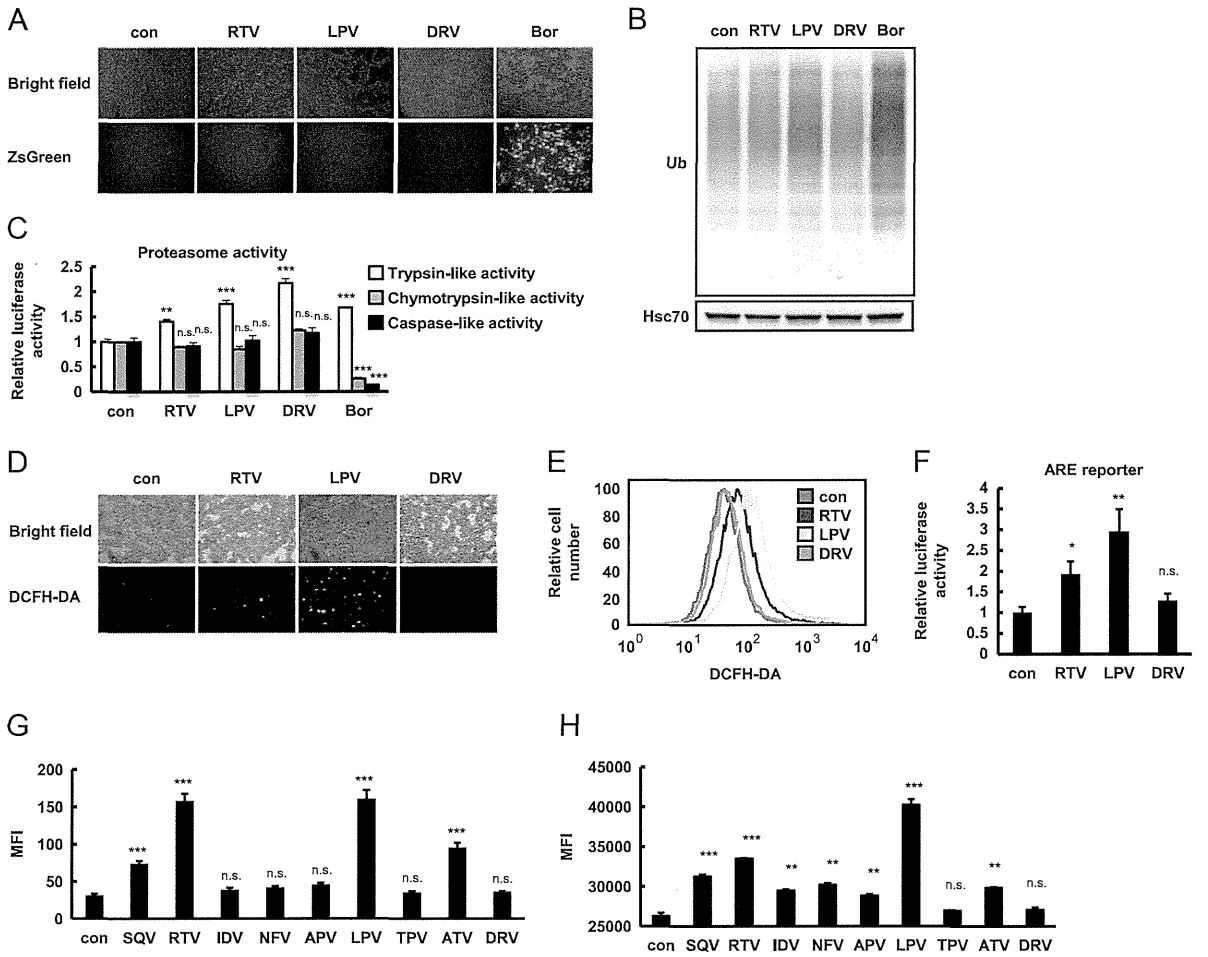

Fig. 4. RTV and LPV but not DRV induce ROS production rather than proteasome inhibition. (A) HEK293 cells were stably transfected with proteasome sensor vector and treated with 40 μM RTV, LPV, and DRV and 10 nM bortezomib (Bor) for 24 h. Bor was used as positive control for proteasome inhibition. Cells were fixed and imaged by using fluorescence microscopy. (B) Ubiquitinated protein expression was examined in HEK293 cells untreated or treated with 40 μM RTV, LPV, and DRV and 10 nM Bor for 6 h. (C) Proteasomal protease activity (trypsin-like, chymotrypsin-like, caspase-like activity) was examined in HEK293 cells treated with 40 μM RTV, LPV, and DRV and 10 nM Bor for 6 h. Luciferase activity was determined and is expressed as fold activation over the DMSO treatment as control.$^{***}P < 0.001$; $^{**}P < 0.01$ vs the control group, assessed by Student's *t* test. (D, E) HEK293 cells were pretreated with 10 μM DCFH-DA for 1 h before untreated or treated with 40 μM RTV, LPV, and DRV for 30 min. DCFH-DA activation was examined by using fluorescence microscopy and flow cytometry after treatment for 30 min. Fluorescence intensity was measured and shown as the indicated colored lines, which represent intracellular ROS production in each sample. (F) HEK293 cells transfected with ARE reporter plasmid and treated with 40 μM RTV, LPV, and DRV for 24 h. Luciferase activity was determined 24 h after treatment and is expressed as fold activation over the DMSO treatment as control. Values are mean ± SE of triplicate platings. $^{**}P < 0.01$; $^{*}P < 0.05$ versus control, determined by ANOVA with Tukey–Kramer.(G, H) ROS production was examined by 10 μM DCFH-DA (G) and Amplex Red (H) activation in HEK293 treated with 40 μM of 9 HIV-PIs for 30 min. Fold induction of ROS production (relative to untreated control) was computed using the mean fluorescence intensity (MFI).$^{***}P < 0.001$; $^{**}P < 0.01$ versus control, determined by ANOVA with Tukey–Kramer.

difference between LPV and DRV on ER stress and apoptosis induction, and might be a determinant of ER stress induction by PIs.

RTV and LPV but not DRV induce ER stress response in mice liver, kidney, and small intestine

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

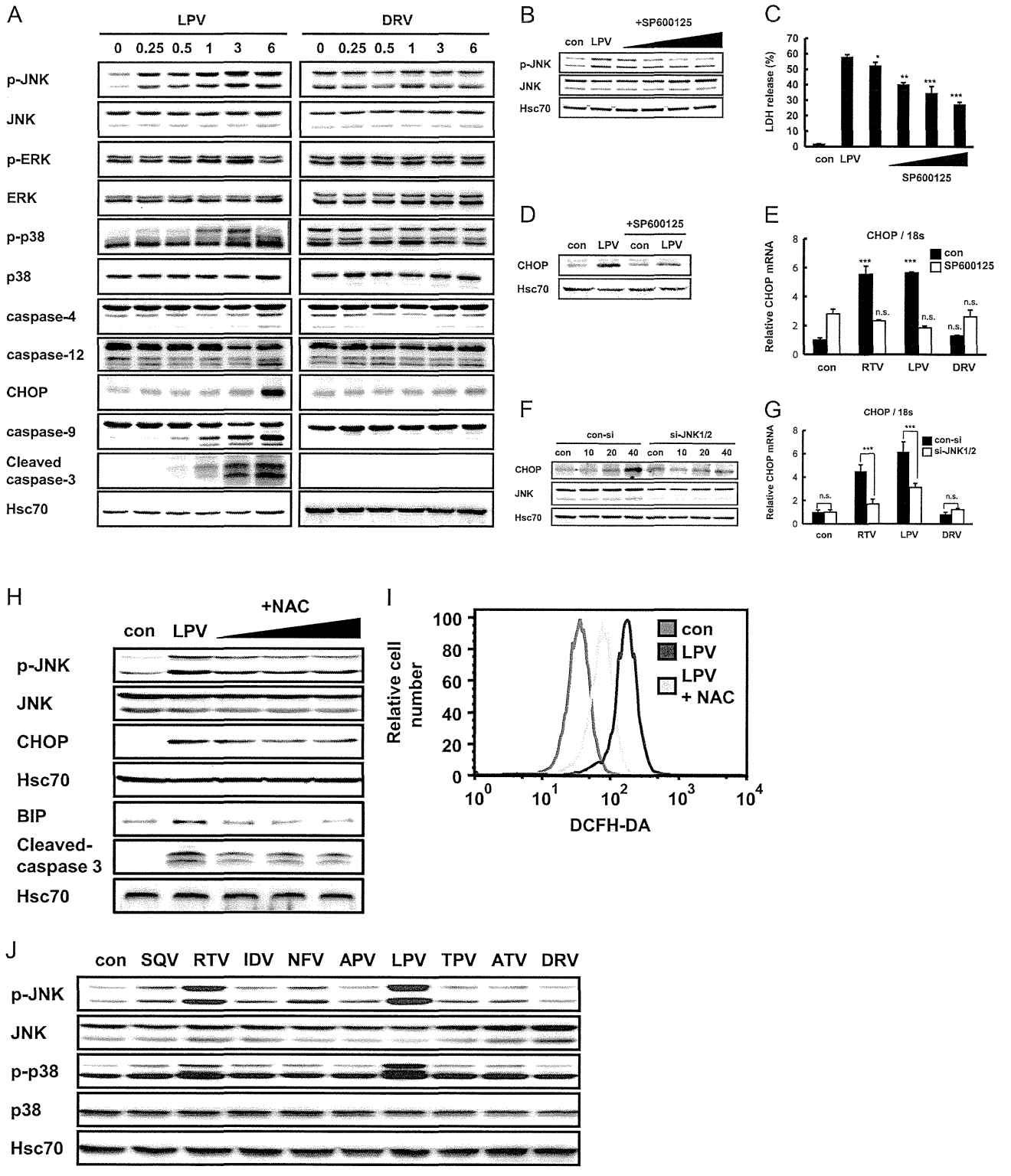

Fig. 5. HIV-PI-induced ER stress is dependent on ROS-induced JNK activation.(A) MAPK (p-JNK, JNK, p-ERK, ERK, p-p38, and p38), caspase-3/4/9/12, CHOP, and Hsc70 expressions were examined in 40 μM LPV or DRV treated for the indicated times in HEK293 cells. (B, C) HEK293 cells were untreated or treated 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. $^{***}P < 0.001$ versus each control, determined by ANOVA with Tukey–Kramer. (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.

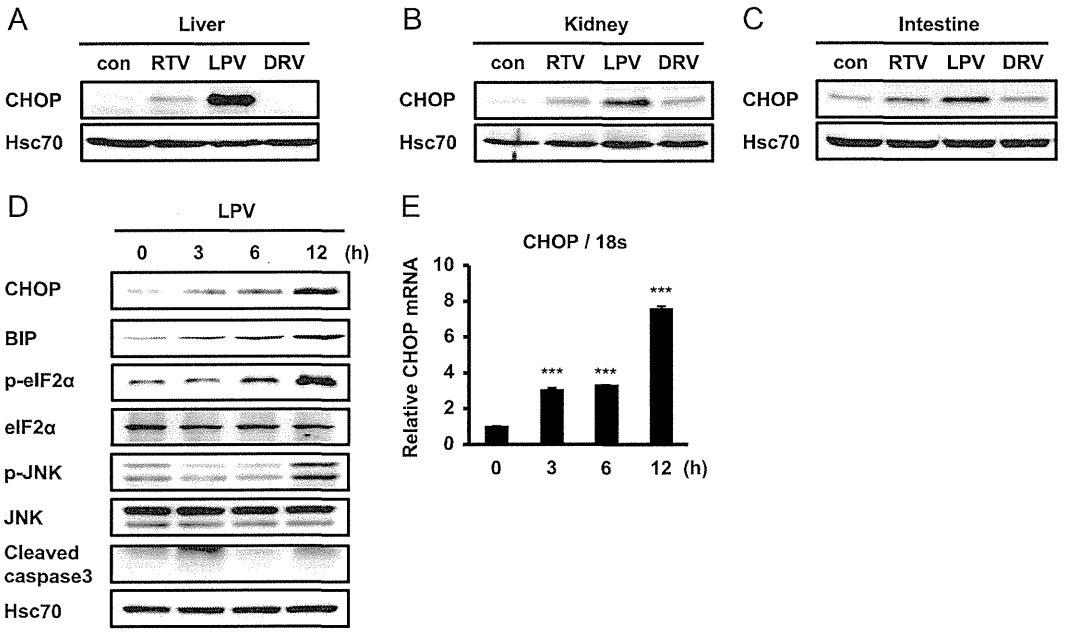

Fig. 6. RTV and LPV but not DRV induce ER stress response in mice tissue. (A, B, C) Mice were intraperitoneally injected with 40 μmol/kg of RTV, LPV, and DRV. Twelve hours after injection, CHOP protein expression was examined in liver (A), kidney (B), and intestine (C). (D, E) Mice were intraperitoneally injected with 40 μmol/kg of LPV. At 3, 6, and 12 h after injection, CHOP, BIP, p-eIF2α, eIF2α, p-JNK, JNK, cleaved-caspase-3 protein (D), and CHOP mRNA (E) expressions were examined in liver. $^{***}P < 0.001$ versus each control, determined by ANOVA with Tukey–Kramer.

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 the critical pathogenic factor 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, 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 and 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

[11,13–15]. 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 clinical therapy [47]. Therefore, it is not enough for clinical study of PIs compared with other therapeutic drugs [48]. 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 cART 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.

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Chapter 1
Development of the First AIDS Drugs: AZT and Other Dideoxynueosides

Robert Yarchoan and Hiroaki Mitsuya

1.1 Introduction

On March 19, 1987, zidovudine (3′-azido-2′3′dideoxythymidine, azidothymidine, AZT) was approved by the United States Food and Drug Administration (FDA) as the first drug to treat acquired immunodeficiency syndrome (AIDS). The initial development of this drug was the result of a collaboration between scientists in the National Cancer Institute (NCI), Burroughs Wellcome Co., and Duke University. Before this, there was no effective treatment for this devastating disease, and the median survival of AIDS patients was measured in months. Since the development of AZT (Mitsuya et al. 1985; Yarchoan et al. 1986), 25 additional antiretroviral drugs have been approved to treat HIV/AIDS, and combination anti-HIV therapy has converted AIDS from a death sentence to a manageable chronic disease. Several years ago, it was recently estimated that advances in AIDS therapy have already saved over 3 million years of life in the United States alone (Walensky et al. 2006), and we continue to see new benefits from these agents in both the developed world and resource-challenged countries. Moreover, the use of antiviral drugs to prevent HIV transmission was identified as the "2011 Breakthrough of the Year" by *Science* magazine (Cohen 2011). Looking back, the rapidity with which the initial AIDS drugs were introduced into the clinic (Table 1.1) (Mitsuya and Broder 1986, 1987; Mitsuya et al. 1985; Yarchoan and Broder 1987a; Yarchoan et al. 1986, 1988, 1989b) and their impact are even more remarkable in light of the skepticism and pessimism surrounding their initial developmental efforts and the fact that we still do not have an effective AIDS vaccine 28 years after the first isolation of the causative agent.

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Table 1.1 Timeline of early HIV drug development

Drug	In vitro activity	Phase I trial started	FDA approval
Zidovudine	February 1985	July 1985	March 19, 1987
Zalcitabine	May–June 1985	October 1986	June 19, 1992
Didanosine	May–June 1985	October 1988	October 9, 1991

To get a sense of the backdrop for the development of AZT, it is worthwhile to briefly review the status of the AIDS epidemic and earlier efforts to deal with this new disorder. Although AIDS was identified as a new disease in May 1981 (CDC 1981a, b), it was some time before the scope of this new pandemic was appreciated. The only cases that could be initially tracked were those with full-blown AIDS, and it was not appreciated that many more patients with early prodromes or even asymptomatic HIV infection were part of this same pandemic and, if untreated, would nearly all eventually progress to AIDS and death. Over the next few years, it became apparent that AIDS was caused by an infectious agent, but efforts to identify this causal agent were initially hindered by the multitude of opportunistic infections that AIDS patients harbor as a result of their profound immunodeficiency. In 1983, Barre-Sinoussi, Montagnier, and coworkers first reported isolation of a novel retrovirus from a patient with a condition related to AIDS that was felt to be an AIDS prodrome (Barre-Sinoussi et al. 1983). However, uncertainty lingered as to whether this new agent was indeed the cause of AIDS until a year later, when Gallo and coworkers published a series of papers in *Science* describing the isolation of the a new virus from patients with AIDS, its propagation in a cell line, and development of an antibody test (Gallo et al. 1984; Popovic et al. 1984). After publication of these papers, it became clear to most scientists working in this area that this new virus was indeed the cause of AIDS.

Soon after AIDS was recognized as a new disease, then NCI Director Vincent T. DeVita Jr. urged Dr. Samuel Broder, who had recently been appointed Clinical Director of the National Cancer Institute (NCI) and whose research had been focused at the intersection of cancer and immunology, to spearhead an effort directed at developing a therapy for this new disorder (Harden 2012). As this effort got under way, there were only three research fellows in the Broder laboratory, and the two coauthors of this paper (HM and RY) turned their attention to this project, benefitting from substantial help from others within and outside the NCI. Immediately after publication of these papers by the Gallo laboratory in *Science*, we concentrated on development of antiviral therapy to treat HIV/AIDS. With what we now know, this approach seems straightforward, but at the time, it was rather controversial and, for a number of reasons, was felt to have a very limited chance of success. Prominent among these was the fact that HIV, like other retroviruses, integrates into host cells as a provirus, and because of this, retroviruses were not considered amenable to treatment by antiviral drugs.

A related concern was that it would not be possible to selectively develop effective anti-HIV drugs, since the virus offered few unique targets. All viruses use the host cellular machinery for most of their replicative steps and thus offer very few unique targets for attack. At the time, in contrast to the development of antimicrobial agents, little progress had been made in the antiviral therapy of any disease, and the only effective drug in widespread clinical use was the anti-herpes drug acyclovir, which had been approved in 1982. And HIV, with only nine genes, was considered a much simpler virus than herpesviruses. Also, because HIV was detectable in only a few T cells in AIDS patients, it was felt that most of the T cell depletion and immunodeficiency was through a secondary indirect mechanism that would not be halted by blocking HIV replication, especially if this replication was not completely inhibited. Lastly, there were also concerns that the immunologic damage, once it occurred, would not be reversible.

1.2 Initial Developmental Efforts

In spite of the pessimism mentioned above, our group in the NCI felt that it might be feasible to develop effective anti-HIV therapy to treat AIDS. Our thinking was that, unlike other retroviruses that often caused cancer by integrating into the target cell genome and causing expansion of the infected cell population, HIV generally caused AIDS by constantly infecting and destroying new cells. Also, the experience with bone marrow transplantation provided evidence that the immune system (including T cells) could reconstitute itself over a period of months under appropriate conditions. We thus hypothesized that if HIV replication could be halted in patients, we should see evidence of immune stabilization and even reconstitution in a matter of weeks or months.

In considering possible viral targets that could be attacked, we soon focused on reverse transcriptase (RT). This enzyme is essential for replication of retroviruses and hepadnaviruses, but not for eukaryotic cells. In addition, there was earlier literature on agents that blocked the activity of RTs of murine leukemia virus and other retroviruses, and these enzymes had some structural similarity to HIV RT (De Clercq 1979; Furmanski et al. 1980; Ostertag et al. 1974; Ting et al. 1972; Yang et al. 1972). To proceed, we needed an assay to assess drug activity, and one of us (HM), building on his recent studies on human T cell leukemia virus type I (HTLV-1), developed one in which agents could be assessed for their ability to block the cytopathic effect of HIV on an HTLV-1-infected cell line (Mitsuya and Broder 1987; Mitsuya et al. 1984). For a drug to be scored as active in this assay, it had to prevent killing of the target $CD4^+$ T cells by HIV; this assay had the substantial advantage that it could simultaneously assess anti-HIV activity and drug toxicity in the same cell line (Fig. 1.1). Because of the urgency of the epidemic, we focused our initial efforts on drugs currently in clinical use for another indication and thus could be rapidly brought into the clinic. If none of these seemed promising, our next focus

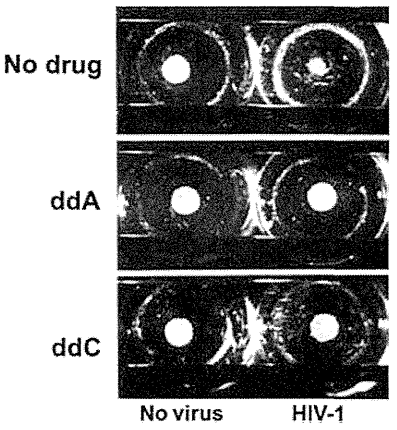

Fig. 1.1 Cytopathic effect of HIV-1 on clone ATH8 and protection by 2′–3′-deoxyadenosine (ddA) and 2′,3′-dideoxycytidine (ddC). ATH8 cell (2×10^5) were exposed to HIV-1 and cultured in test tubes in the presence or absence of dideoxynucleosides. By day 7 of culture, ATH8 cells were almost completely destroyed by the virus. In the absence of drug, the cytopathic effect is seen as a small disrupted pellet, which contains debris of cells (*top right*). In the presence of ddA (40 mM) or ddC (2 mM), ATH8 cells were virtually completely protected and continued to grow, which can be seen as large cell pellets (*middle right* and *bottom right*) comparable to that of HIV-1-unexposed and drug-unexposed ATH8 population (*top left*). ATH8 cells exposed to only drug (*middle left*, *bottom left*) formed large pellets comparable to the virus-unexposed and drug-unexposed cell pellet (*top left*) (Reproduced from Mitsuya et al. 1987b with permission)

was on drugs that we could develop in collaboration with a pharmaceutical drug company with the idea that this would accelerate preclinical development, clinical testing, and manufacture.

One of the first compounds we studied was suramin, an old drug synthesized as an outgrowth of Paul Ehrlich's work on trypanosomiasis that had been developed as a treatment for onchocerciasis. Suramin had been reported by Eric De Clercq to be active against the RT of RNA tumor viruses (De Clercq 1979) and was found to be effective against HIV in the cytopathic screening assay (Mitsuya et al. 1984). Within 3 months of the Gallo *Science* articles on HIV, we had developed an assay to test drugs, identified the in vitro activity of suramin against HIV, obtained approval of an Investigational New Drug (IND) Application from the FDA to test suramin in the clinic, and initiated a clinical trial to test suramin in patients with AIDS or AIDS-related complex (Broder et al. 1985; Mitsuya et al. 1984; Yarchoan et al. 1985). While there was a suggestion that it was more difficult to isolate HIV from some patients on suramin, there was no evidence from this study that it had clinical activity or could reverse HIV-associated immunosuppression (Broder et al. 1985), and we soon turned our attention to other approaches. Nonetheless, the experience in bringing suramin into the clinic and testing it in patients greatly facilitated the subsequent development of AZT.

1.3 Preclinical Development of AZT and Other 2′,3′-Dideoxynucleosides

While this work proceeded, we searched for pharmaceutical companies that might have compounds to screen. However, most companies that we approached were unwilling to work on anti-HIV drugs, because there were still relatively few reported cases of AIDS and this was not viewed as a profitable area. One company that did express a willingness to collaborate, however, was Burroughs Welcome Co., whose scientists, although they had considerable expertise in nucleoside analogues and had started working on murine retroviruses as a model for HIV, had no experience or ability to work with HIV. Burroughs Wellcome scientists submitted to us a number of compounds to test, and one, AZT, was identified in February 1985 to be quite potent in the HIV cytopathic assay (Fig. 1.1, Table 1.1) (Mitsuya et al. 1985). At this stage, Dr. Dani Bolognesi's group in Duke University, which was also working with HIV, joined this collaborative effort.

AZT was not a new drug and in fact was initially synthesized and studied as a possible anticancer drug by Jerome Horwitz on an NCI grant in 1964 (Fig. 1.2) (Horwitz et al. 1964). Although AZT was unsuccessful as a cancer drug, Wolfram Ostertag et al. published a decade later that it interfered with murine retrovirus replication in a complex system consisting of both spleen focus-forming and leukemia viruses (Ostertag et al. 1974). At the same time, however, they found that AZT increased the intracisternal A-type particles in Friend erythroleukemic cells infected with Friend virus, suggesting that it might interfere with viral release (Krieg et al. 1978). While the relevance of this system to HIV was unclear, AZT did in fact prove to have activity against HIV (Mitsuya et al. 1985) and became the first of these drugs that we brought into the clinic (Yarchoan et al. 1986).

Our group continued to test other compounds, based on the postulate that dideoxynucleosides could serve as proviral DNA chain terminators and thereby block HIV infectivity (Fig. 1.2). Such agents had been synthesized some years earlier,

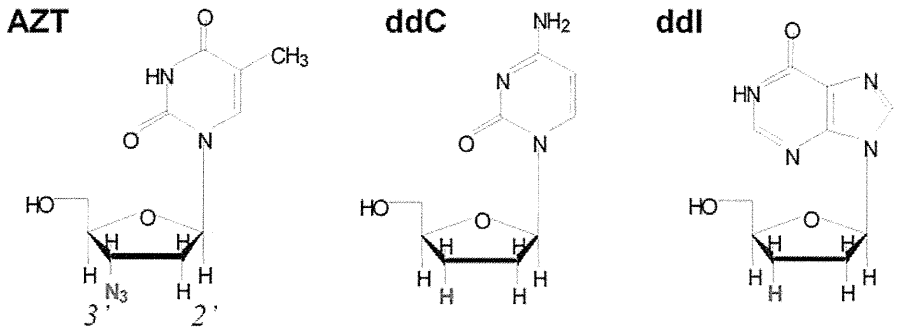

Fig. 1.2 Structures of AZT (zidovudine), ddC (zalcitabine), and ddI (didanosine)

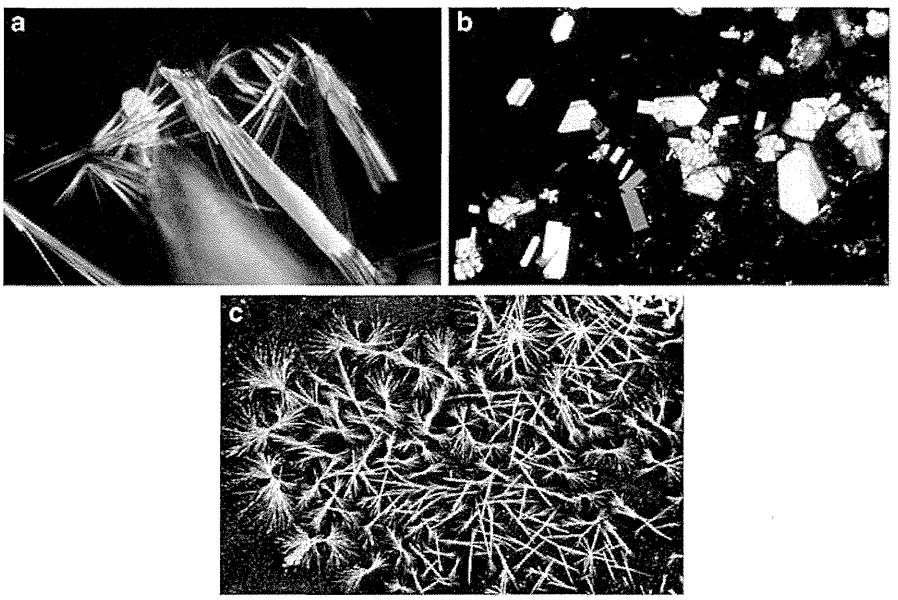

Fig. 1.3 Crystals of AZT (Panel a), ddC (Panel b), and ddI (Panel c)

e.g., 2′,3′-dideoxycytidine (ddC) had been synthesized by J. Horwitz et al. (Horwitz et al. 1967), and 2′,3′-dideoxyadenosine (ddA) had been synthesized in 1964 (Fig. 1.3) (Robins and Robins 1964). Also, previous reports had suggested that certain of these drugs might have activity against murine retroviruses in cell lines from some, but not other species, and only at high concentrations. In particular, Furmanski et al. had reported that high concentrations (50–100 μM) of dideoxythymidine (ddT) had activity against type C murine leukemia/sarcoma viruses in mouse and human cells, but not in rat cells (Furmanski et al. 1980). Also, Waqar et al. had reported that infection of murine embryonic fibroblasts by murine leukemia virus was inhibited by high concentrations (50 μM or so) of certain dideoxynucleosides (Waqar et al. 1984). In a very short period of time, using the cytopathicity inhibition assay, Mitsuya and Broder found that a number of 2′,3′-dideoxynucleosides, including ddC, ddA, and 2′,3′-dideoxyinosine (ddI), had potent in vitro activity against HIV (Mitsuya and Broder 1986). Subsequent studies revealed a logical pattern of structure-function relationships (Mitsuya and Broder 1987; Mitsuya et al. 1987a, 1990). These agents were not active against HIV by themselves, but had to be phosphorylated to their 5′-triphosphate moiety by cellular enzymes to be effective (Furman et al. 1986; Mitsuya and Broder 1987; Mitsuya et al. 1987a). Phosphorylation of these drugs varied greatly from species to species, which greatly complicated the use of animal models (or even cell lines derived from different species) for activity or toxicity testing. In the triphosphate moiety, they inhibited HIV reverse transcription by acting both as proviral DNA chain terminators and competitive RT inhibitors. In general, active agents had a modification of their 3′-hydroxy group so that

once added to a growing chain of proviral DNA by HIV reverse transcriptase, no further 5′->3′ phosphodiester linkages could be formed and the growing proviral DNA chain was terminated (Mitsuya and Broder 1987; Mitsuya et al. 1990; Yarchoan et al. 1989a). They could also act as competitive inhibitors for reverse transcriptase. However, such 2′,3′-dideoxynucleoside-5′-triphosphates were relatively poor substrates for cellular DNA polymerases, especially DNA polymerase α.

ddC, ddA, ddI, and related compounds found to be active in vitro against HIV had been purchased from chemical supply houses for laboratory use, and thus moving them into the clinic would require substantial animal toxicity and other studies. Much of this testing had already been completed for AZT, since Burroughs Wellcome Co. had been considering it as an antibiotic for bacterial infections. It thus made sense to prioritize AZT for clinical testing. Another advantage of testing AZT first was that we already had an established drug company as a partner, which would facilitate the manufacture, large-scale clinical testing, and marketing of the drug if it was found to be promising in initial clinical testing.

1.4 Clinical Development of Zidovudine (AZT)

1.4.1 Early Clinical Development of AZT

Following the in vitro observation that AZT had activity against HIV, a concerted effort was made to rapidly move it into clinical trials. There was considerable concern at that time that because AZT, as well as the other dideoxynucleosides, was a DNA chain terminator, thus its use might cause cancer or severe cumulative toxicity. However, because of lethality of AIDS, it was felt that this risk was worth taking. Over the next few months, a phase I clinical protocol, based on the suramin study, was drafted and approved by the relevant Institutional Review Boards, and an IND Application was filed and approved by the FDA. Generation of the protocol was a collaborative effort of the NCI, Burroughs Wellcome Co., and investigators at Duke University. On July 3, 1985, less than 5 months after the initial laboratory observation of AZT activity, the first AIDS patient was administered AZT on the NCI wards of the NIH Clinical Center in Bethesda, MD. As the study was originally designed, patients received a test dose, followed by 2 weeks of intravenous dosing; as experience was gained, the protocol was amended so that patients could receive oral dosing (Yarchoan et al. 1986).

This study showed that AZT was well tolerated at doses that could achieve plasma levels that had been shown to be effective in vitro and that it penetrated into the cerebrospinal fluid (Klecker et al. 1987; Yarchoan et al. 1986). Initially, 19 patients were entered at increasing doses, ranging from 2 mg/kg every 8 h (orally) to 10 mg/kg every 4 h (orally). While it would seem that the best way of assessing a beneficial effect would be to measure its effect on HIV, we had very limited tools at hand, and technologies to measure HIV viral load by RT-PCR or even serum p24 antigen had not been developed. Perhaps the best developed test to assess HIV was

the ability to isolate HIV from peripheral blood mononuclear cells; while this was somewhat decreased at higher doses of AZT, results were inconsistent and hard to interpret (Yarchoan et al. 1986). Surprisingly to some, the best evidence that AZT was doing something of benefit came from immunologic and clinical assessments. Fifteen of the 19 patients had increased CD4 counts during the 6 weeks of treatment, and six patients who were anergic at entry developed positive delayed-type hypersensitivity skin test reactions during treatment. Also, two patients had clearing of chronic nail bed infections, one patient reported an increased ability to concentrate, and several patients reported other clinical improvements, and the patients as a group had a mean weight gain of 2.2 kg. Overall, AZT was well tolerated at these doses with the main side effects being headaches and bone marrow suppression (Yarchoan and Broder 1987a; Yarchoan et al. 1986).

1.4.2 Randomized Double-Blind Efficacy Trial and Approval of AZT

There was considerable skepticism at the time whether the increased CD4 count and other findings in this study were indicative of a beneficial effect. In fact, an abstract describing the trial was rejected outright when submitted to the AFCR/ASCI/AAP National Meeting (Yarchoan Personal communication). Even so, many of us were convinced of its activity, and Burroughs Wellcome scientists moved ahead to conduct a multicenter double-blind, placebo-controlled trial in 282 patients to assess the efficacy of oral AZT in patients with AIDS or severe AIDS-related complex (Fischl et al. 1990; Richman et al. 1987). This trial was conducted in 12 centers throughout the United States that had substantial experience with AIDS patients. Patients received 250 mg of AZT every 4 h (145 patients) or placebo (137 patients). The principal endpoints were survival and development of AIDS-related complications. The trial was initiated in June 1986 and terminated by the Data Safety Monitoring Board in September of the same year because of clear evidence that it had clinical activity. At the time of termination, 19 patients on the placebo arm had died, compared to only one patients on the AZT arm, a result that was highly significant ($P<0.001$). In addition, patients receiving AZT had greater CD4 counts, Karnofsky scores, weight, and more frequent reversal of skin test anergy (Fischl et al. 1990; Richman et al. 1987). The principal toxicities seen in the AZT recipients were bone marrow suppression, nausea, myalgia, insomnia, and headaches.

At the time, the design of this randomized trial was highly controversial, with much of this centering on the use of a placebo arm and the deaths that occurred on this arm as one of the principal assessed endpoints (Yarchoan 2012). Nonetheless, the trial greatly accelerated the general acceptance that AZT was in fact active against AIDS, as well as its approval and widespread use. Indeed, from this vantage point, perhaps it would be fair to say that if this trial had not been conducted in this way, many more people would have died because they could not obtain AZT. Even with this trial, many persons held on to the belief for years that AZT toxicity far outweighed the benefits. As an extreme example, a group of so-called AIDS denialists, the most prominent of

whom was the scientist Peter Duesberg, claimed that AIDS was not caused by HIV but in fact was caused by a variety of drugs, including AZT (Clayton 1993; Duesberg 1992). This argument has had devastating consequences; it is cited, for example, as being a main contributor to the adoption by the South African government of the viewpoint that HIV was not the cause of AIDS and the opposition of that government to the use of antiretroviral therapy until 2005 (Specter 2007). It has been estimated that as a result of this policy, more than 330,000 lives, or approximately 2.2 million person-years, were lost in South Africa (Chigwedere et al. 2008).

Getting back to AZT's development, on the basis of this phase II study, AZT was almost immediately made widely available to AIDS patients in the United States through a Treatment IND and was approved on March 19, 1987. The time from the initial observation of the in vitro activity of AZT to its approval was 25 months, arguably the fastest time of any drug in the modern era.

While the multicenter trial of AZT was ongoing, our group in the NCI explored higher doses of AZT on a continuation of the phase I trial. We found that the maximum tolerated dose for short-term (6-week) administration was 90 mg/kg/day orally (Yarchoan and Broder 1987b, c). Interestingly, we found that some patients with Kaposi's sarcoma (KS) who received the higher dose of AZT had partial or complete responses of their KS (Yarchoan and Broder 1987b, c). KS has since been shown to be caused by a new herpesvirus, Kaposi's sarcoma-associated herpesvirus (KSHV) (Chang et al. 1994; Moore and Chang 1995), and a lytic gene of this virus has been shown to phosphorylate AZT to a moiety that is toxic to cells (Davis et al. 2007; Gustafson et al. 2000). It is thus possible that the KS responses were in part as a result of AZT's phosphorylation by KSHV.

Also, we had noticed that one patient on the phase I trial had improved cognitive function and we recruited additional patients with AIDS dementia or other neurologic disease to explore this further. We found that each of the three patients with dementia had substantial improvement in their cognitive function on AZT (Yarchoan et al. 1987). The principal cells infected with HIV in the brain are monocytes and related microglial cells, and we found that AZT blocked HIV replication in monocytes (Perno et al. 1988). The claim that AZT could reverse dementia, which was previously felt to be irreversible, was initially met with substantial skepticism by the neurologic community. However, some cognitive testing was done on the multicenter placebo-controlled trial of AZT, and an analysis of those results confirmed that cognitive function did in fact improve in those receiving AZT (Schmitt, et al. 1988). Also, in a subsequent trial of intravenous AZT in HIV-infected children conducted at the NCI, a number had dramatic improvement in HIV-associated neurodevelopmental abnormalities (Pizzo et al. 1988). AZT penetrates into the cerebrospinal fluid better than other AIDS drugs and arguably remains the best drug to treat AIDS-associated neurocognitive disorder.

During early in vitro testing of potential anti-HIV drugs, it was found that the anti-herpes drug acyclovir had little in vitro anti-HIV activity by itself but could potentiate the activity of AZT (Mitsuya and Broder 1987; Mitsuya et al. 1987b). We explored this combination in the clinic and conducted a pilot study of the combination of acyclovir and AZT (Surbone et al. 1988). This small study showed that the regimen was well tolerated and that patients had increased CD4 counts. This study

and a second at about the same time of AZT alternating with 2′,3′-dideoxycydine (ddC) (Yarchoan et al. 1988) were the two first combination studies involving effective anti-HIV drugs. Interestingly, there has been renewed interest in the potential of acyclovir as an anti-HIV drug in recent years (McMahon et al. 2011; Vanpouille et al. 2012, 2009).

Finally, during this period, we continued to follow patients on AZT and noticed that their CD4 counts tended to decrease after about 4–6 months on therapy (Yarchoan and Broder 1987c). Several years later, it was found that this was because of the ability of HIV to develop resistance to antiviral drugs (Larder et al. 1989; Larder and Kemp 1989; Mitsuya et al. 1990), which remains one of the principal challenges to prolonged and effective anti-HIV therapy.

Finally, it should be mentioned that when AZT was first approved, the retail price was approximately $8,000 to $12,000 per year, which was at the time one of the highest prices ever for a prescription drug (Zonana 1989). This was called an "inhuman cost" and was highly criticized by AIDS patients, advocates, and government officials. A major factor that helped reduce the price of AZT over the next few years was the anticipation that a competing drug would come onto the market as ddC and ddI were developed.

1.5 Development of Zalcitabine (2′,3′-Dideoxycytidine, ddC)

While clinical testing of AZT was ongoing, our group turned to the other dideoxynucleosides that had been shown to have in vitro activity against HIV (Mitsuya and Broder 1986; Mitsuya et al. 1987a, 1990). We initially had no corporate partner for these drugs, and their initial preclinical and early clinical development was completely done within the NCI. The first of these drugs that we chose for clinical development was ddC, which demonstrated potent in vitro activity and a good in vitro activity/toxicity ratio. Animal toxicology was done by Dr. Joseph Tomascewski's group in NCI's Developmental Therapeutics Program, and the IND Application was then filed by Dr. Dale Shoemaker and colleagues in the Regulatory Affairs Branch of the NCI. At the same time, the rights to develop ddC were licensed by the NIH to Hoffmann-La Roche Co., which then became a partner in development of the drug.

The initial ddC phase I trial commenced at the NCI in October 1986 (Yarchoan et al. 1988). By the time this trial was complete, an early version of the HIV p24 antigen assay had been developed, and the evaluable patients receiving ddC at doses of 0.03 mg/kg every 8 h to 0.09 mg/kg every 4 h were found to have a decrease in their serum HIV p24 antigen that was most pronounced at week 2 but was still significantly decreased through week 6 (Yarchoan et al. 1988). The patients also had a slight increase in their CD4 count at week 2, but this did not persist beyond this point. This trial also revealed that ddC was more toxic than AZT at doses that appeared to have anti-HIV activity. Primary toxicities were cutaneous eruptions, fever, mouth sores, arthralgias, thrombocytopenia, and, after several weeks, a painful peripheral neuropathy.