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# Development of a Novel Self-Microemulsifying Drug Delivery System for Reducing HIV Protease Inhibitor-Induced Intestinal Epithelial Barrier Dysfunction

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**Abstract:** The development of HIV protease inhibitors (PIs) has been one of the most significant advances of the past decade in controlling HIV infection. Unfortunately, the benefits of HIV PIs are compromised by serious side effects. One of the most frequent and deleterious side effects of HIV PIs is severe gastrointestinal (GI) disorders including mucosal erosions, epithelial barrier dysfunction, and leak-flux diarrhea, which occurs in 16−62% of patients on HIV PIs. Although the underlying mechanisms behind HIV PI-associated serious adverse side effects remain to be identified, our recent studies have shown that activation of endoplasmic reticulum (ER) stress response plays a critical role in HIV PI-induced GI complications. The objective of this study was to develop a novel self-microemulsifying drug delivery system (SMEDDS) using various antioxidants as surfactants and cosurfactants to reduce the GI side effects of the most commonly used HIV PI, ritonavir. The biological activities of this SMSDDS of ritonavir were compared with that of Norvir, which is currently used in the clinic. Rat normal intestinal epithelial cells (IEC-6) and mouse Raw 264.7 macrophages were used to examine the effect of new SMEDDS of ritonavir on activation of ER stress and oxidative stress. Sprague−Dawley rats and C57/BL6 mice were used for pharmacokinetic studies and *in vivo* studies. The intracellular and plasma drug concentrations were determined by HPLC analysis. Activation of ER stress was detected by Western blot analysis and secreted alkaline phosphatase (SEAP) reporter assay. Reactive oxygen species (ROS) was measured using dichlorodihydrofluorescein diacetate as a probe. Cell viability was determined by Roche's cell proliferation reagent WST-1. Protein levels of inflammatory cytokines (TNF-$\alpha$ and IL-6) were determined by enzyme-linked immunosorbent assays (ELISA). The intestinal permeability was assessed by luminal enteral administration of fluorescein isothiocyanate conjugated dextran (FITC−dextran, 4 kDa). The pathologic changes in intestine were determined by histological examination. The results indicated that incorporation of antioxidants in this new SMEDDS not only significantly reduced ritonavir-induced ER stress activation, ROS production and apoptosis in intestinal epithelial cells and macrophages, but also improved the solubility, stability and bioavailability of ritonavir, and significantly reduced ritonavir-induced disruption of intestinal barrier function *in vivo*. In conclusion, this new SMEDDS of ritonavir has less GI side effects compared to Norvir. This new SMEDDS can be used for other HIV PIs and any insoluble antiviral drug with serious GI side effects.

**Keywords:** Ritonavir; antioxidants; SMEDDS; ER stress; inflammation



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## Introduction

The development of HIV PIs has been one of the most significant advances of the past decade in controlling HIV infection. Incorporation of HIV PIs in highly active antiretroviral therapy (HAART) has markedly decreased mortality and morbidity of patients with HIV infection. Despite the clinical successes of HIV PIs, accumulating clinical evidence suggests that the benefits of HIV PIs are compromised by various undesirable side effects.[1−7] One of the most frequent and deleterious side effects of HIV PIs is their necrotizing effects on gut mucosa including the impairment of epithelial barrier function, which occurs in 16−62% of patients on HAART.[8,9] At present, the relationship between HAART and GI complications, and the underlying mechanisms behind HIV PI-associated intestinal barrier dysfunction, are unknown. Therapeutic and preventive strategies are currently unavailable.

The endoplasmic reticulum (ER) plays a major role in regulating the synthesis, folding, and orderly transport of proteins.[10] The ER has evolved highly specific signaling pathways, collectively termed the unfolded protein response (UPR), to ensure that its protein-folding capacity is not overwhelmed under various stresses. It has been reported that the UPR can be activated by disruption of intraluminal calcium homeostasis, alteration of protein glycosylation, glucose deprivation, virus infection, and changes in redox status.[11] The protein folding capacity of ER is significantly impacted by the reactive oxygen species (ROS) levels. Persistent generation of ROS in the ER results in the activation of the UPR and plays fundamental roles in the pathogenesis of multiple human diseases including various liver diseases, inflammatory bowel disease (IBD), and drug-induced tissue injury.[12] We have previously reported that HIV PIs activated the UPR and disrupted lipid metabolism in macrophages and hepatocytes.[13,14] Our most recent studies have shown that ER stress activation plays a critical role in HIV PI-induced disruption of intestinal barrier integrity,[15] suggesting that inhibition of the ER stress response represents a potential therapeutic target for HIV PI-induced GI side effects.

Self-emulsifying drug delivery systems (SEDDS) are isotropic mixtures of oils, surfactants, solvents and cosolvents/surfactants and have been used for oral delivery of various hydrophobic drugs including HIV PIs, Norvir (ritonavir) and Fortovase (saquinavir).[16] A self-microemulsifying drug delivery system (SMEDDS) is a novel drug delivery system based on the intrinsic chemical property of the drug formulation. SMEDDS offers numerous advantages such as spontaneous formation, thermodynamic stability, improved bioavailability, and ease of manufacture. The main objective of this study was to develop a novel SMEDDS for ritonavir using antioxidants as surfactants and cosurfactants to inhibit ritonavir-induced oxidative stress and the UPR

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*Table 1.* Solubility Studies in Various Vehicles

| name of vehicles | solubility in mg/mL ± SD |
|---|---|
| ethyl linoleate | 5.5 ± 0.2 |
| ethanol with 1% VC | >150 mg |
| cremorphol EL | 97.3 ± 2.2 |
| oleic acid | >150 mg |
| ethyl linoleate/oleic acid (3/1) | 141.7 ± 2.5 |
| ethyl linoleate/cremorphol EL (1/1) | 79.3 ± 1.8 |

activation and reduce the GI side effects associated with currently used SEDDS for ritonavir, Norvir.

## Materials and Methods

**Materials.** Antibodies against C/EBP homologous protein (CHOP), lamin B, and horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody against $\beta$-actin was from Calbiochem (San Diego, CA). Bio-Rad protein assay reagent, Criterion XT Precast Gel, HRP-conjugated goat anti-rabbit IgG and Precision Plus Protein Kaleidoscope Standards were obtained from Bio-Rad (Hercules, CA). Ritonavir was purchased from XIAMEN MCHEM laboratories LTD (Xiamen, China). Norvir was Abbott Laboratories (Abbott Park, IL). Biomax MS films were obtained from Eastman Kodak (Rochester, NY). Great EscAPe SEAP Reporter Assay Kit was obtained from Clontech Laboratories (Mountain View, CA). Mouse TNF-α and IL-6 ELISA Max Set Deluxe Kits were from BioLegend (San Diego, CA). Dichlorodihydrofluorescein diacetate (DCF-DA) and cell culture media were from Invitrogen, Ltd. (Paisley, U.K.). All other chemical reagents were from Sigma (St. Louis, MO).

**Solubility Studies.** The solubility of ritonavir in various oils and surfactants was determined. A total of 1 mL of each selected vehicle was added into a screw capped test tube containing 25 mg of ritonavir and mixed by vortexing. After visual assessment of solubility, additional ritonavir was added. The mixtures were shaken with a shaker at 37 °C for 24 h. After reaching equilibrium, the mixtures were centrifuged at 3,000 rpm for 5 min, and excess insoluble ritonavir was discarded by filtration using a Whatman filter. The free drug concentration of ritonavir was determined by HPLC. In order to save ritonavir, we stopped the solubility test in ethanol and oleic acid after the amount of ritonavir added was up to 150 mg. Table 1 shows the results of the solubility studies.

**Preparation of Ritonavir SMEDDS.** A series of SMEDDS were prepared in each formulation by varying the ratio of oil, surfactant, cosurfactant based on results of *in vitro* antioxidant effects and inhibition of the ER stress response. A SMEDDS was prepared with oil (ethyl linoleate, vitamin E, oleic acid), surfactant (cremophor EL), cosurfactant (1% vitamin C in ethanol), and ritonavir. The level of ritonavir was kept constant (10% w/v) in the total volume. Briefly, ritonavir was dissolved in cosurfactant in a glass vial. Oil and surfactant were accurately weighed and added into the glass vial containing ritonavir. Then, the components were mixed by gentle stirring and vortexing until ritonavir was completely dissolved. The blank SMEDDS was prepared by the same method as described above without ritonavir. The mixtures of ritonavir SMEDDS and blank SMEDDS were stored at 4 °C until further use.

**Characterization of SMEDDS: Particle Size Analysis and Zeta Potential.** Twenty-five microliters of SMEDDS formulation was diluted to 2.5 mL of deionized water in a beaker and gently mixed using a glass rod. The particle size and zeta potential of the resulting microemulsion were determined by the dynamic light scattering technique with a NICOMP particle sizing system (ZPW388 version 1.75). The intensity-WT Gaussian distribution mean diameters and polydispersity index were determined. For the zeta potential, the microemulsion was placed in the electrophoretic cell and the average surface charge was recorded.

**Stability of Ritonavir SMEDDS.** The ritonavir SMEDDS was stored at 4 °C for three months. The ritonavir content of the samples was analyzed by HPLC and the particle size was determined as described in the previous section.

**Cell Culture and Treatment.** The IEC-6 cell line was purchased from the American Type Culture Collection (ATCC) at passage 13 and used at passages 15−20 for the current experiments. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al.[12] They are nontumorigenic and retain the undifferentiated character of epithelial stem cells. IEC-6 cells were cultured in DMEM supplemented with 10% FBS, 0.1U/mL insulin, 100 U/mL penicillin, and 100 $\mu$g/mL streptomycin at 37 °C with 5% $CO_2$. Mouse RAW264.7 macrophages from ATCC were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 $\mu$g/mL streptomycin at 37 °C with 5% $CO_2$. Cells from passages six to nine were used in these studies. The ritonavir SMEDDS, blank SMEDDS and Norvir were diluted in DMEM and ritonavir was dissolved in DMSO. The diluted ritonavir SMEDDS, Norvir or ritonavir was added directly into the cell culture medium to a final concentration of 15 or 25 $\mu$M and incubated for 4−24 h.

**Quantification of Ritonavir Using High Performance Liquid Chromatography (HPLC) Assay.** HPLC was used to quantify ritonavir concentrations in IECs and mouse serum according to the method developed by Granda et al. with some modifications.[17] An Aglient 1200 series HPLC system and a Beckman C18 reverse phase column (5 $\mu$m, 4.6 mm × 25 cm) were used to quantify the ritonavir in cells and medium. The mobile phase was acetonitrile:1% acetic acid (50:50 to 60:40 v/v in 15 min). The samples were extracted with acetonitrile with a ratio of 40:60 (v/v) followed by centrifugation. The ritonavir was detected at a wavelength of 205 nm. A standard curve of ritonavir was constructed

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using weighted linear regression of peak area ratio values of the calibration standards.

IEC-6 cells were seeded on 6-well plates at a density of $6 \times 10^5$ for 24 h, and then treated with 15 $\mu$M of ritonavir in SMEDDS, Norvir or DMSO for various time periods 0.25, 0.5, 1, 2, 4, 8, and 24 h. Ritonavir in the cells was extracted using methanol and subjected to HPLC analysis. The intracellular ritonavir was normalized with the total protein amount.

**Cell Viability Assay.** The cytotoxicities of ritonavir in DMSO, ritonavir SMEDDS and Norvir in IEC-6 cells were assessed using cell proliferation reagent WST-1 from Roche Molecular Biochemicals. IEC-6 cells were seeded onto 96-well plates for overnight, then treated with ritonavir (0−50 $\mu$M) in different formulations for 24 h. At the end of treatment, 10 $\mu$L of cell proliferation reagent WST-1 was added into each well. Cells were incubated at 37 °C for 1 h. The absorbance of the samples at 450 nm was measured against a background control using a 96-well plate reader. The percentage of viable cells under each treatment condition was determined relative to the negative control.

**Western Blot Analysis.** Total nuclear proteins were prepared and the protein levels of CHOP and lamin B or $\beta$-actin were detected and analyzed as previously described.[14]

**Construction of IEC-6 Stable Cell Line Expressing Secreted Alkaline Phosphatase (SEAP) and SEAP Enzyme Activity Assay.** SEAP is secreted from the ER and its activity is rapidly downregulated by ER stress independent of transcriptional regulation.[14] IEC-6 stable cell line expressing SEAP was established as described previously.[15] The SEAP enzyme activity was measured by chemiluminescent assays using Great EscAPe SEAP Detection Kit according to the manufacturer's instructions (Promega).

**Enzyme-Linked Immunosorbent Assays (ELISA) of Cytokines.** Mouse macrophages were treated with 15 $\mu$M ritonavir in different formulations or vehicle control for 24 h. At the end of the treatment, the culture media were collected and centrifuged at 13,000 rpm for 5 min. The supernatants were collected and stored in aliquots at −70 °C until use. The protein levels of TNF-α and IL-6 in the media were determined by ELISA as described previously.[6] The total protein concentrations of the viable cell pellets were determined using Bio-Rad Protein Assay reagent. Total amounts of the TNF-α and IL-6 in media were normalized to the total protein amounts of the viable cell pellets.

**Measurement of Intracellular ROS.** Dichlorodihydrofluorescein diacetate ($H_2$DCFDA) is a cell-permanent nonfluorescent dye until the acetate groups are removed by intracellular esterases and oxidation occurs within the cells. Cells were cultured in 12-well plates and treated with 15 $\mu$M ritonavir with or without various antioxidants (all dissolved in DMSO), ritonavir SMEDDS, Norvir or blank SMEDDS (diluted in medium) for 4 h. Hydrogen peroxide ($H_2O_2$, final concentration, 300 $\mu$M) was used as positive control. Following the treatment, cells were washed once with PBS and loaded with 10 $\mu$M $H_2$DCFDA in serum free DMEM for 30 min at 37 °C. After washing with PBS three times, cells were collected and divided into two fractions. One was resuspended in PBS for measuring protein concentration; the other was resuspended in 0.5 mL of lysis buffer (0.1 N NaOH in 50% methanol). The total cell lysate was centrifuged at 5,000 rpm for 5 min. The supernatant was used to determine the fluorescence intensity using a 96-well plate reader with excitation and emission wavelengths of 485 and 520 nm. The fluorescence values were normalized to protein levels in each sample.

**Animal Studies.** Sprague−Dawley rats (male, 220 to 260 g) were used for *in vivo* pharmacokinetic studies. C57BL/6 wild type (male, 6−8 weeks old, Jackson Laboratories, Bar Harbor, ME) were used to assess the *in vivo* intestinal epithelia function and tissue distribution of ritonavir. Rats and mice were housed and fed standard mouse chow and tap water *ad libitum* throughout the study following protocols approved by the IACUC at Virginia Commonwealth University.

**Pharmacokinetic Studies of Ritonavir SMEDDS.** Sprague−Dawley rats (220 to 260 g) were used to compare the pharmacokinetics of ritonavir SMEDDS and Norvir. Rats were randomly divided into the following ritonavir SMEDDS and Norvir groups ($n = 3$). Drugs were orally administrated at a dose of 50 mg/kg. The blood samples (0.2 mL) were collected from the tail vein at 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 h. The ritonavir concentrations in plasma were determined by HPLC. Drug and Statistics for Windows (DAS ver1.0) was utilized to analyze the pharmacokinetic parameters of the area under the plasma concentration−time curve ($AUC_{0-\infty}$), the apparent volume of distribution ($V_d$), total body clearance (CL), elimination half-life ($t_{1/2\beta}$) and mean residence time (MRT) of ritonavir for each formulation.

**The Effect of Ritonavir SMEDDS on Intestinal Barrier Function.** Mice were randomly assigned to the following four groups: Control, blank SMEDDS, ritonavir SMEDDS, and Norvir. Mice were gavaged daily with ritonavir SMEDDS or Norvir (50 mg/kg) or control solution for 4 weeks. Animals were monitored daily for appearance of diarrhea, body weight loss and other distress. At the end of treatment, mice were gavaged with FITC−dextran (4 kDa) at a dose of 600 mg/kg body weight 4 h before harvest. Blood was collected from the hepatic portal vein. The serum concentration of the FITC−dextran was determined using a fluorescence plate reader with an excitation wavelength at 490 nm and an emission wavelength of 530 nm.[15] The plasma concentration of ritonavir was determined by HPLC analysis. The intestines were removed and sections were taken for formalin fixation and histological examination as described previously.[15]

**Statistical Methods.** Values are means ± SD from three to six samples. Western blot results were repeated at least three times. One-way ANOVA was employed to analyze the data. Statistics were performed using Prism 5 (GraphPad, San Diego, CA). The $p < 0.05$ values were considered statistically significant.

## Results

**Effect of Individual Antioxidant on Ritonavir-Induced ROS Production in IEC-6 Cells.** It has been reported that HIV PIs augment production of ROS in various types of cells including macrophages, vascular smooth muscle cells, umbilical vein endothelial cells, adipocytes, and pancreatic $\beta$-cells, suggesting that oxidative stress plays a role in HIV PI-induced adverse side effect.[18−22] Our most recent studies found that HIV PI-induced ER stress contributed to disruption of intestinal barrier integrity.[15] First, we tested whether the commonly used antioxidants in drug formulations were able to prevent ritonavir-induced ROS production in IEC-6 cells. As shown in Figure 1, the vitamin C, ethyl linoleate and oleic acid were able to reduce ritonavir-induced ROS production in IEC-6 cells. Similar results were obtained in mouse macrophages (Figure 1 in the Supporting Information).

**Effect of Individual Antioxidant on Ritonavir-Induced ER Stress in IEC-6 Cells.** Oxidative stress and ER stress are closely related events. It has been shown that oxidative stress induces UPR activation[23] and antioxidants reduce ER stress and improve the protein folding capacity of ER.[24] In order to assess the effect of individual antioxidant on ritonavir-induced ER stress activation, we constructed stable IEC-6 cells overexpressing SEAP as described previously. The SEAP is secreted from the ER and its activity is rapidly downregulated by ER stress independent of transcriptional regulation. As shown in Figure 2A, ritonavir markedly inhibited SEAP activity (>80%), which was significantly prohibited by vitamin C, vitamin E, ethyl linoleate and oleic acid. We further examined whether these antioxidants were also able to reduce ritonavir-induced UPR activation in IEC-6 cells by detecting the expression of CHOP, the downstream transcriptional factor of the UPR, using Western blot analysis. As shown in Figure 2B, ritonavir-induced CHOP expression was significantly reduced by vitamin C, ethyl linoleate and oleic acid, while vitamin E had less effect. Similarly, vitamin C, ethyl linoleate and oleic acid also inhibited ritonavir-induced CHOP expression in macrophages (Figure 2 in the Supporting Information). These results suggested that these antioxidants were of great potential in the development of a novel ritonavir formulation.

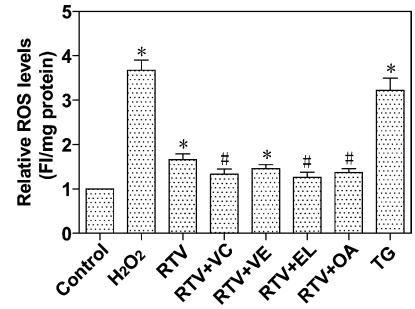


***Figure 1.*** Effect of antioxidants on ritonavir-induced ROS production in IEC-6 cells. Cells were treated with vehicle control (DMSO), positive control ($H_2O_2$, 10 $\mu$M), thapsigargin (TG, a known ER stress inducer, 100 nM), ritonavir (RTV, 15 $\mu$M), or RTV with vitamin C (VC 15 $\mu$g/mL), with vitamin E (VE 20 $\mu$g/mL), with ethyl linoleate (EL, 20 $\mu$g/mL) or with oleic acid (OA, 10 $\mu$g/mL) for 4 h. The intracellular ROS levels were measured as described in Materials and Methods. Values are mean ± SD of 3 independent experiments and analyzed using one-way ANOVA, statistical significance relative to control, $*p < 0.05$ or relative to RTV, $\#p < 0.05$.

**Preparation and Characterization of Ritonavir SMEDDS.** Our previous studies have shown that ritonavir-induced dyslipidemia, inflammation and gastrointestinal barrier dysfunction are linked to ER stress and the UPR activation.[6,13−15] It also has been shown that persistent oxidative stress can initiate ER stress response and polyunsaturated fatty acids have beneficial effects on lipid metabolism.[11,25] Based on the results of *in vitro* studies, we selected vitamin C, vitamin E, ethyl linoleate, and oleic acid, together with cremophor EL, to prepare the ritonavir SMEDDS. A series of ritonavir SMEDDS formulations were prepared. The composition of ritonavir SMEDDS was optimized based on the concentration of oil and emulsifier and particle size. The final formulation of ritonavir SMEDDS consisted of ritonavir (10% w/v), ethyl linoleate (30% v/v), vitamin E (5% v/v), 1% vitamin C in ethanol (20% v/v), cremophor EL (25% v/v) and oleic acid

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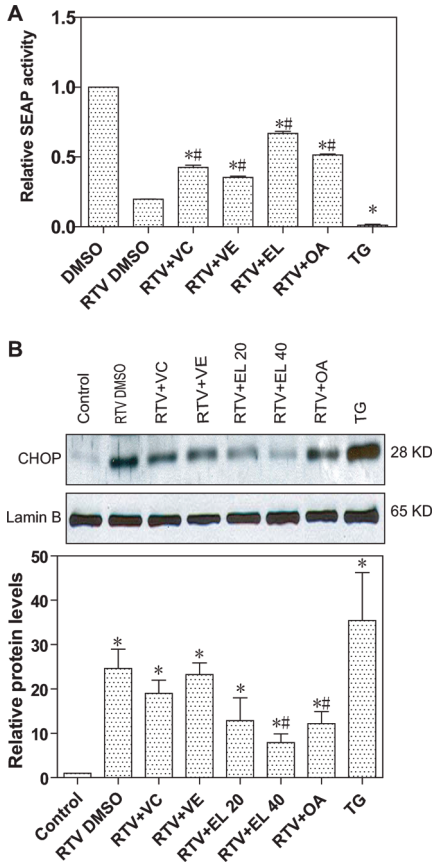


***Figure 2.*** Effect of antioxidants on ritonavir-induced ER stress and UPR activation in IEC-6 cells. (A) IEC-6 cells stably transfected with pSEAP plasmid were treated with vehicle control (DMSO); ritonavir (RTV, 15 $\mu$M) in the absence or presence of individual antioxidant, vitamin C (VC 15 $\mu$g/mL), vitamin E (VE 20 $\mu$g/mL), ethyl linoleate (EL, 20 $\mu$g/mL), or oleic acid (OA, 10 $\mu$g/mL); or TG (100 nM) for 24 h. Activity of SEAP was measured as described in Materials and Methods and expressed as percent of control. Values are mean ± SD of 3 independent experiments. Statistical significance relative to vehicle control, $*p < 0.05$ or relative to RTV, $\#p < 0.05$. (B) Representative immunoblots against CHOP and lamin B from the nuclear extracts of IEC-6 cells treated with ritonavir (RTV, 15 $\mu$M) in the absence or presence of individual antioxidant, vitamin C (VC 15 $\mu$g/mL), vitamin E (VE 20 $\mu$g/mL), ethyl linoleate (EL, 20 $\mu$g/mL or 40 $\mu$g/mL), or oleic acid (OA, 10 $\mu$g/mL) for 4 h. Lamin B was used as a loading control of nuclear protein. Relative protein levels of CHOP from three independent experiments. Statistical significance relative to control, $*p < 0.05$ or relative to RTV, $\#p < 0.05$.

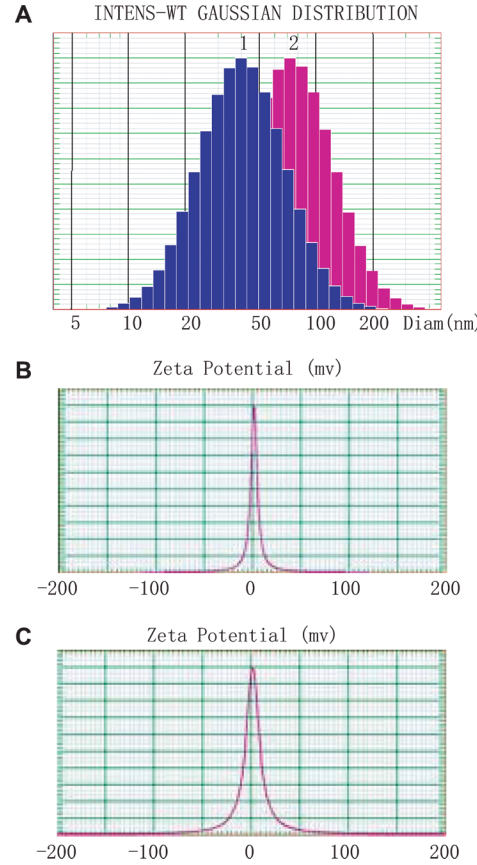


***Figure 3.*** Characterization of ritonavir SMEDDS. (A) Particle size distribution of SMEDDS in water: (1) blank SMEDDS 46.1 nm; (2) RTV SMEDDS 81.6 nm. (B, C) Zeta potential of SMEDDS: (B) blank SMEDDS 1.94 mV; (C) RTV SMEDDS 1.70 mV.

(10% v/v). The blank SMEDDS was prepared with the same amount of each component except ritonavir. Both ritonavir SMEDDS and blank SMEDDS were easily self-emulsified in water.

We further determined the particle size and zeta potential to characterize the particles of ritonavir SMEDDS. The zeta potential is a good estimate of the surface charge of the particles, which is determined from the particle electrophoretic mobility in an electrophoretic cell.[26] As shown in Figure 3A, the intensity-WT Gaussian distribution means of the diameters for blank SMEDDS and ritonavir SMEDDS were 46.1 and 81.6 nm, respectively. The incorporation of ritonavir in the SMEDDS increased particle size, but it was less than 100 nm. As shown in Figures 3B and 3C, the surface charges of blank SMEDDS and ritonavir SMEDDS were similar (1.70 mV and 1.94 mV). In addition, both blank SMEDDS and ritonavir SMEDDS were narrowly distributed and their polydispersity indexes were under 0.3.

**Stability and Pharmacokinetic Studies of Ritonavir SMEDDS.** To determine whether this new formulation affected the stability of ritonavir, we compared the stability of ritonavir SMEDDS and Norvir in culture medium at 37 °C. As shown in Figure 4A, ritonavir SMEDDS was more stable than Norvir. We further examined the uptake of ritonavir in IEC-6 cells; as shown in Figure 4B, this SMEDDS more efficiently delivered the encapsulated ritonavir into IEC-6 cells. The *in vivo* pharmacokinetic studies

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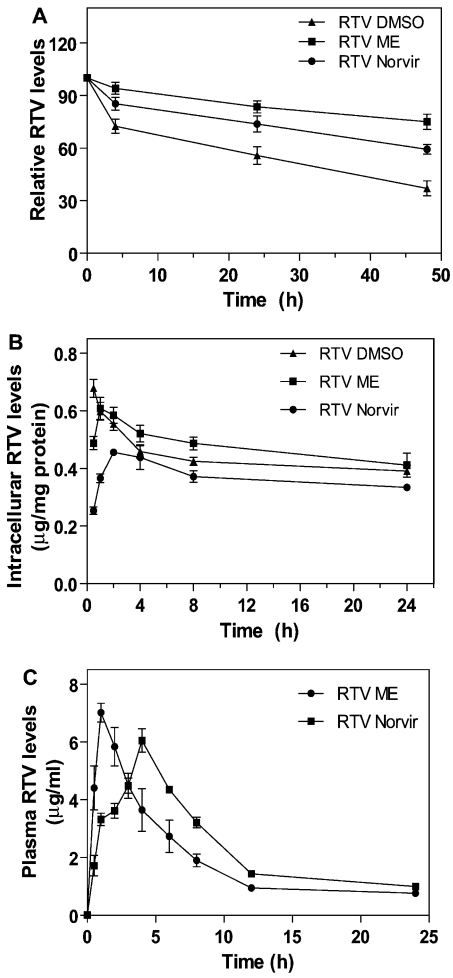


***Figure 4.*** Stability and pharmacokinetics of ritonavir SMEDDS. (A) The stability of ritonavir SMEDDS (RTV ME), and Norvir in medium. RTV ME and Norvir (25 $\mu$M) were incubated in cell culture medium at 37 °C for 4, 24, and 48 h. The remaining ritonavir in medium was determined by HPLC as described in Materials and Methods. (B) IEC-6 cells were treated with 15 $\mu$M ritonavir SMEDDS (RTV ME), Norvir or ritonavir in DMSO (RTV DMSO) for 0.25, 0.5, 1, 2, 4, 8, and 24 h. The intracellular ritonavir concentration was determined by HPLC as described in Materials and Methods and normalized to total protein amount. Each data represents mean ± SE of three experiments. (C) The *in vivo* pharmacokinetics of RTV ME. Sprague−Dawley rats were gavaged with RTV ME or Norvir at a dose of 50 mg/kg. The plasma concentration of ritonavir was determined as described in Materials and Methods.

indicated that the bioavailability of ritonavir SMEDDS was better than that of Norvir as indicated by higher Cmax and shorter Tmax (Figure 4C).

**Effect of Ritonavir SMEDDS on Cell Viability in IEC-6 Cells.** Our previous studies have shown that ritonavir induces cell apoptosis in macrophages, primary hepatocytes and IEC-6 cells.[13−15] In order to determine whether incorporation of antioxidants in ritonavir SMEDDS is able to prevent ritonavir-induced cell death, we assessed the viability of IEC-6 cells after treatment with ritonavir SMEDDS and

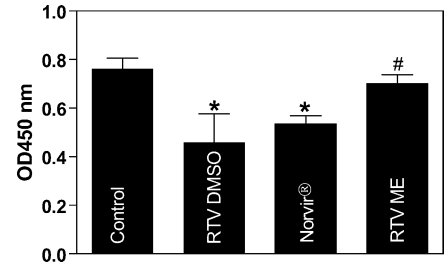


***Figure 5.*** Cell viability assay in IEC-6 cells. IEC-6 cells were seeded on 96-well plates overnight and then treated with 25 $\mu$M of ritonavir SMEDDS (RTV ME), Norvir, or ritonavir in DMSO (RTV DMSO) for 24 h. The cell viability was determined using cell proliferation reagent WST-1 as described in Materials and Methods. Each data represents mean ± SD of three experiments. Statistical significance relative to control, $*p < 0.05$ or relative to RTV, $\#p < 0.05$.

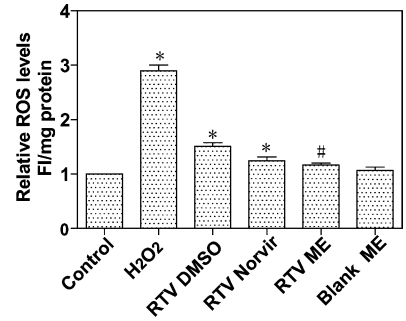


***Figure 6.*** Effect of ritonavir SMEDDS and Norvir on ROS production in IEC-6 cells. Cells were treated with vehicle control (DMSO), positive control ($H_2O_2$, 10 $\mu$M), ritonavir in DMSO (RTV DMSO, 15 $\mu$M), blank SMEDDS (blank ME), ritonavir SMEDDS (RTV ME, 15 $\mu$M), and Norvir (15 $\mu$M) for 4 h. The intracellular ROS levels were determined using H2DCFDA as described in Materials and Methods. Each bar represents mean ± SD of three experiments. Statistical significance relative to DMSO control, $*p < 0.05$, and relative to RTV DMSO, $\#p < 0.05$.

Norvir for 24 h. As shown in Figure 5, ritonavir SMEDDS had less effect on cell viability compared to Norvir in IEC-6 cells.

**Effect of Ritonavir SMEDDS on ROS Production in IEC-6 Cells.** To determine whether incorporation of antioxidants in ritonavir SMEDDS was able to quench ritonavir-induced ROS production, IEC-6 cells were treated with 15 $\mu$M ritonavir in DMSO, ritonavir SMEDDS or Norvir for 4 h; the intracellular levels of ROS were measured as described in Materials and Methods. As shown in Figure 6, ritonavir-induced ROS production was inhibited by ritonavir SMEDDS, suggesting that incorporation of antioxidants in ritonavir SMEDDS helped reduce ritonavir-induced oxidative stress. Similar results were obtained in macrophages (data not shown).

**Effect of Ritonavir SMEDDS on ER Stress and UPR Activation in IEC-6 Cells.** In order to assess the effect of ritonavir SMEDDS on ER stress and UPR activation, we

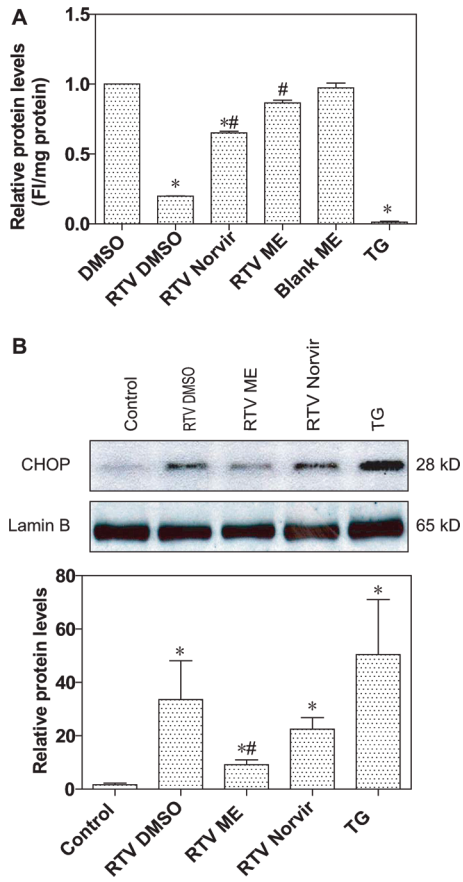


***Figure 7.*** Effect of ritonavir SMEDDS on ER stress and UPR activation in IEC-6 cells. (A) IEC-6 cells stably transfected with pSEAP plasmid were treated with vehicle control (DMSO), ritonavir in DMSO (RTV DMSO, 15 $\mu$M), ritonavir SMEDDS (RTV ME, 15 $\mu$M) or Norvir (15 $\mu$M), or TG (25 nM) for 24 h. Activity of SEAP was measured as described in Materials and Methods and expressed as percent of control. Values are mean ± SD of 3 independent experiments. Statistical significance relative to vehicle control, $*p < 0.05$, or relative to RTV DMSO, $\#p < 0.05$. (B) Representative immunoblots against CHOP and lamin B from the nuclear extracts of IEC-6 cells treated with vehicle control (DMSO), ritonavir in DMSO (RTV DMSO, 15 $\mu$M), ritonavir SMEDDS (RTV ME, 15 $\mu$M) or Norvir (15 $\mu$M), or TG (25 nM) for 4 h. Lamin B was used as a loading control of nuclear protein. Relative protein levels of CHOP from three independent experiments were analyzed. Statistical significance relative to control, $*p < 0.05$, or relative to RTV DMSO, $\#p < 0.05$.

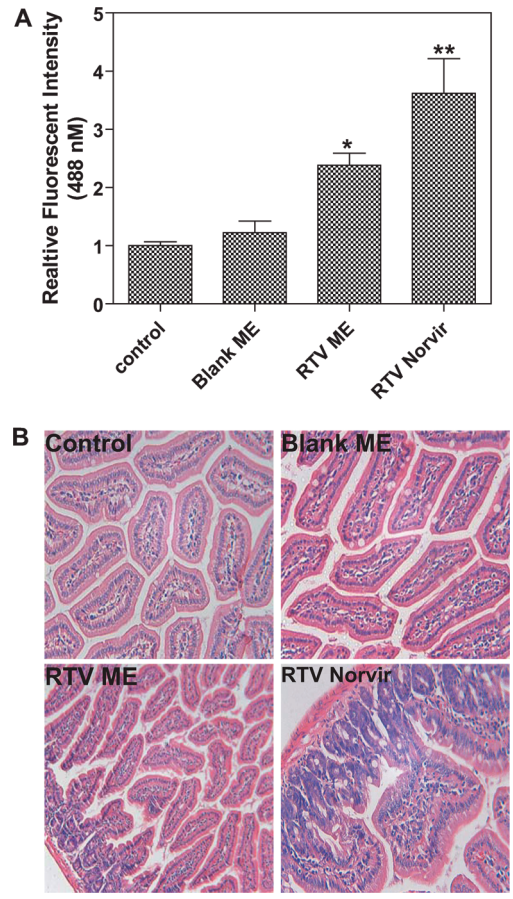


***Figure 8.*** Effect of ritonavir SMEDDS on intestinal permeability and tissue damages *in vivo.* Wild type C57BL/6 mice were treated with control solution (PBS), blank SMEDDS (Blank ME), ritonavir SMEDDS (RTV ME) or Norvir (50 mg/kg) for 4 weeks. (A) The intestinal permeability was measured using FITC−dextran as described in Materials and Methods. Statistical significance relative to vehicle control ($n = 5$), $*p < 0.05$, $**p < 0.01$. (B) Representative images of HE staining for each treatment group are shown.

compared the effects of ritonavir SMEDDS and Norvir on SEAP secretion and CHOP expression in IEC-6 cells as described in previous sections. As shown in Figure 7A, Norvir inhibited SEAP activity by 35%, but ritonavir SMEDDS had little effect. Similarly, ritonavir SMEDDS had less effect on CHOP expression. Both ritonavir in DMSO and Norvir significantly induced CHOP expression in IEC-6 cells (Figure 7B). Similarly, ritonavir SMEDDS had no significant effect on UPR activation in macrophages (Figure 3 in the Supporting Information).

**Effect of Ritonavir SMEDDS on Intestinal Epithelial Integrity *in Vivo*.** We have recently shown that UPR activation greatly contributes to ritonavir-induced disruption of intestinal barrier function.[15] Ritonavir-induced intestinal epithelial damage was significantly reduced in CHOP knockout mice. To further evaluate the effect of ritonavir SMEDDS on intestinal epithelial integrity, we examined the effects of ritonavir SMEDDS and Norvir on intestinal permeability and intestinal tissue damage in wild type C57/BL6 mice. As shown in Figure 8A, after 4-week treatment, Norvir and ritonavir SMEDDS increased intestinal permeability by 260% and 140%, respectively. Incorporation of antioxidants significantly prevented ritonavir-induced increase of intestinal permeability. Histological examination

further confirmed that ritonavir-induced intestinal tissue damage was also significantly reduced in ritonavir SMEDDS-treated mice (Figure 8B).

## Discussion

HIV PIs are currently among the preferred components used in combination with other drugs as part of HAART, which has changed HIV infection from invariably lethal to a manageable chronic condition. However, the benefits of HIV PIs are compromised by a number of clinically significant adverse side effects.[27] HIV PI-induced dysfunction of gastrointestinal barrier has recently been recognized as a paramount factor in the pathogenesis of HIV progression and HIV PI-associated cardiovascular complication.[9,28] The elevated plasma endotoxin, a consequence of microbial translocation due to leaky gut, and subsequent activation of systemic inflammatory response are the hallmarks of progressive HIV infection and better predict disease outcome than plasma viral load.[29−31] A number of studies have shown that induction of oxidative stress and ER stress contributes to the HIV PI-induced various side effects such as dysregulation of lipid metabolism, insulin resistance, inflammation and disruption of intestinal barrier function.[2] Development of novel strategies to prevent HIV PI-induced clinical complications is of great importance.

Ritonavir is the most commonly used HIV PI and is presently sold in a soft gelatin capsule dosage form for oral administration under the trade name Norvir. Norvir uses a self-emulsifying drug delivery system (SEDDS), and it forms a milk-like emulsion in water. The current study developed a novel SMEDDS for ritonavir using various antioxidants, evaluated its potential effects on reducing ritonavir-induced oxidative stress and UPR activation in intestinal epithelial cells, and further studied its pharmacokinetics and effect on intestinal barrier function *in vivo*.

Of many drug delivery systems, SMEDDS have attracted growing interest due to their excellent efficiency in improving the drug solubility and have been successfully used for oral delivery of hydrophobic drugs.[16,32−34] SMEDDS form fine and stable oil-in-water microemulsions upon aqueous dilution owing to the gentle agitation of the gastrointestinal fluids.[16] In this study, we used an oily antioxidant mixture (ethyl linoleate, oleic acid and vitamin E) as the oil phase and successfully developed a novel SMEDDS for ritonavir. The results indicate that incorporation of ritonavir in SMEDDS significantly improved its solubility in the aqueous phase. After dilution in an aqueous solution, nanoscale droplets (<100 nm) were formed (Figure 3). Also, there was no precipitation of ritonavir observed when the SMEDDS were diluted in the cell culture media or deionized water for over 24 h. It is important to note that the formation of microemulsions depends on multiple factors including physicochemical characteristics of the drug, surfactant selection, usage of cosolvent and proportion of oil and surfactant. The inclusion of drugs within SMEDDS will affect their performance if the drug is able to compete with water for hydrogen bonding interactions.[35] We found that the inclusion of ritonavir enlarged the mean particle size up to 35 nm (Figure 3) compared to the blank microemulsion, but it was still within the 100 nm scale. We further examined the zeta potential of ritonavir SMEDDS. The zeta potential is often used to estimate the surface charge of the particles, which is determined from the particle electrophoretic mobility in an electrophoretic cell. The microemulsions of ritonavir show low but positive surface charge by absorption of ions from medium. The positive surface charge benefits the small droplets of microemulsion penetrating into the intestinal mucus layers, which show negative charge and enhance oral absorption.[36] Both *in vitro* and *in vivo* pharmacokinetic studies have shown that this SMEDDS significantly improved the uptake of ritonavir indicated by higher Cmax and faster Tmax. The higher plasma concentration tends to more efficiently distribute the drug into target tissues. In addition, the shorter Tmax indicates the faster onset of action.

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Oxidative stress and ER stress greatly contribute to HIV PI-induced side effects. Overproduction of ROS is closely related to the UPR activation in ER. Numerous studies have shown that antioxidants can reduce ER stress and improve ER function.[24] Our studies demonstrated that incorporation of antioxidants in this SMEDDS not only significantly reduced ritonavir-induced ROS formation but also inhibited the UPR activation both in IEC-6 cells (Figures 6 and 7) and macrophages (Figures 1−3 in the Supporting Information). Our recent studies have shown that upregulation of CHOP expression plays a critical role in HIV PI-induced intestinal tissue damage.[15] The *in vivo* studies further demonstrated that this new ritonavir SMEDDS causes less damage to intestinal epithelia compared to Norvir (Figure 8).

Inflammation plays a very important role in various diseases including cardiovascular diseases and drug-induced tissue damage.[37−40] We have previously shown that HIV PI-induced ER stress is associated with the increase of TNF-α and IL-6 expression in macrophages.[6] The inflammatory cytokine expression was associated with the ROS production in macrophages.[41] We also found that antioxidants used in ritonavir SMEDDS was able to inhibit ritonavir-induced TNF-α and IL-6 expression in macrophages (data not shown). The ritonavir SMEDDS had less effect on TNF-α and IL-6 expression compared to Norvir (Figure 4 in the Supporting Information). In addition to the direct effect of HIV PI on macrophages, gut microbial translocation due to the intestinal barrier dysfunction plays a critical role in activating systemic inflammatory response. Our preliminary studies indicate that Norvir significantly increased plasma endotoxin levels, but ritonavir SMEDDS has less effect (Figure 5 in the Supporting Information).

In summary, the intestinal epithelium constitutes the largest and most important barrier against the external environment. Drug-induced disruption of the intestinal barrier function occurs commonly in the clinic, especially in HIV-infected patients. The current study provided important evidence for the potential application of pharmaceutical formulations in reducing HIV PI-induced intestinal barrier dysfunction. This novel SMEDDS using antioxidants as surfactants and co-surfactants represents a new strategy to improve the drug bioavailability and reduce drug-induced tissue damage. The application of this new SMEDDS is not limited to HIV PIs. It also can be used for any other lipophilic drug with serious GI side effects.

## Abbreviations Used

CHOP, C/EBP homologous protein; DCFDA, dichlorodihydrofluorescein diacetate; ELISA, enzyme-linked immunosorbent assays; ER stress, endoplasmic reticulum stress; FITC−dextran, fluorescein isothiocyanate conjugated dextran; GI, gastrointestinal; HAART, highly active antiretroviral therapy; IBD, inflammatory bowel disease; IEC-6, intestinal epithelial cells; IL-6, interleukin-6; ROS, reactive oxygen species; SEAP, secreted alkaline phosphatase; SMEDDS, self-microemulsifying drug delivery system; TNF-α, tumor necrosis factor-alpha; UPR, unfolded protein response.

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**Supporting Information Available:** Additional figures depicting effects of antioxidants on ritonavir-induced ROS production and UPR activation in macrophages, of ritonavir SMEDDS on UPR activation and TNF-α and IL-6 expression in macrophages, and of ritonavir SMEDDS and Norvir on gut microbial translocation in vivo. This material is available free of charge via the Internet at http://pubs.acs.org.

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