UNIVERSITY OF CALIFORNIA, SAN DIEGO

HIV Protease Inhibitors Cause Apoptosis in Intestinal Epithelium Via Autophagy Induction

A thesis submitted in partial satisfaction of the requirements

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in

Biology

by

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2010

The Thesis of Jonathan Kain Brick is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2010

DEDICATION

To the great people of Dr. Raz's lab whose support and collaboration have made this all possible.

To my family and friends for their unconditional love and support.

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VITA

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PUBLICATIONS

“ERK activation drives intestinal tumorigenesis in Apc(min/+) mice.” Nature Medicine, vol. 16, no. 6, pp 665-670, June 2010.

ABSRACT OF THE THESIS

HIV Protease Inhibitors Cause Apoptosis in Intestinal Epithelium
Via Autophagy Induction

by

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Master of Science in Biology

University of California, San Diego, 2010

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HIV protease inhibitors (HPIs) are a major treatment option within highly active antiretroviral therapy (HAART) for HIV patients. Diarrhea is a common side effect found in 16-62% of patients using HPIs. Little is known about the mechanism that mediates the HPI-induced diarrhea. It has been suggested that intestinal barrier disruption is the cause of HPI-induced diarrhea. Recent studies have shown that HPIs disrupt intestinal barrier function via cell death in intestinal epithelial cells (IECs). Using an *in-vitro* human IEC line, HCA7, we

show that the HPI, Ritonavir, induces apoptosis of IECs via an autophagic/apoptotic pathway. Importantly, we also identify an alternative pathway for intestinal barrier disruption through a Ritonavir-induced downregulation of tight junction proteins. These findings implicate two independent pathways for HPI-induced intestinal barrier disruption. Future study may provide therapeutic solutions that exploit these pathways to reduce diarrhea in HAART patients and increase patient compliance to treatment.

INTRODUCTION

HIV Protease Inhibitors (HPIs) are a major component within highly active anti-retroviral therapy (HAART) for effectively suppressing HIV replication and increasing HIV/AIDS patient survivability [1,2,3]. However, patients with HPI-based treatment regimens have increase incidence of gastrointestinal side effects, such as diarrhea, vomiting, and nausea [2,3]. The HPI-based side effects have been shown to pose a greater risk to discontinuation of treatment than in patients taking non-HPI based HAART [4,5].

Diarrhea affects 16-62% of HIV patients using HPI-based HAART [6,7]. Although the reason for HPI-induced diarrhea is unclear, possible explanations include abnormal intestinal motility, malabsorption, the increase in active ion secretion (secretory diarrhea), and/or a leaky epithelial barrier (leak-flux diarrhea) [6]. Leaky epithelial barrier refers to a loss of the barrier between the luminal environment and lamina propria of the intestinal tract, which provides regulation of passive diffusion of solutes between the two environments. This barrier functions due to the network of tight junction proteins that hold and polarize intestinal epithelial cells (IECs). It has been suggested that barrier disruption by HPIs may be due to an induction of apoptosis or other forms of cell death in IECs, as well as down-regulation of tight junction proteins [8,9,10]. Studies conducted by *Bode et al* [6,11,12] showed evidence for HPIs inducing apoptosis and decreasing barrier function in human intestinal

epithelial cells (IECs), suggesting that HPI-induced apoptosis of IECs is responsible for the loss of IEC barrier function and the subsequent diarrhea experienced by patients on HPI-based HAART [6,11,12]. However, the data is not clear as to how apoptosis is induced or as to whether it is solely responsible for barrier disruption.

Autophagy is a process where intracellular organelles and other parts of the cytoplasm are sequestered within a double membrane vacuole (autophagosomes) and delivered to lysosomes for degradation. When there are degenerative organelles, the cell will undergo autophagy to preserve cell homeostasis. However, in times of starvation or high stress conditions, autophagy activation leads to catabolism of macromolecules to meet the bioenergetic needs of the cell. Both autophagy and apoptosis have a complex functional relationship whereby they share the same stimuli and can both activate and suppress each other in different situations [13]. Previous studies have shown activation of autophagy along with apoptosis in epithelial cells by HPI administration [14,15]. Whether these two processes are connected with HPI-induction still remains unclear.

In our study, we investigated how the HPI, Ritonavir, induces barrier disruption in IECs monolayers via the autophagy, apoptosis, and tight junction protein pathways.

METHODS

Reagents and Antibodies: Anti-occludin antibody was purchased from Invitrogen (Carlsbad, CA): All the claudin antibodies were obtained from Lab Vision (Fremont, CA). Anti-β-actin came from Sigma (St. Louis, MO). Anti-Cleaved caspase-3, anti-PARP, anti-β-Tubulin, and anti-LC3B antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-ATG16L antibody was obtained from MBL International (Woburn, MA). Anti-mouse and anti-rabbit IgG secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). All HIV protease inhibitors (HPIs) were obtained from the National Institute of Health.

Cell Culture: The HCA-7 (Human Colonic Adenocarcinoma) cell line was used for all *in-vitro* experiments. Culture conditions and measurement of Trans-Epithelial Electrical Resistance (TEER) of HCA-7 cells have been previously described [16]. In detail, HCA-7 cells were grown in low glucose (1g/L) Dulbecco's modified Eagle medium (Mediatech, Manassas, VA) and supplemented with 10% heat-inactivated fetal calf serum, L-glutamine, sodium pyruvate, and pen strep on T75 flask surfaces until 80-90% confluency. Cells were trypsinized, pelleted, resuspended in media, and seeded 1×10^6 to 1.5×10^6 on the microporous filter inserts in Transwell plates (0.4-μm pore size, Transwell; Costar, Cambridge MA), which permit epithelial cell monolayers to

form and polarize as seen *in-vivo.* In this *in-vitro* model and culture condition, the cells were manipulated from the apical surface (representing IECs facing the intestinal lumen) and from the basolateral surface (representing the serosal compartment). Cells were allowed to polarize for 3-5 days and were used after the TEER reached at least 600 Ω/cm^2 as measured with the Millicell-ERS Resistance System (Millipore, Billerica, MA). Cells were maintained in incubator at 95% O_2, 5% CO_2 and 37℃ and the medium was changed every 48 hours.

Barrier Function Assay: TEER was measured with the Millicell-ERS Resistance System (Millipore, Billerica, MA) to track the barrier function of the HCA-7 monolayers.

HPI Administration: The powder formulations were dissolved in DMSO and added to the apical chamber of cultured Transwell (final concentration 0.1-100mM). The liquid Ritonavir formulation was at a stock concentration of 80mg/ml in a 43% ethanol vehicle and was used for all *in-vitro* experiments, unless otherwise noted. Ritonavir was added to the Transwell HCA-7 IEC monolayers via the apical chamber, directly to culture medium (final concentrations 10-120 μg/ml) and incubated overnight (18 to 24 hours).

Western Blot Analysis: Total cell lysates, cytoplasmic, or nuclear proteins were extracted and run on NuPAGE 12% BIS-Tris gels (Invitrogen). The proteins were transferred to Immobilon-FL transfer membranes (Millipore) and blocked with 1X PBS, 2% BSA, 0.1% sodium azide solution. Immunodetection was used to detect protein levels of Cleaved Caspase-3, PARP, LC3B, ATG16L, C/EBP β, Occludin, Claudin-1, Claudin-3, Claudin-5, β-tubulin, and β-actin.

Flow Cytometry: Apoptosis was detected via flow cytometry using Vybrant Apoptosis Assay Kit #2 (Invitrogen) according to manufacturers' instructions.

Confocal Imaging: IECs were fixed with 4% paraformaldehyde and washed with PBS/TritonX-100. Cells were incubated with the LC3 primary antibody (1:200) overnight at room temperature. After 2 washes, the cells were incubated for 1 hour with Anti-Rabbit-IgG-Alexafluor-488 (1:500, Invitrogen), Phalloidin-AF546 (1:500, Invitrogen), and Hoechst 33258 visualized in the blue channel (1:1000, Invitrogen). Images were obtained with an Olympus FV1000 confocal microscope at the UCSD Neuroscience Microscopy Shared Facility.

RNA Interference: ATG16L was silenced with Atg16 shRNA lentiviral particles (sc-72580-V) in HCA-7 cells according to manufacturers' instructions (Santa

Cruz, CA). Successful knockdown was determined with immunoblotting of ATG16L.

Chemical Inhibition: HCA-7 IECs in transwells were pre-treated basolaterally with Z-VAD, a pan-caspase inhibitor, at a final concentration of 6μM and treated apically with Norvir (0-120 μg/ml) overnight.

RESULTS

HIV protease inhibitors (HPIs) disrupt epithelial barrier

To investigate how HPIs affect barrier function of intestinal epithelium, we applied the drugs on the apical (luminal) surface of polarized IECs, HCA-7, and monitored the barrier function by measuring trans-epithelial electrical resistance (TEER). Among the drugs tested, Nelfinavir, Ritonavir, Saquinavir, Lopinavir, and Tipranavir decreased TEER in a dose dependent manner **(Fig. 1)**.

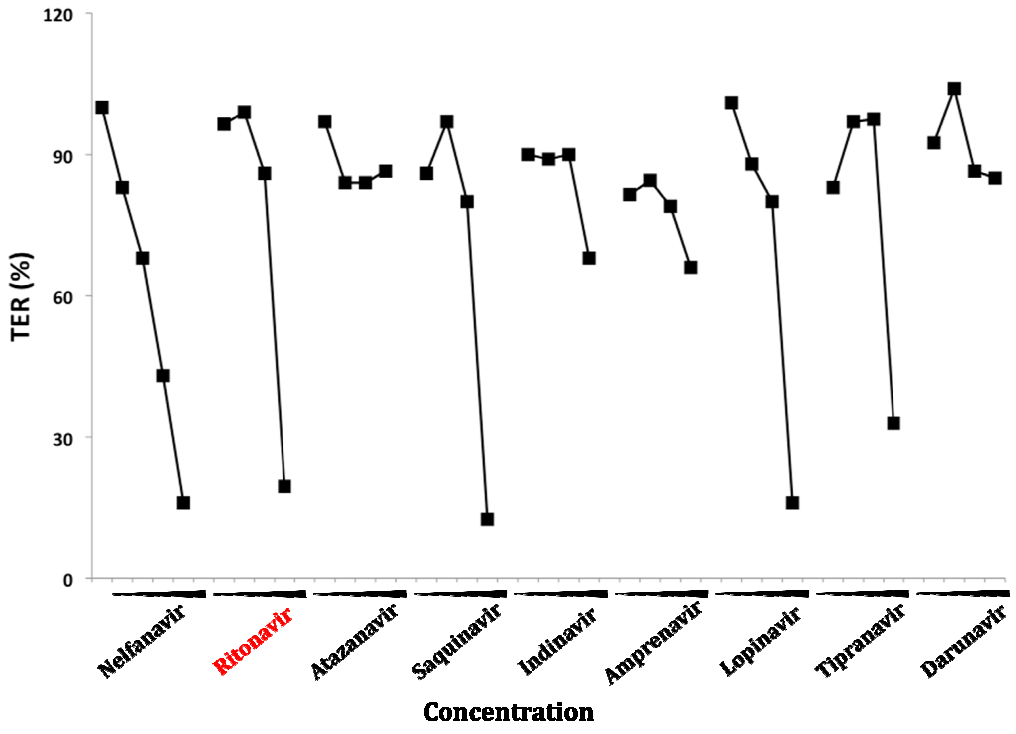

Figure 1: HIV protease inhibitors (HPIs) induce barrier disruption *in-vitro*.

Ritonavir, Nelfinavir, and Lopinavir have been reported to cause moderate to severe diarrhea in HIV patients **(Fig. 2)**.

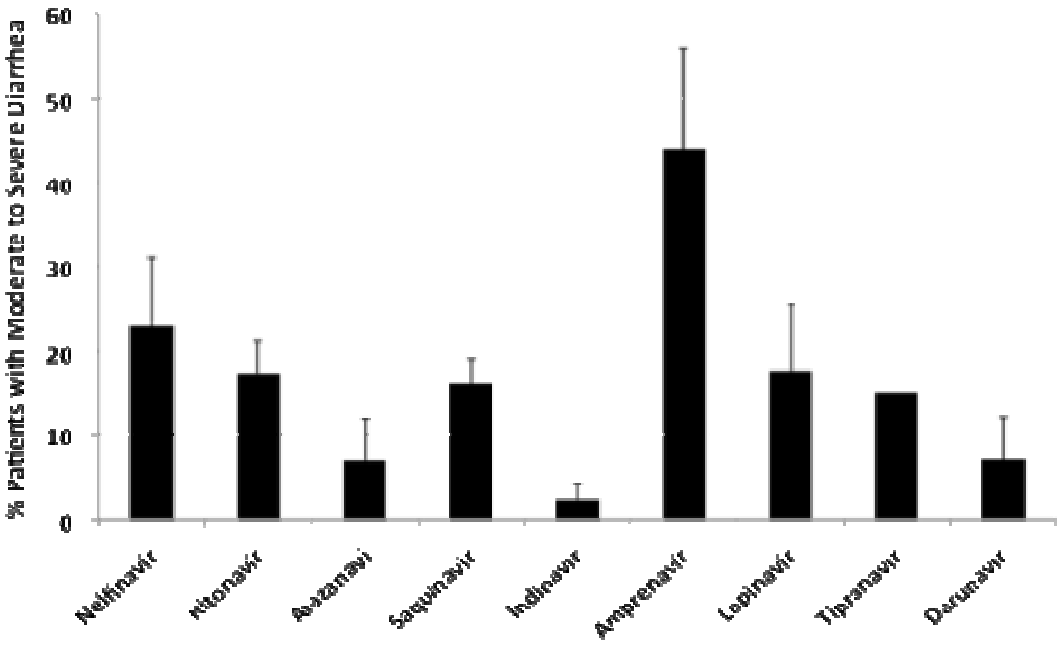

Figure 2: Reported percentages of patients with HPI-associated diarrhea.

Our data suggest that HPIs may cause enteropathy by disrupting the intestinal epithelial barrier. We focus the ensuing study mostly on Ritonavir (also known as Norvir) since it is prescribed most in combination with other HPIs for HAART [17]. Ritonavir induced barrier disruption in both a dose and time dependent manner **(Fig. 3 & 4)**.

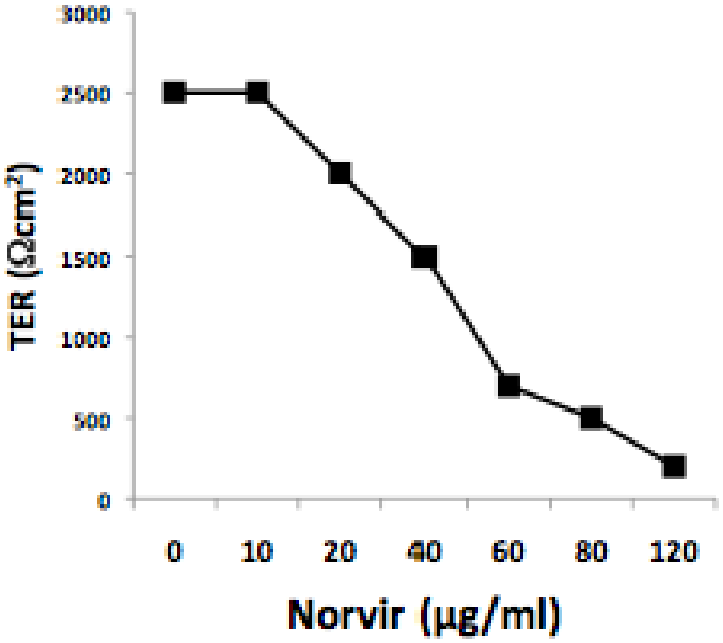

Figure 3: Norvir induces dose-dependent epithelial barrier disruption in HCA-7 cells.

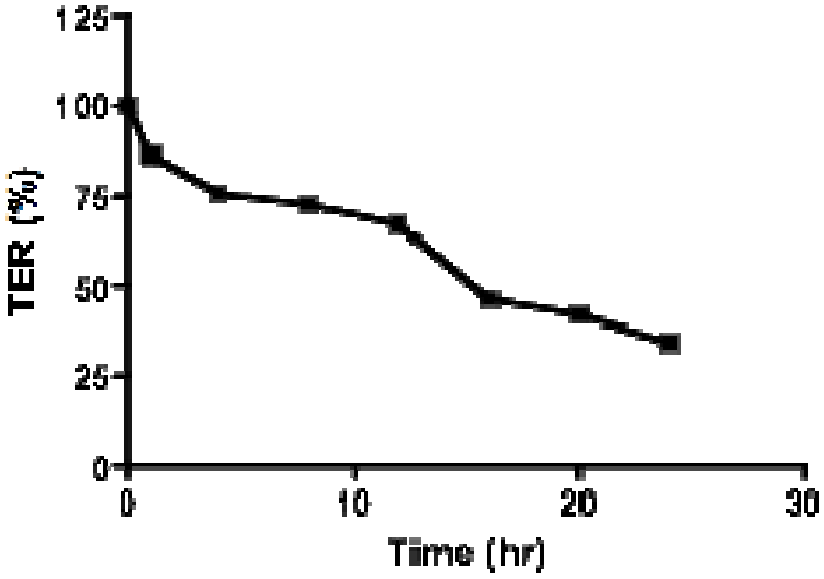

Figure 4: Norvir induces time-dependent epithelial barrier disruption in HCA-7 cells.

Ritonavir induces apoptosis in IECs

We next investigated how HPIs disrupt epithelial barrier. A previous study suggested that Ritonavir, among other HPIs, disrupt barrier function via apoptosis in intestinal epithelial cells [6]. Indeed, Ritonavir induced Caspase-3 activation and cleavage of PARP, a substrate of Caspase-3, in a dose and time-dependent manner in IECs **(Fig. 5 & 6)**.

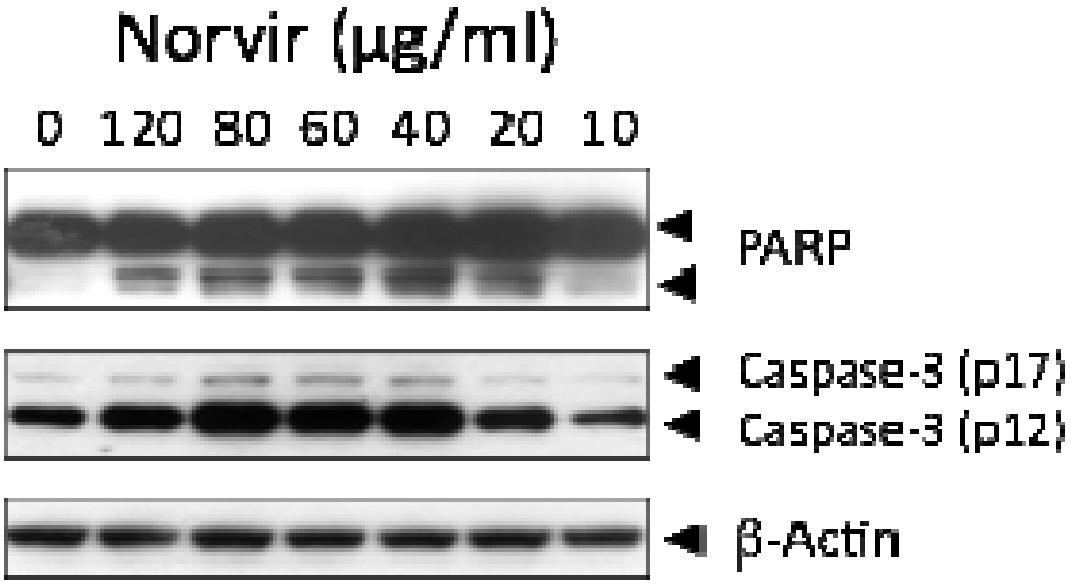

Figure 5: Norvir induces dose-dependent apoptosis in HCA-7 cells.

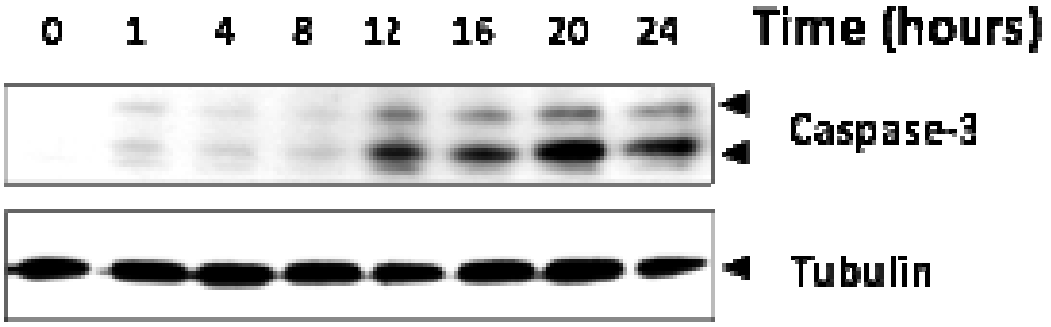

Figure 6: Norvir induces time-dependent apoptosis in HCA-7 cells.

At a concentration of 80 μg/ml, Ritonavir-induced apoptotic cells were 23.4% compared to 10.9% in the control cells **(Fig. 7)**.

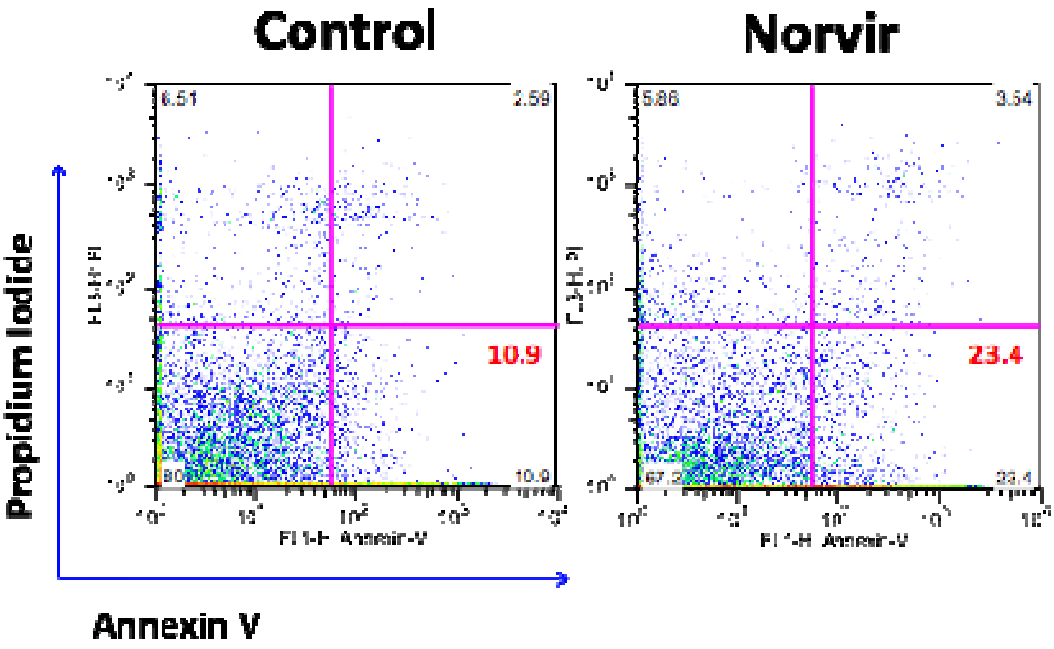

Figure 7: Norvir induces apoptosis in HCA-7 cells (FACS plot).

Ritonavir induces apoptosis via autophagy in IECs

A recent report demonstrated that HPIs induce autophagy in epithelial cells [14]. Indeed, ritonavir induced autophagy in IECs measured by LC3 containing autophagosomes **(Fig. 8)** and LC3 lipidation **(Fig. 9)**.

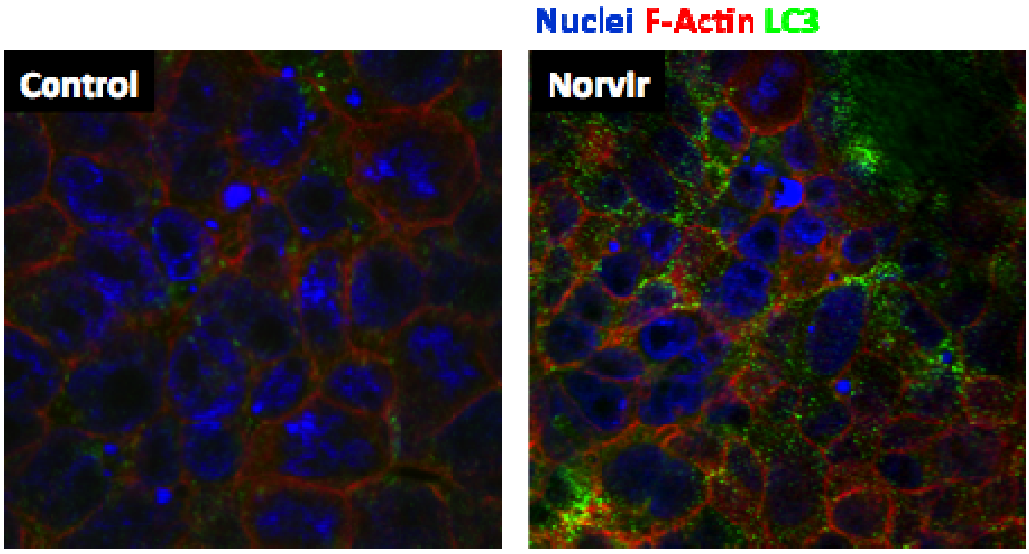

Figure 8: Norvir induces autophagy in HCA-7 cells (confocal image).

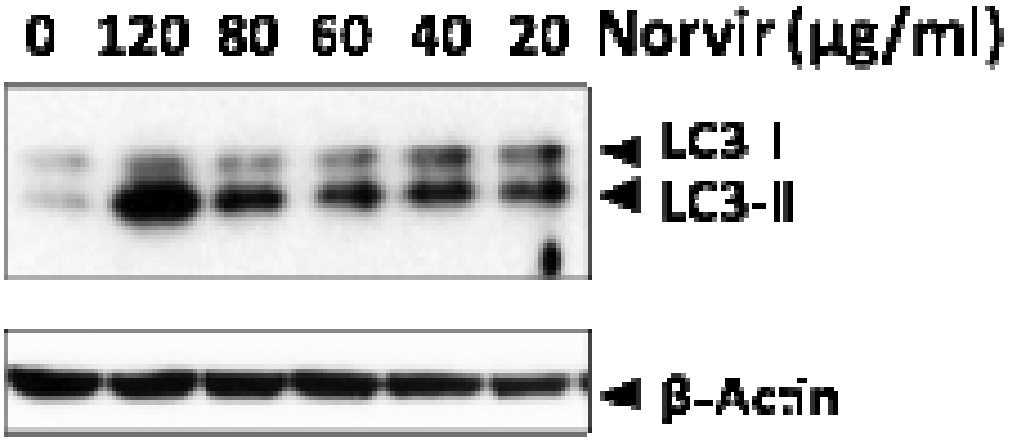

Figure 9: Norvir induces autophagy in HCA-7 cells (Western Blot).

To investigate the role of autophagy induction in ritonavir-induced barrier disruption and apoptosis, we generated a stable cell line expressing ATG16L siRNA. We confirmed that autophagy was not inducible in ATG16L-depleted cells upon nutrient depravation or administration of Ritonavir **(Fig. 10)**.

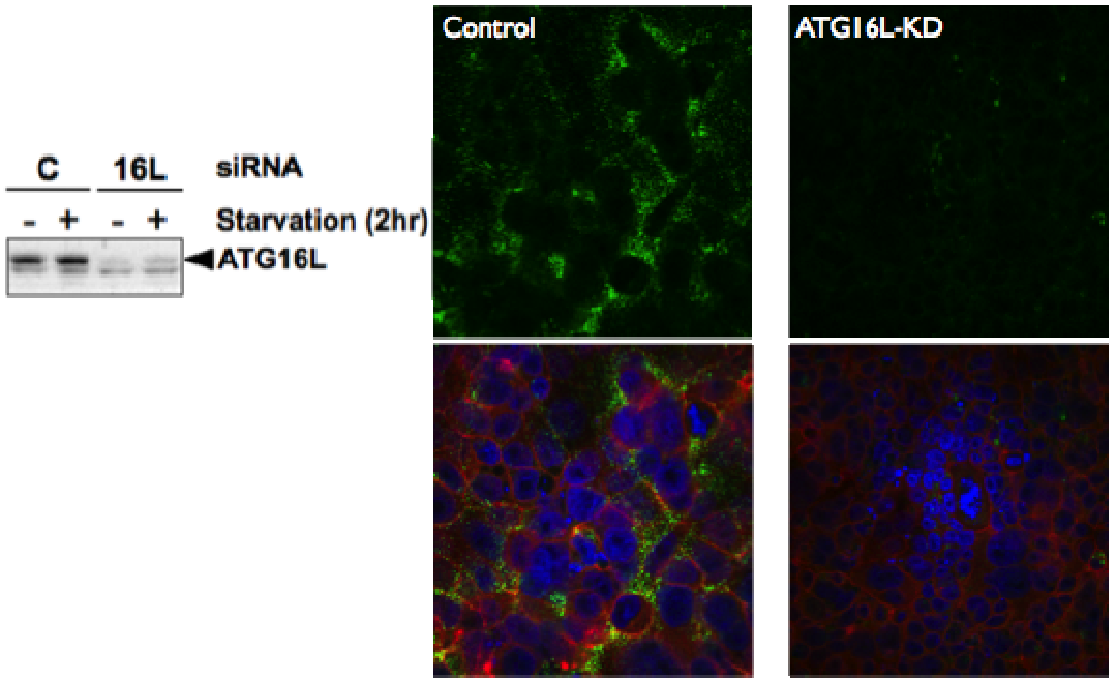

Figure 10: Atg16L is essential for autophagy induction by starvation.

Ritonavir-induced activation of caspase-3 was strictly dependent on autophagy formation since ritonavir failed to activate caspase-3 in ATG16L-depleted IECs **(Fig. 11)**.

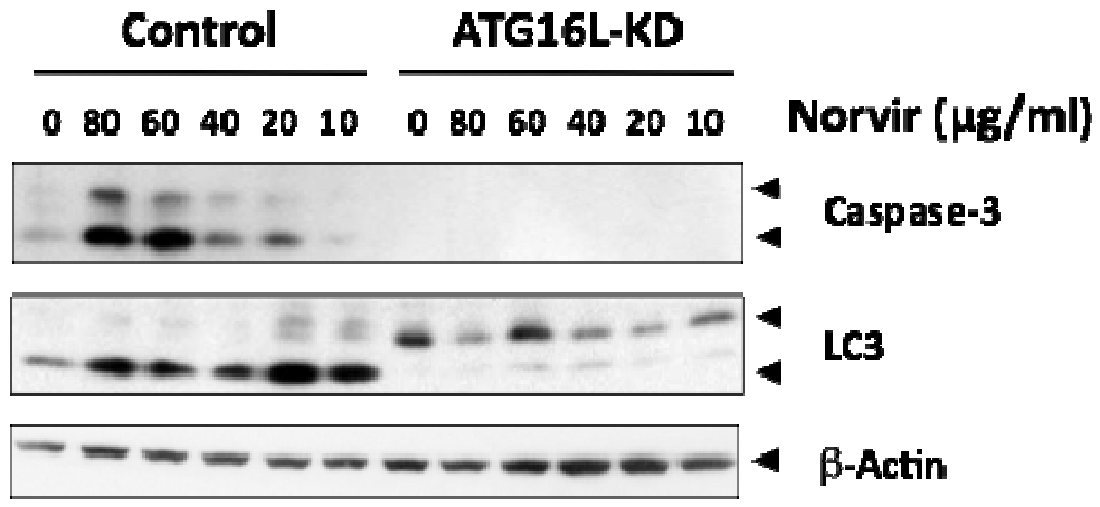

Figure 11: Autophagy is required for Norvir-induced apoptosis.

Disruption of epithelial barrier by ritonavir is independent of autophagy/apoptosis induction

While autophagy induction was essential for Ritonavir-induced apoptosis in IECs **(Fig. 11)**, Ritonavir disrupted IEC barrier in an autophagy-independent manner **(Fig. 12)**.

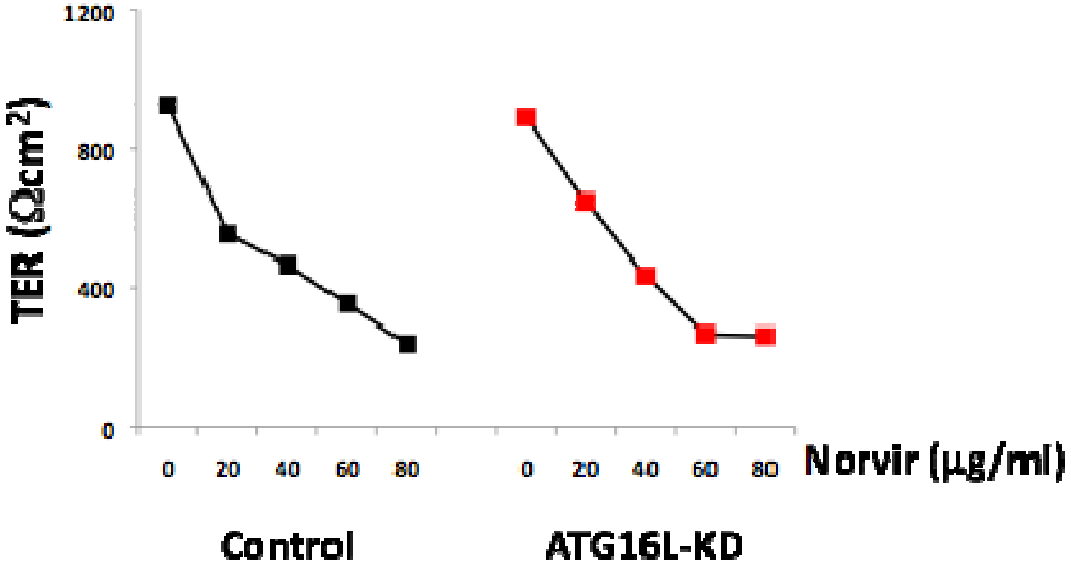

Figure 12: Inhibition of autophagy does not prevent barrier dysfunction caused by Norvir.

To further confirm whether apoptosis was the cause of Norvir-induced barrier disruption, a pan-caspase inhibitor, Z-VAD, was used to inhibit Ritonavir-induced apoptosis **(Fig. 13)**.

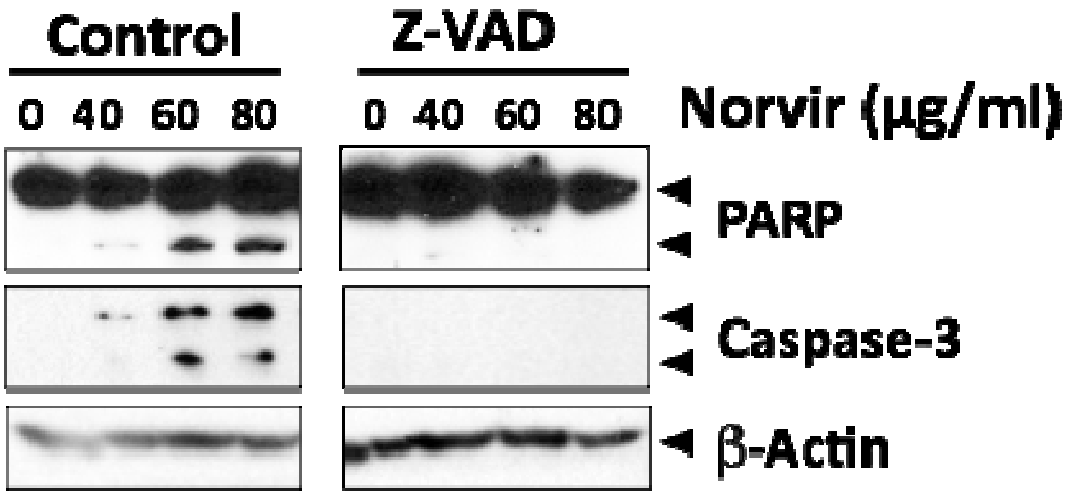

Figure 13: Caspase inhibitor (Z-VAD) prevents Norvir-induced apoptosis.

Although Z-VAD prevented activation of caspase 3 and cleavage of PARP, it failed to suppress barrier disruption **(Fig. 14)**.

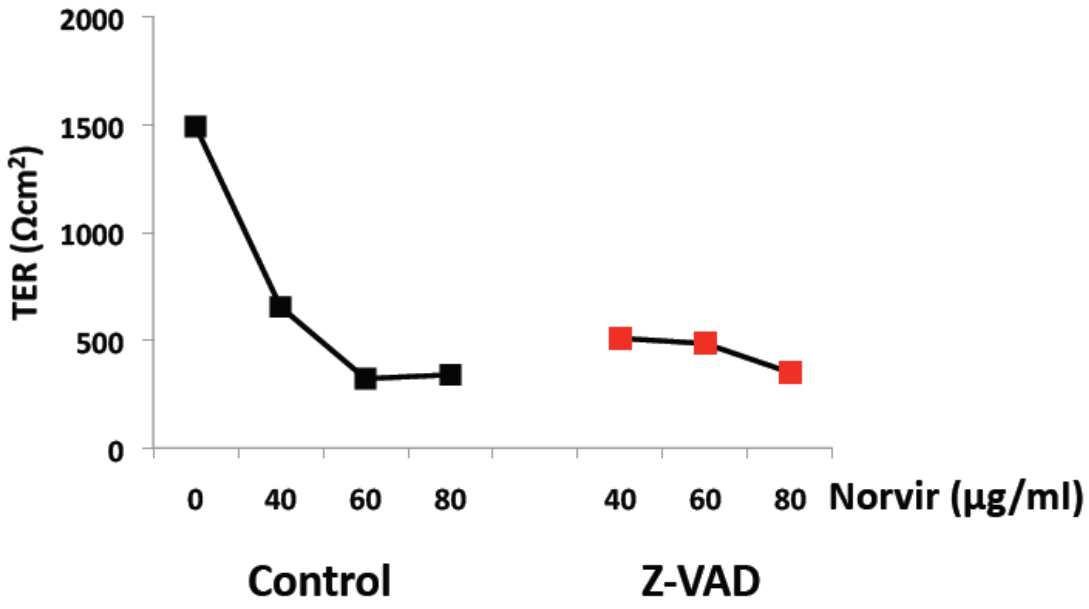

Figure 14: Z-VAD does not prevent Norvir-induced barrier disruption.

Ritonavir down-regulates tight junction proteins independently of autophagy induction

We next investigated how Ritonavir disrupts IEC barrier function. Tight junctions (TJ) are the structural seals that prevent the passage of molecules and ions through the space between cells. Major components of TJ are extracellular proteins including claudins (CLDs) and occludin. The treatment of polarized IECs with Ritonavir in a dose and time dependent manner decreased the levels of CLD-1, -3, -5, and occludin **(Fig. 15 & 16)**.

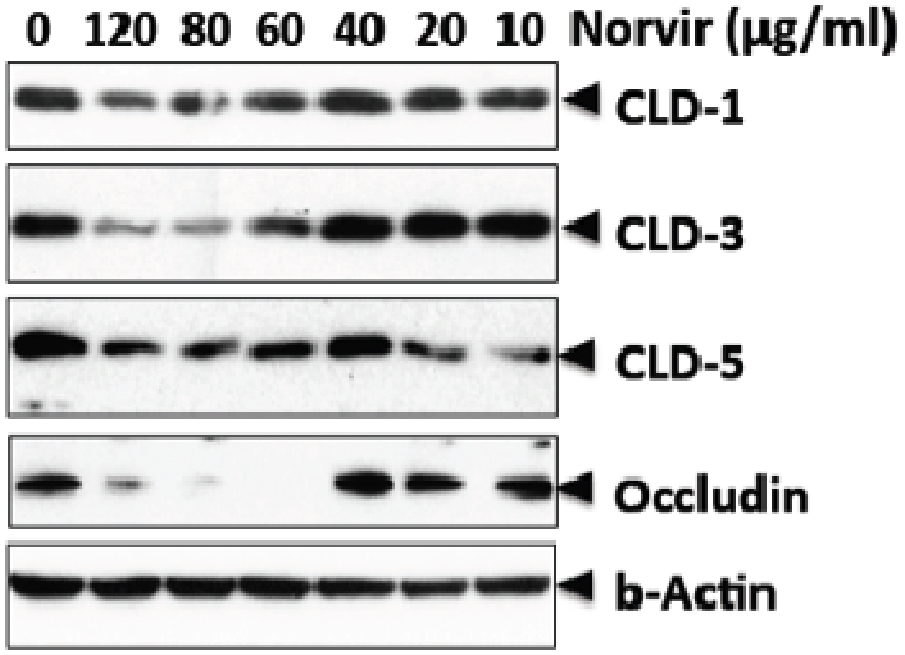

Figure 15: Norvir downregulates tight junction proteins (dose-dependent).

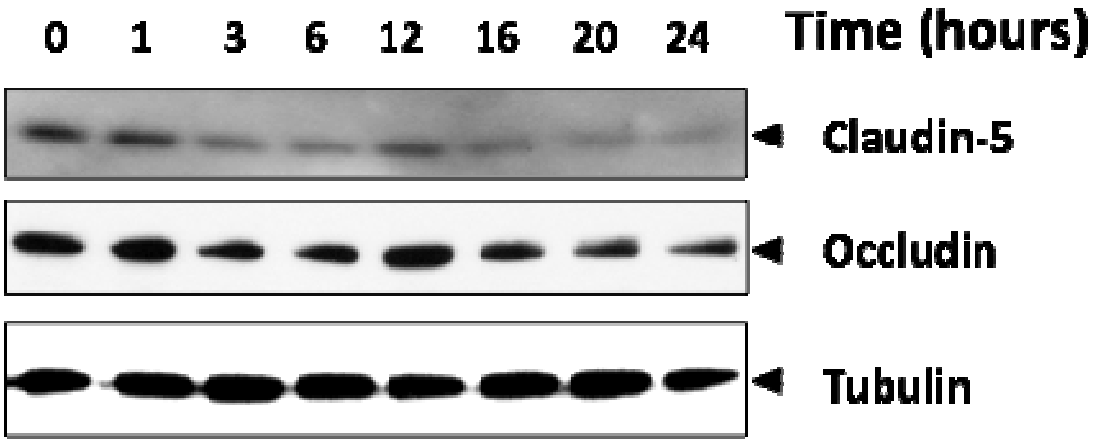

Figure 16: Norvir downregulates tight junction proteins (time-dependent).

However, autophagy induction was not involved in down-regulation of TJ proteins by Ritonavir **(Fig. 17).**

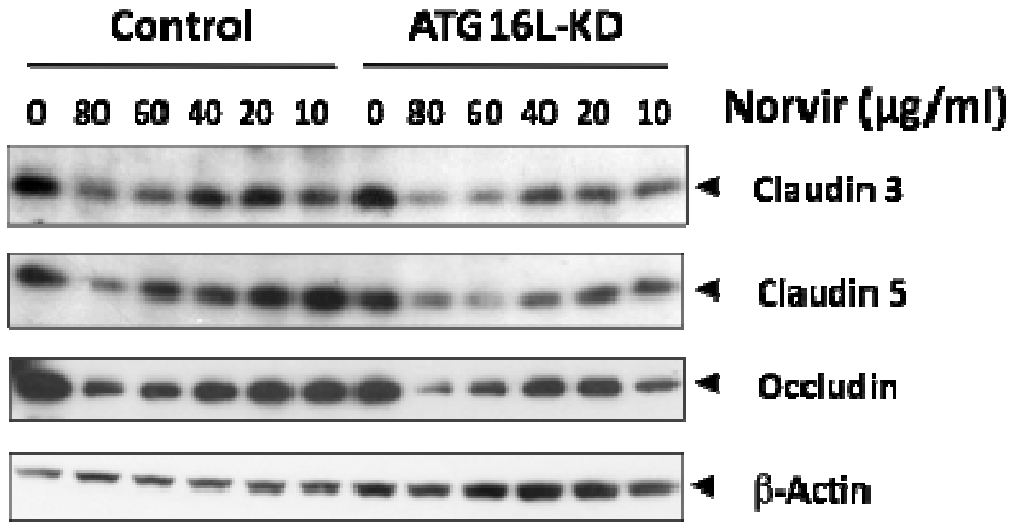

Figure 17: Norvir downregulates tight junction proteins independently of autophagy induction.

These data indicate that Ritonavir induces downregulation of tight junction proteins via a mechanism independent of autophagy/apoptosis pathway.

DISCUSSION

For our investigation, we established an HCA-7 cell line within Transwells to form confluent IEC monolayers similar to that seen *in-vivo*. This epithelium model helped to determine the process by which HPIs induce barrier disruption in IECs. Early indications from our administration of 9 HPIs to IEC monolayers pointed to Nelfinavir, Ritonavir (Norvir), Saquinavir, Lopinavir, and Tipranavir being inducers of barrier dysfunction. These 5 tested HPIs showed significant decreases in TEER and correlated with increasing percentages of diarrhea cases found in patients treated with the corresponding HPI [Fig. 2]. This correlation suggests that the aforementioned HPIs and their disruption of IEC barrier function are responsible for the leak-flux diarrhea in patients receiving HPI-based HAART.

Barrier function in IECs relies on both cell polarity and interactions between cell adhesion complexes and tight junctions with the cells' actin cytoskeletons. A tight junction seals the paracellular side of the cells to invading pathogens and solute diffusion, thereby forming a physical barrier between the intestinal lumen environment and lamina propria. Tight junctions are made up of intracellular and membrane-spanning proteins such as occludins and claudins that link cells and form tight seals between each IEC [18]. Both a down-regulation of tight junction proteins or cell death by HPIs, whether necrotic or apoptotic in nature, would reasonably be assumed to disrupt barrier function in an IEC monolayer by creating holes in the barrier

allowing solutes in. Previous reports by *Bode et al* argue that the cause for barrier disruption by HPIs was cell death. Following up these studies, we found that Norvir does induce apoptosis in IECs in a dose and time-dependent manner. In further exploration of how Norvir activates apoptotic pathway, we looked towards other possible pathways that were induced by Norvir. Autophagy and apoptosis were reported being induced in ovarian epithelial cancer cell lines in multiple studies [14,15]. Autophagy and apoptosis often have similar stimuli and can suppress/activate each other during different situations[13]. We found that Norvir did indeed induce autophagy in IECs and that autophagy was responsible for Norvir-induced apoptosis.

However, the autophagy/apoptosis pathway that was activated by Norvir was not the major contributor to barrier disruption in our experiments. Both genetic and pharmacological suppression of autophagic/apoptotic pathways in HPI treated IECs did not prevent barrier disruption. Tight junction proteins were measured and were found to be reduced as a result of Norvir treatment in an independent manner to autophagy/apoptosis induction. Ritonavir may therefore participate in barrier disruption and subsequent leak-flux diarrhea via activation of two independent pathways (autophagy/apoptosis induction and tight junction protein down-regulation).

Further study into how Norvir activates autophagy, apoptosis and tight junction downregulation may provide a therapeutic solution to further patient compliance to treatment and reduce diarrhea in patients.

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