In-vitro effects of protease inhibitors on *BAX, BCL- 2* and apoptosis in two human breast cell lines

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Currently, the treatment of choice of HIV/AIDS in South Africa is the multidrug combination regimen known as HAART (highly active antiretroviral treatment). HAART, which commonly consists of nucleoside or non-nucleoside reverse transcriptase inhibitors and protease inhibitors, has radically decreased mortality and morbidity rates among people living with HIV/AIDS. The emphasis of the original development of the antiretroviral drugs was on clinical effectiveness (reducing mortality). Presently, emphasis has shifted from the initial short- term considerations to the long-term undesirable or harmful effects induced by this treatment regimen. Whether antiretroviral compounds are oncogenic is widely speculated, which led to this investigation into the effects of protease inhibitors on the expression of key apoptotic regulatory genes, *BAX* and *BCL-2,* in two human breast cell lines, MCF-7 and MCF-10A by real-time qPCR gene expression and immunofluorescence. The anti-apoptotic effects of the protease inhibitors – LPV/r were also investigated by cell death detection ELISA and acridine orange staining. This study also evaluated the cytotoxicity of the antiretroviral drugs in normal and cancer cell lines of the breast (at clinically relevant concentrations of the drugs and at different time points, 24–96 h), employing the neutral red uptake assay. The drugs and combinations tested did not alter *BAX* and *BCL-2* gene expression and protein expression and localisation in both cell lines. In addition, the protease inhibitors–LPV/r did not inhibit camptothecin-induced apoptosis in both cell lines. We have shown that the protease inhibitors demonstrated varying degrees of cytotoxicity in the breast cells. The resulting DNA damage associated with cytotoxicity is strongly implicated in the processes of tumour initiation.

Introduction

The HIV/AIDS pandemic has had a catastrophic effect on the world population. In 2011, approximately 34 million people were recorded as living with HIV globally. Sub-Saharan Africa, which is home to just 10% of world's population contains a huge 69% of people living with HIV or AIDS.[1] South Africa has the highest number of people living with HIV in sub-Saharan Africa and in the world.[1] It is for this reason that in November 2003, South Africa started the world's largest public sector rollout of highly active antiretroviral treatment (HAART) to date.[2]

HAART has radically decreased mortality and morbidity among people living with HIV and AIDS[3,4,5] The emphasis of the original development of the antiretroviral (ARV) drugs was on clinical effectiveness (reducing mortality) with all other considerations secondary.[6] Presently, emphasis has shifted from the initial short-term considerations to the long-term undesirable or harmful effects induced by this treatment regimen.[6] Whether antiretroviral compounds are oncogenic remains to be fully elucidated.[7] Torres et al.[8] suggest that the clinical use of nucleoside/nucleotide reverse transcriptase inhibitor (NRTI) drug pairs may lead to additive or synergistic effects compounding long-term risk for cancer gene mutations and potential carcinogenesis.

Breast cancer is the most common cancer seen in women worldwide.[9] HIV-infected patients on antiretroviral treatment are reported to survive longer due to the treatment and therefore are at danger of developing breast cancer because its risk increases with age.[9,10,11] Other studies suggest that the use of ARV drugs might increase breast cancer risk by fat redistribution from peripheral and gluteal tissues to the breast as a result of increased production of oestrogen.[9] The total influence of HAART on the risk for non-AIDS defining malignancies (NADM) is far from concluded. In a meta-analysis,[12] the standardised incidence ratio of some non-AIDS defining malignancies, including breast cancer, was substantially increased prior to and in the HAART era. In a Swiss HIV cohort study, in which a mega prospective cohort of people were tracked for an average of about 5 years after HAART, no definite influence of HAART on standardised incidence ratios of NADMs was revealed.[13] However, another HIV/AIDS cohort conducted in England[7], showed that a substantial total rise in risk for NADMs objectively accompanied HAART treatment. Non-AIDS defining cancers now generally account for more deaths in HIV-infected individuals than AIDS defining cancers. Although traditional risk factors may account for some of this discrepancy, they certainly do not explain the explosion of these cancers in HIV-affected people on HAART.[14] Engels et al.[15] found that non-AIDS defining cancers comprise 58% of all cancer deaths post-HAART (1996–2002) in comparison to 31.4% in the pre- HAART era (1991–1995). These data strongly suggests that antiretroviral drugs may influence cancer development and progression.

In many cancers, there is a consistent pattern of apoptosis inhibition and deregulation of cell and tissue homeostasis. Numerous studies implicate apoptosis-related genes and their products in the development of cancer.[16-18] The induction of apoptosis occurs via two major pathways: the extrinsic (death receptor) pathway and the intrinsic (mitochondria) pathway.[16] The extrinsic pathway is activated by the attachment of the Fas plasma-membrane death receptor (and related receptors like tumour necrosis receptor 1 and its family) to its extracellular ligand, Fas-L. The intrinsic pathway also leads to apoptosis but under the control of mitochondrial pro-enzymes. The majority of cellular death in vertebrates follows the mitochondrial pathway of apoptosis.[17] This pathway to cell death is controlled by the B-cell CLL/lymphoma 2 *(BCL-2)* family of proteins. They operate to regulate the integrity of the mitochondrial outer membrane. When apoptosis arises as a result of cooperation among these proteins, the two pro-death BCL-2 effector proteins; BCL-2-associated X protein (BAX) and BCL-2 antagonistic killer (BAK), disrupt this membrane in a process known as mitochondrial outer membrane permeabilisation.[17] If mitochondrial outer membrane permeabilisation takes place, proteins located in the mitochondrial

inter-membrane compartment enter the cytosol and activate caspases and cysteine proteases that coordinate the disassembling of the cell.[17] The process of mitochondrial outer membrane permeabilisation is opposed by the pro-survival *BCL-2* proteins, such as BCL-2, BCL-W and BCL-xL, which impedes the capacity of BAX and Bak to permeabilise. The relevance of this impact in cancer is highlighted by the remark that oncogenes, like *Myc*, which advances proliferation, also encourage cell death that is inhibited by the anti-apoptotic *BCL-2* proteins.[17] The widely studied genes associated with apoptosis include *BCL-2*; the anti-apoptotic gene and *BAX*; a pro-apoptotic gene.[18] Hypothetically *BCL-2* over-expression renders a survival advantage for cancer cells; in contrast, *BAX* stimulation restores sensitivity to apoptosis induced by drugs or radiation.[18] In this study, we hypothesised that HIV protease inhibitors might inhibit the pro-apoptotic *BCL-2* family member *BAX*, or activate the anti-apoptotic *BCL-2* family member thereby promoting cell survival and lead to cancer development and/or progression.

In the majority of published studies, the in-vitro effects of antiretroviral drugs on immune cells were investigated. Few studies on non-immune cells were found. In the current study, we therefore investigated the effects of some of the drugs in the South African HAART treatment guidelines, individually and in combination, on *BAX* and *BCL-2* gene expression in two breast cell lines at clinically relevant concentrations. BAX and BCL-2 proteins were localised with immunofluorescence. We also determined whether the protease inhibitors – LPV/r, reported to have anti-apoptotic effects,[19-20] can inhibit drug-induced apoptosis in these non-immune cell lines. We also evaluated the cytotoxicity of the antiretroviral drugs in normal and cancer cell lines of the breast at clinically relevant concentrations of the drugs and at different time points (24 h, 48 h, 72 h and 96 h) by employing the neutral red uptake assay.

Materials and methods

Cells

Human breast carcinoma MCF-7 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in DMEM medium (Gibco BRL, Gaithersburg, MD, USA) containing 10% foetal bovine serum (Gibco).

Immortalised human breast epithelial cells, MCF-10A (American Type Culture Collection) were cultured in mammary epithelium growth medium (MEGM; Lonza, Walkersville, MD, USA) containing human recombinant epidermal growth factor, hydrocortisone, insulin and bovine pituitary extract (Lonza).

Cells were cultivated as a stationary monolayer in plastic tissue-culture dishes (Nunclon, Denmark) and were incubated at 37 °C in a 5% CO_2 humidified environment. The cells were incubated with the drugs individually and in combination at the indicated physiologically relevant concentrations for 24–96 h. The normal breast MCF-10A cells were cultured in serum-free media. MCF-7 cells were synchronised by serum starvation for 24 h[21,22] prior to all experiments. After being cultured in complete medium for overnight attachment, MCF-7 cells were incubated with serum-free media for 24 h. The cells were then treated with the antiretroviral drugs (as described in Table 1) in medium supplemented with serum for the indicated periods.

This study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (Clearance Certificate reference: M10905).

Table 1: Treatment groups and drug concentrations administered for each cell line

GROUP	TREATMENT
1	Growth medium
2	0.01% Methanol
3	9.8 μg/mL LPV
4	0.6 μg/mL RTV
5	LPV/r (Kaletra®)

LPV, lopinavir; RTV, ritonavir

Drugs and treatment

The drugs were administered at clinically relevant concentrations, which reflect their steady-state plasma peak concentration.[23,24] Lopinavir (LPV) and ritonavir (RTV) were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada) and were dissolved in methanol. The cells were incubated with the drugs individually and in combination at the indicated concentrations (Table 1) for 24 h, 48 h, 72 h and 96 h. Normal and vehicle control groups were exposed to growth medium and vehicle, respectively (Groups 1 and 2).

The cells were exposed to 9.8 μg/mL LPV (Group 3) and LPV/r (Kaletra® Group 5). Even though the absolute bioavailability of ritonavir had not been determined in humans at the time of this study[25], across studies, daily administration of Kaletra® 400/100 mg yields mean steady-state lopinavir plasma concentrations 15–20 times higher than those of ritonavir in individuals living with HIV-1.[26] Therefore a 9.8 μg/mL LPV:0.6 μg/mL RTV ratio was used. In Group 4, cells received 0.6 μg/mL RTV alone.

Kits, antibodies and reagents

The neutral red TOX-4 kit, methanol, acridine orange (AO), ethylenediaminetetraacetic acid (EDTA) and camptothecin (CPT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit polyclonal anti-BAX antibody and mouse monoclonal anti-BCL-2 were purchased from DAKO (DK-2600, Glostrup, Denmark). Secondary antibodies, goat anti-rabbit rhodamine-conjugated and goat anti-mouse FITC-conjugated were purchased from Abcam (Cambridge, UK). The GeneJET RNA Purification Kit, DNase I, RNase-free kit and O'GeneRuler Low Range DNA Ladder were purchased from Thermo Scientific (Pittsburgh, PA, USA). High Capacity cDNA Reverse Transcription Kit and Power SYBR® Green PCR Master Mix were purchased from Life Technologies (California, CA, USA). Oligos for quantitative polymerase chain reaction (qPCR) was purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). Agarose D-1 Low EEO-GQT was purchased from Conda Laboratories (Madrid, Spain).

Neutral red uptake assay

The neutral red uptake cytotoxicity assay is a precise, easy and reproducible assay that allows for distinguishing between viable and non-viable cells.[27] Neutral red is a weak cationic supravital dye that is actively transported across the undamaged cell membrane of viable cells and accumulates in the lysosomes. Alterations to the lysosomal membrane integrity arising from the toxicity of substances reduces neutral red uptake thereby allowing the distinction between viable and damaged cells.[27] After the viable cells have incorporated the dye, the dye is subsequently liberated from the cells and the degree of cytotoxicity, quantified by a spectrophotometer, is a measure of how many cells excluded the dye.[27] This assay was used to evaluate the cytotoxic effects of the protease inhibitors used in this study. MCF-7 and MCF-10A cells were cultured in the presence of the drugs, or diluent at the indicated concentrations for periods of time ranging from 24 h to 96 h. For each cell line, a 96-well Falcon tissue culture plate was seeded with cells in replicates for each group including negative and vehicle controls (Table 1). After 24 h, 48 h, 72 h and 96 h of treatment with antiretroviral drugs, the medium was removed and the manufacturer's protocol[27] was followed for the assay. Cells were rinsed in phosphate buffered saline (PBS). Neutral red medium was added to each well and plates were incubated for 2 h at 37 °C for the neutral red dye to be taken up by viable cells. Thereafter, the neutral red solution was removed and cells were rinsed with PBS. The solubilising solution (1% acetic acid in 50% ethanol) was added to each well for 10 min in order to extract the dye. The absorbance of the extracted dye was measured in a microplate reader (Anthos 2010 Model 17-550, Salzburg, Austria) at a wavelength of 540 nm. Background absorbance was read at 690 nm and subtracted from the 540 nm measurement. All experiments were repeated three times on three different days.

Total RNA extraction, cDNA synthesis, and real-time qPCR analysis

Cells were exposed to the drugs for up to 96 h and total RNA extraction was performed using the GeneJET RNA Purification kit according to the manufacturer's instructions. RNA concentration and purity were

determined by using the Nanodrop-1000 spectrophotometer. RNA integrity was checked by gel electrophoresis. According to the manufacturer's instructions, genomic DNA was removed from total RNA using the DNase I, RNase-free kit. The DNAase I treated RNA was again cleaned with the GeneJET RNA Purification kit, re-quantified and stored at -80 °C until used. According to the manufacturer's instructions, cDNA was synthesised using the MultiScribe™ Reverse Transcriptase from 700 ng RNA. The reverse transcriptase reaction was carried out in a GeneAmp® PCR System 9600 Thermal Cycler for 10 min at 25 °C, 120 min at 37 °C and then the enzyme was deactivated for 5 min at 85 °C.

The cDNA aliquots were then utilised in qPCR reactions for *BAX and BCL-2*, with TBP, RPLP0 and TFRC used as the endogenous reference genes. PCR reactions were amplified for 40 cycles prior to which the AmpliTaq Gold® DNA polymerase was activated for 10 min at 95 °C. Each cycle consisted of a denaturing step for 15 s at 95 °C, and annealing/extension step for 1 min at 60 °C. PCR amplification was performed in a final volume of 20 μL using the Power SYBR® Green PCR Master Mix with the ABI 7500 real-time PCR machine. Primer sequences and PCR product sizes are indicated in Table 2. To confirm the absence of nonspecific amplification, PCR products were separated on 3% agarose gels, stained with ethidium bromide and images acquired with the BioRad Gel Doc® XR (Model 170-8170 Segrate, Milan, Italy). Melt curves were generated for each PCR product using the Applied Biosystems ABI 7500 software. The relative mRNA expression levels of target genes in each sample were calculated using the qbasePLUS software (Biogazelle, Zulte, Belgium). The expression stability of the reference genes was evaluated using qbasePLUS version 2.3. This software uses a pair-wise comparison model to calculate the stability of each reference gene, and selects the two or more most stable genes from a panel of reference genes for normalisation.[28] Genes are ranked based on a gene stability parameter M, where a low M value indicates high expression stability. To further indicate how stable a gene is expressed, the qbasePLUS software also calculates a coefficient of variation. PCR baseline quantification cycle (Cq) values were exported from the ABI 7500 software as an Excel file (Microsoft, Redmond, WA, USA) and imported into the qbasePLUS software. The data was analysed with the default settings and the arithmetic mean of replicates was used. Data from standard curve experiments from the ABI 7500 software, imported into the qbasePLUS software, was used to generate amplification efficiencies and standard errors that are used downstream by the qbasePLUS software to determine normalised gene expression levels. The relative quantity of each target/sample combination was scaled to the average Cq of corresponding target (scale set to untreated control in the qbasePLUS software). The relative expression of specific genes in the experiment were normalised as a ratio to the amount of the two most stably expressed reference genes according to the $-^{\Delta\Delta C}$T method of Livak and Schmittgen[29].

Table 2: Oligonucleotide sequences used for qPCR

Gene	Sequences (5'–3' direction)	Product size (bp)
BAX	F: CCT TTT CTA CTT TGC CAG CAA AC R: GAG GCC GTC CCA ACC AC	148
BCL-2	F: ATG TGT GTG GAG AGC GTC AAC C R: GCA TCC CAG CCT CCG TTA TC	122
TBP	F: TGA TGC CTT ATG GCA CTG GAC TGA R: CTG CTG CCT TTG TTG CTC TTC CAA	86
RPLP0	F: TGC AGC TGA TCA AGA CTG GAG ACA R: TCC AGG AAG CGA GAA TGC AGA GTT	178
TFRC	F: GGC ACC ATC AAG CTG CTG AAT GAA R: GTT GAT CAC GCC AGA CTT TGC TGA	133

Immunofluorescence of BAX and BCL-2

The ABCAM® double labelling procedure was followed for immunofluorescence analysis. $1x10^4$ cells were plated on cover slips 1 day before the experiment. Cells were treated with the drugs at the indicated combinations and concentrations for up to 96 h. Following treatment, cells were washed three times with 0.5% BSA in PBS, followed by fixation in 10% phosphate buffered formalin for 10 min. The fixed cells were rinsed in PBS and permeabilised with 0.05% Triton-X 100, washed and blocked with 10% normal goat serum in PBS for 60 min to eliminate non-specific binding of the secondary antibody. Cells were then incubated with polyclonal rabbit anti-human BAX (1:1000 DAKO) and monoclonal mouse anti-human BCL2 (1:100 DAKO) overnight at 4 °C in a moist chamber. Following overnight incubation, the cells were washed and incubated for 2 h with a FITC-conjugated goat anti-mouse antibody (1:500 ABCAM) and rhodamine-conjugated goat anti-rabbit antibody (1:1000 ABCAM) in the dark. Slides were rinsed, nuclei counterstained in 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) 300 nM for 5 min, rinsed and mounted with fluoromount (Sigma). Negative control groups were set up to ensure that the secondary antibodies were specific for their primary antibodies. The primary antibodies were substituted with 0.1% BSA/PBS and the normal protocol was carried out. In a second negative control, the cells were treated in the same way as the experimental slides, but the secondary antibodies were substituted with PBS. The positive controls used in this study were HeLa cells, which had been previously shown to express BAX and HepG2 cells, shown to express BCL-2.

Cells were visualised using a Zeiss Laser Scanning Confocal Microscope 780 under a Zeiss 100X oil immersion objective. Slides were kept dark once fluorescent antibodies had been added, to prevent bleaching. As fading of the fluorochromes will take place, images were analysed within the same time period. The image acquisition settings remained constant for all exposures. Images were taken and, using the ZEN 2010 (Carl Zeiss, Jena, Germany) image analysis software, the intensity of the fluorescence of each micrograph was analysed. This analysis was done by using the software to initially define the regions of interest – the nucleus and cytoplasm. The mean intensity of each region of interest from the treatment groups was then analysed with the statistics software JMP® (Version 10.0 SAS Institute Inc., Cary, NC, USA). Data are reported as mean$\pm$SEM. After verifying the normal distribution and the homogeneity of the variance using an F test ($p<0.05$), a one-way analysis of variance (where a significance level of $p<0.05$ was set) was used to compare the results.

Acridine orange staining

Fluorescence light microscopy with differential uptake of fluorescent DNA binding dyes such as AO provides a simple, rapid, and accurate method for measuring apoptosis and cell membrane integrity.[30] Acridine orange permeates all cells, making the nuclei appear green. Live cells therefore have a normal green nucleus; early apoptotic cells have a bright green nucleus with condensed or fragmented chromatin; late apoptotic cells display condensed and fragmented chromatin and membrane blebbing.[30,31] This procedure was carried out to determine whether the protease inhibitors inhibit drug-induced apoptosis in the breast cell lines. MCF-7 and MCF-10A cells were routinely grown on 22-mm square coverslips placed into 35-mm culture dishes (Costar, Cambridge MA, USA). Cells were treated with methanol and the protease inhibitor based combinations, LPV/r, for 96 h before being stimulated[32] to undergo apoptosis with 1 μM CPT (MCF-7) and 30 μM CPT (MCF-10A) for 6 h. After induction of apoptosis, cells were stained with the AO dye mix for 5 min. The dye mix was 100 μg/mL AO in PBS.[33] After AO staining, culture dishes were inverted and fixed with formaldehyde vapour for 1 min to prevent the photo-damaging effects of continuous excitation on living cells because of the photosensitising effects of most fluorescent dyes.[34] A total of 600 cells per cell line per group was counted from three independent experiments to determine the percentage of apoptotic cells.

Cell death detection

The Cell Death Detection ELISA^{plus} kit (Roche Molecular Biochemicals, Mannheim, Germany) is used to measure changes in apoptosis.[35,36] In this procedure, internucleosomal DNA fragmentation is quantitatively

assayed by antibody mediated capture and detection of cytoplasmic mononucleosome and oligonucleosome associated histone-DNA complexes. Cells were cultured (as described) in 96-well plates in duplicate. Cells were treated with 0.01% methanol (vehicle) and the protease inhibitor based combination LPV/r (in the appropriate medium) for 96 h before being stimulated[32] to undergo apoptosis with 1 μM CPT (MCF-7)[37] and 30 μM CPT (MCF-10A)[38] for 6 h.

According to the protocol described by Liu et al.[35] and Tu et al.[36], cells were centrifuged (200 *g*), resuspended in 200 μL of the lysis buffer supplied in the kit and incubated for 30 min at room temperature. Nuclei were then pelleted at 200 *g* for 10 min and 20 μL of the supernatant (cytoplasmic fraction) from the treated group, untreated group, positive control and background control were transferred into the corresponding streptavidin-coated wells for the enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's standard protocol.[36] Then, 80 μL of the immunoreagent was added to each well containing the 20 μL supernatant or controls. The wells were covered with adhesive foil and incubated on a microplate shaker under gentle shaking (300 rpm) for 2 h at room temperature. The solution was then removed by gentle pipetting; wells were rinsed three times with 250 μL incubation buffer and removed. Next, 100 μL of the ABTS solution was pipetted into the wells and incubated on a plate shaker at 250 rpm until the colour development was sufficient for photometric analysis (approximately 10–20 min). Then 100 μL of the ABTS stop solution was added to each well. Absorbance was measured at 405 nm and 490 nm (reference wavelength) with a microplate reader (Anthos 2010 Model 17-550). Signals in wells containing the substrate only were subtracted as background. Data were analysed from three independent experiments. Data analysis was performed using JMP® (Version 10.0). Data are reported as mean$\pm$SEM. After verifying the normal distribution and the homogeneity of the variance using an F test ($p<0.05$), a one-way analysis of variance (significance level $p<0.05$) was used to compare the results. If there was a significant difference between the means ($p<0.05$), then a Tukey–Kramer post-hoc analysis was performed to determine which treatment groups were significantly different from each other.

Statistical analysis

Statistical analysis was performed using JMP® (Version 10.0). Data are reported as mean$\pm$SEM. After verifying the normal distribution and the homogeneity of the variance using an F test ($p<0.05$), a one-way analysis of variance (where a significance level of $p<0.05$ was set) was used to compare the results. All means were then compared using the Tukey–Kramer honestly significant different test.

Results

Effects of ARV drugs on the viability of MCF-7 and MCF 10A cells

The effects of the antiretroviral (ARV) drugs on the viability of MCF-7 and MCF-10A breast cells were investigated by the neutral red uptake assay. LPV significantly ($p<0.05$) reduced the viability of the normal breast MCF-10A cells at 24 h when compared to the vehicle control (Figure 1a). Also at 24 h, LPV significantly ($p<0.05$) reduced the percentage viability of the normal breast cells when compared to the cancer breast MCF- 7 cells (Figure 1a). With continuous exposure, the normal breast cells recovered from the initial cytotoxic effects of LPV, such that from 48 h to 96 h, there were no significant differences between the treated cells and their vehicle controls (Figure. 1a). LPV did not significantly ($p<0.05$) alter the percentage viability of the breast cancer MCF-7 cells from 24 h to 96 h when compared to their vehicle controls (Figure 1a The reduction in % viability of MCF-7 cells at 48 h was not statistically significant. RTV significantly ($p<0.05$) reduced the viability of the normal breast MCF-10A cells when compared to the vehicle control and the breast cancer MCF-7 cells at 24 h (Figure 1b). At 48 h, there was no significant ($p<0.05$) alteration in percentage viability between RTV treated and untreated in either cell line. At 72 h, RTV treated MCF-10A cells demonstrated significantly ($p<0.05$) increased viability, while at 96 h, there was no statistical significance in the difference between RTV treated and untreated MCF-10A cells (Figure 1b). RTV did not alter the percentage viability of the breast cancer MCF-7 cells from 24 h to 96 h (Figure 1b). The apparent increase in % viability of RTV treated MCF-7 cells at 24 h did not achieve statistical significance. The combination LPV/r interestingly did not significantly ($p<0.05$) alter the percentage viability of the normal (MCF-10A) and cancer (MCF-7) breast cells from 24 h to 96 h when compared to their respective vehicle controls and to each other at the respective time points (Figure 1c).

Effects of ARV drugs on BAX/BCL2 gene expression in MCF-7/MCF10A cells

The effects of the protease inhibitors on *BAX* and *BCL-2* mRNA expression in both cell lines were investigated after exposure for 24 h to 96 h (data shown for 96 h). In both cell lines treated with LPV, the differences in fold changes were not significantly ($p<0.05$) different from the untreated controls for *BAX* and *BCL-2* (Figure 2a). In both cell lines treated with RTV and LPV/r, the differences in fold changes were not significantly ($p<0.05$) different from the untreated controls for *BAX* and *BCL-2* (Figures 2b and 2c). Results were analysed with the qBASE^{PLUS} software and normalised to the expression levels of *TBP*, *TFRC* and *RPLP0*.

Effects of ARVs on BAX/BCL2 immunofluorescence in MCF-7/10A cells

Figure 3a–c is a graphical representation of immunofluorescence intensities illustrating the effects of LPV, RTV and LPV/r on expression of BAX and BCL-2 in MCF-7 and MCF-10A cell cultures after 96 h of exposure. In the untreated group of MCF-7 cells (micrographs not shown), BAX and BCL-2 proteins were expressed and co-localised in the nuclei and cytoplasm. Mean fluorescence intensities of BAX and BCL-2 in the nuclei and cytoplasm were statistically analysed (ANOVA) between the untreated/vehicle control and the treated groups (graphical representation in Figure 3a–c).There are no differences in protein staining, localisation and intensity of vehicle (0.01% methanol) treated group when compared to growth medium alone. BAX and BCL-2, localisations remain unaltered across treatment groups. The untreated group of MCF-10A cells showed the expression and localisation of BAX and BCL-2 (micrographs not shown). Mean fluorescence intensities of BAX and BCL-2 in the nuclei and cytoplasm were statistically analysed (ANOVA) between the untreated (vehicle) control and the treated groups. (graphical representation in Figure 3a–c). There were no differences in protein staining, localisation and intensity of vehicle (0.01% methanol) treated group as compared to growth medium alone. BAX and BCL-2 localisations remain unchanged across treatment groups.

Protease inhibitors and apoptosis inhibition

Numerous studies[19,20,32,39] have shown that protease inhibitors have potent anti-apoptotic effects in a variety of cell systems, the majority of which are immune cells. We assessed the anti-apoptotic effects of LPV/r to test whether these generalised effects are seen in the currently investigated human breast cell lines and to test whether they alter apoptosis via a pathway other than the *BAX/BCL-2* pathway, since they had no significant effects on *BAX* and *BCL-2* mRNA expression (Figure 2). From an evaluation of the many methods currently used to analyse apoptosis, chromatin condensation and nuclear fragmentation remain the hallmarks of apoptotic cells.[30] Because it had been suggested that as a rule, classification of cell death in a given model should always include morphological examination coupled with at least one other assay,[40] we chose the acridine orange staining for morphological evaluation and an ELISA based method for the quantitative analysis of DNA fragmentation. From previous optimisation experiments, it was determined that 1 μM and 30 μM CPT significantly induced apoptosis in the MCF-7 and MCF10-A cells respectively for 6 h. Figures 4 and 5 represent data from AO staining showing that a 96 h exposure to LPV/r did not significantly ($p<0.05$) inhibit apoptosis in both breast cell lines. Figure 6 represents data quantifying DNA fragmentation between groups. These ELISA results also confirm that LPV/r did not significantly inhibit drug-induced apoptosis in the breast MCF-7 and MCF-10A cells.

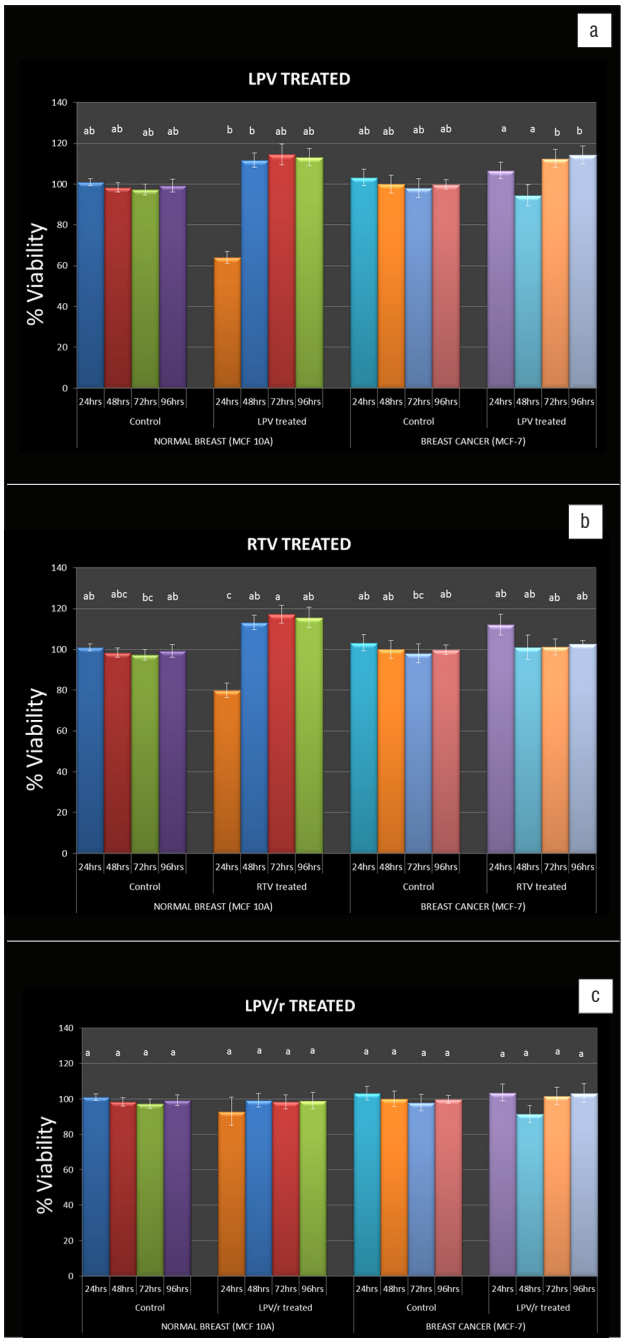

Figure 1: Effects of (a) LPV, (b) RTV and (c) LPV/r on the percentage viability of normal breast (MCF-10A) and breast cancer (MCF- 7) cells. Cells were incubated with 9.8 μg/mL LPV, 0.6 μg/mL RTV and 9.8 μg/mL LPV/0.6 μg/mL for 24–96 h. Graphs were plotted with mean+SEM percentage viability from a mean of three.

Discussion and conclusion

This study investigated the possibility that the use of protease inhibitors could be a risk factor for the initiation or development of breast cancer in patients receiving HAART. An analysis of the incidence of non-AIDS defining cancers in HIV-infected patients suggests that the incidence of NADM in these patients has significantly increased since the introduction and implementation of the combined antiretroviral therapy,[12] creating the need to effectively establish or rule out the possibility that candidate antiretroviral drugs may promote breast cancer. The breast was chosen as a model of non-AIDS-defining malignancies.

The findings reported here show that the antiretroviral drugs: lopinavir, ritonavir and their combination (all at clinically relevant concentrations, which reflect their steady-state peak plasma concentrations in patients receiving these drugs)[41,42] demonstrated some form of cytotoxicity in the breast cells, which may alter the activity of the nuclear and mitochondrial genome of cells and may cause genotoxic effects largely related to the tumour initiation processes.[43-45] LPV showed an initial cytotoxic effect on the MCF-10A cells after 24 h of exposure. Even though this cytotoxic effect disappeared after 48 h, 72 h and 96 h of exposure, this initial cytotoxicity can produce genomic rearrangements that may act as the primary step toward carcinogenesis. RTV was significantly cytotoxic to the normal breast MCF-10A cells at 24 h, when compared with the control group and the breast cancer MCF-7 cells. The cytotoxic effects of RTV on the normal breast disappeared with continuous administration but could lead to DNA damage and may trigger the processes of cancer initiation in normal breast cells. RTV demonstrated opposing effects on normal breast and breast cancer cells at 24 h. It generally appeared to promote survival in normal breast after an initial toxic effect. Interestingly, the combination of the two protease inhibitors (lopinavir and ritonavir), as combined in Kaletra®, did not significantly alter the percentage viability of both breast cell lines.

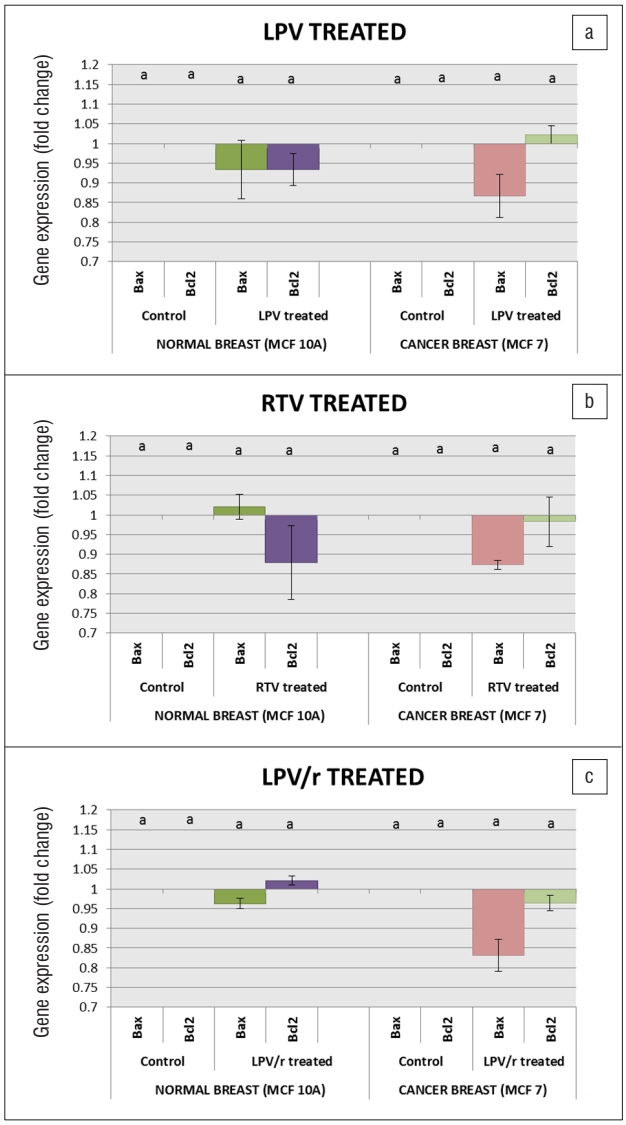

Figure 2: Effects of (a) LPV, (b) RTV and (c) LPV/r on *BAX* and *BCL-2* mRNA expression in normal breast (MCF-10A) and breast cancer (MCF-7) cells. Cells were incubated with 9.8 μg/mL LPV, 0.6 μg/mL RTV and 9.8 μg/mL LPV/0.6 μg/mL for 96 h. Data (mean±SEM) are represented as fold changes of gene expression relative to values from the vehicle-treated cells (defined as 1) and representative of three independent experiments for RNA extraction. Groups not connected by the same letter are significantly ($p<0.05$) different from each other.

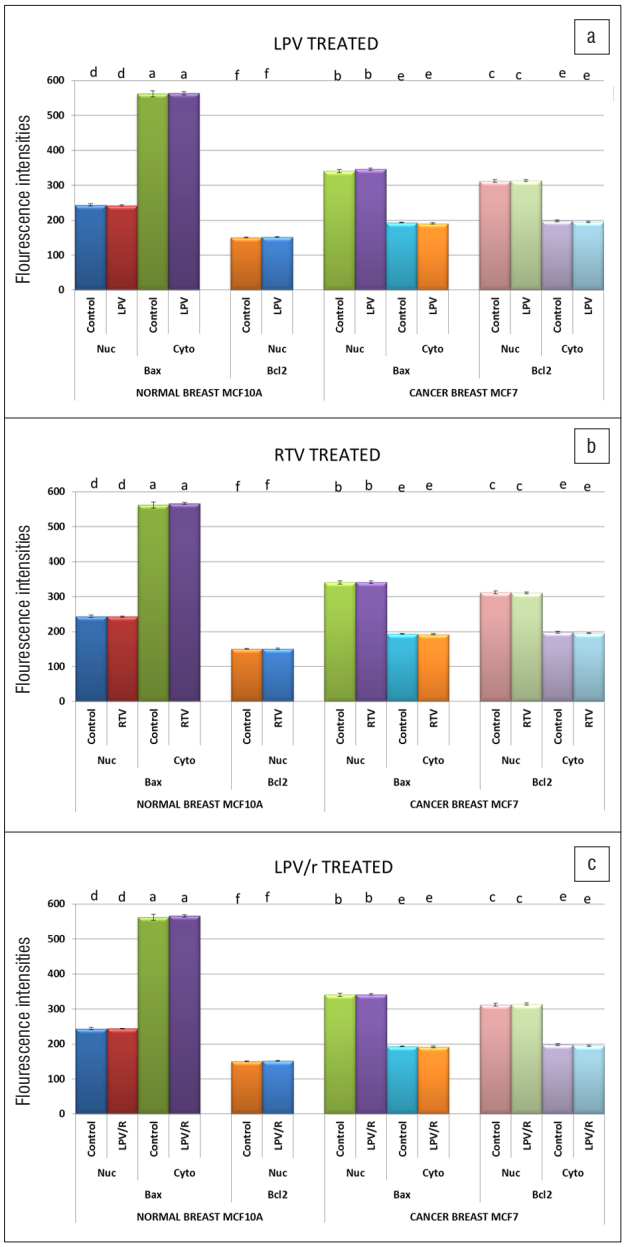

Figure 3: Effects of (a) LPV, (b) RTV and (c) LPV/r on BAX and BCL- 2 protein expression in normal breast (MCF-10A) and breast cancer (MCF-7) cells. Cells were incubated with 9.8 μg/mL LPV, 0.6 g/mL RTV and 9.8 μg/mL LPV/0.6 μg/mL for 96 h. Data (mean±SEM), are represented as fluorescence intensities of protein expression relative to values from the vehicle-treated cells and representative of 3 independent experiments for immunofluorescence staining. Groups not connected by the same letter are significantly ($p<0.05$) different from each other.

To promote cancer, the antiretroviral drugs, either individually or in combination at the clinically relevant concentrations tested were expected to up-regulate the anti-apoptotic *BCL-2* mRNA and or protein and down- regulate the pro-apoptotic *BAX* mRNA and or protein. Testing the effects of protease inhibitors (individually and in combination), our findings show that the antiretroviral drugs; lopinavir, ritonavir and Kaletra®, all at clinically relevant concentrations (which reflect their steady-state peak plasma concentrations), do not alter the mRNA expression of apoptosis related *BAX* and *BCL-2*. Neither do they alter the localisation of their proteins in the human breast cancer cell line MCF-7 and non-tumorigenic immortalised breast cell line MCF-10A. These results are consistent with the findings of other researchers. Reports by Gomez-Sucerquia et al.[46] showed that efavirenz at similar concentrations did not alter the mRNA expression of *BAX* and *BCL-2 like 1* genes following a 24 h treatment period in human Hep3B cells. Phenix et al.[32], Badley[19] and Rizza and Badley[20] reported that protease inhibitors, including lopinavir, did not alter the mRNA expression and protein synthesis of *BAX, BCL-2* and some other key apoptotic genes following a 3-day exposure in immune cells. Some other proteins and/or genes of this or another pathway, however, might be involved.

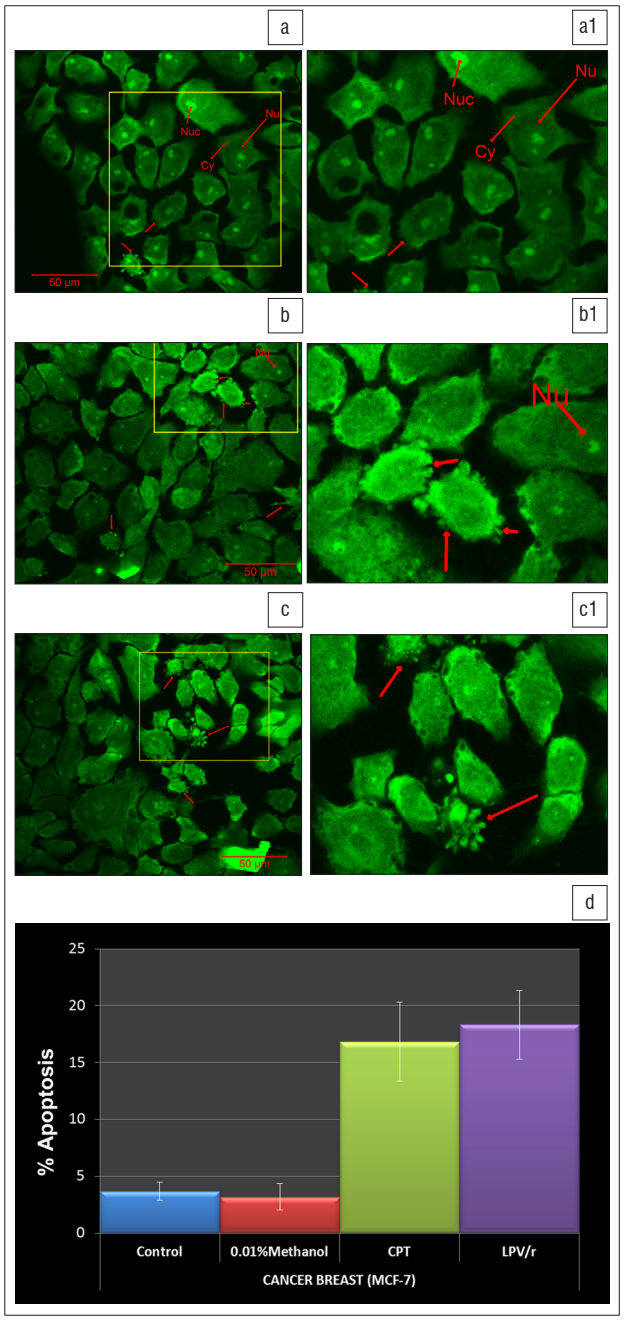

Figure 4: Effects of LPV/r on CPT mediated apoptosis in MCF-7 cells. (a) Untreated control MCF-7 cells. (b) CPT treated MCF-7 cells. MCF-7 cells were stimulated with 1 μM CPT for 6 h without prior exposure to LPV/r. (c) MCF-7 cells incubated with LPV/r prior to apoptosis induction. Apoptosis was assessed using acridine orange staining. Arrows point to apoptotic bodies and cells undergoing apoptosis with the characteristic feature of membrane blebbing. Areas within the box are magnified to the right (a1, b1, c1). (d) A total of 600 cells per group was counted from three independent experiments to determine the percentage of apoptotic cells. LPV/r had no significant inhibitory effects on CPT induced apoptosis in MCF-7 cells.

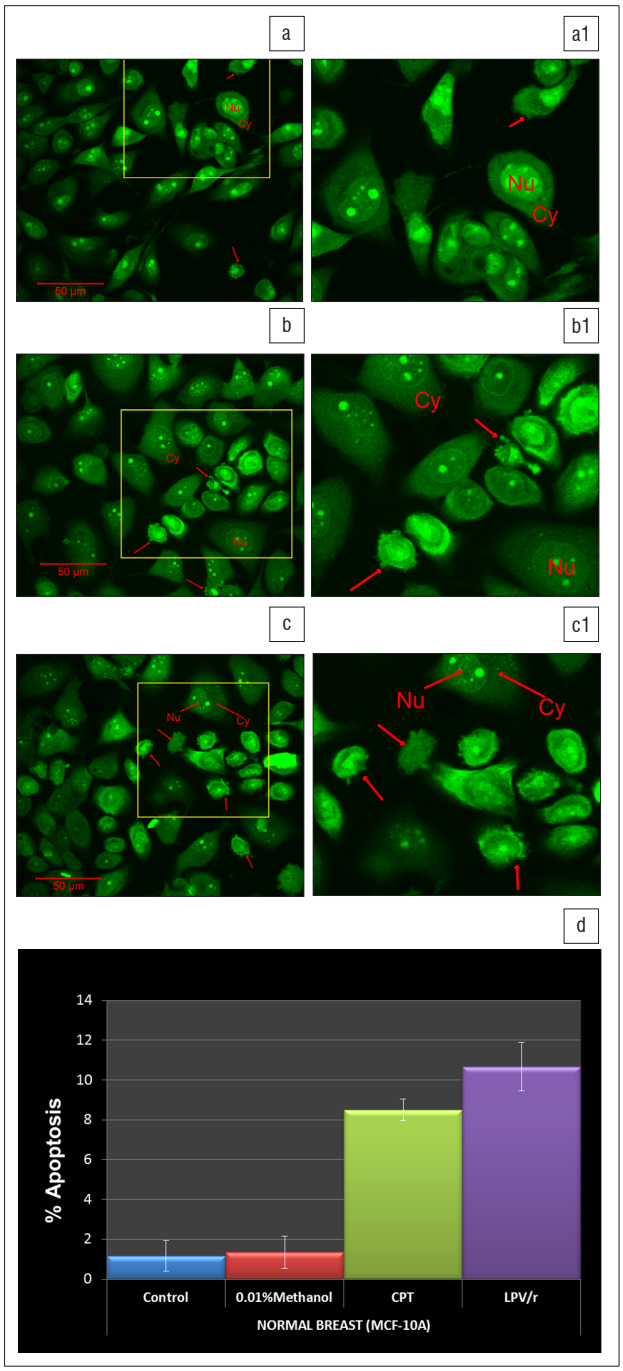

Figure 5: Effects of LPV/r on CPT mediated apoptosis in MCF-10A cells. (a) Untreated control MCF-10A cells. (b) CPT treated MCF-10A cells. MCF-10A cells were stimulated with 30 μM CPT for 6 h without prior exposure to LPV/r. (c) MCF-10A cells incubated with LPV/r prior to apoptosis induction. Apoptosis was assessed using acridine orange staining. Arrows point to apoptotic bodies and cells undergoing apoptosis with the characteristic feature of membrane blebbing. Areas within the box are magnified to the right (a1, b1, c1). (d) A total of 600 cells per group was counted from three independent experiments to determine the percentage of apoptotic cells. LPV/r had no significant inhibitory effects on CPT induced apoptosis in MCF-10A cells.

It has been shown previously that protease inhibitors have potent anti-apoptotic effects in different cellular systems, the majority of which are immune cells.[19,20,32,39] These studies reported that the protease inhibitors investigated inhibited drug induced apoptosis. This anti-apoptotic property, (exhibited by protease inhibitors at concentrations similar to those levels achieved in people receiving the drugs) is an important phase in cancer development. The anti-apoptotic effect of LPV/r was therefore assessed to test whether these generalised effects are seen in the currently investigated cell lines and to test whether they alter apoptosis via a pathway other than the *BAX*/*BCL-2* pathway, because they had no significant effects on *BAX* and *BCL-2* mRNA expression. At a dose of 1 μM CPT significantly increased the percentage of apoptotic MCF-7 cells from 3-4% (spontaneous apoptosis) to 15–18% following a 6-h exposure, while the percentage of apoptotic MCF-10A cells increased from 1–2% to 8–9% following a 6 h exposure to 30 μM CPT. Pre-incubation with LPV/r did not significantly ($p>0.05$) inhibit CPT-induced apoptosis in both breast cell lines. The cell death ELISA assessment of DNA fragmentation showed similar results. These findings conflict with the reports of Phenix et al.[32] which demonstrated that the HIV protease inhibitor nelfinavir inhibited Jurkat T-cell apoptosis induced by a variety of different stimuli, including CPT and with our previous report that pre-incubation with LPV/r for 96 h significantly inhibited the development of apoptosis in the cervical cancer HCS-2 cell line.[47] These disparities might be because different cell lines react differently to the same target molecules. The variety of responses depends on the set of receptor proteins the cell possesses, which determines the specific subset of signals it can respond to, and also depends on the intracellular machinery by which the cell integrates and interprets the signals it receives.[48] However, Phenix et al.[32] and Adefolaju et al.[47] also reported that inhibition of apoptotic death was not associated with alterations in mRNA expression of a variety of pro- and anti-apoptotic factors, and was not dependent on protein synthesis.

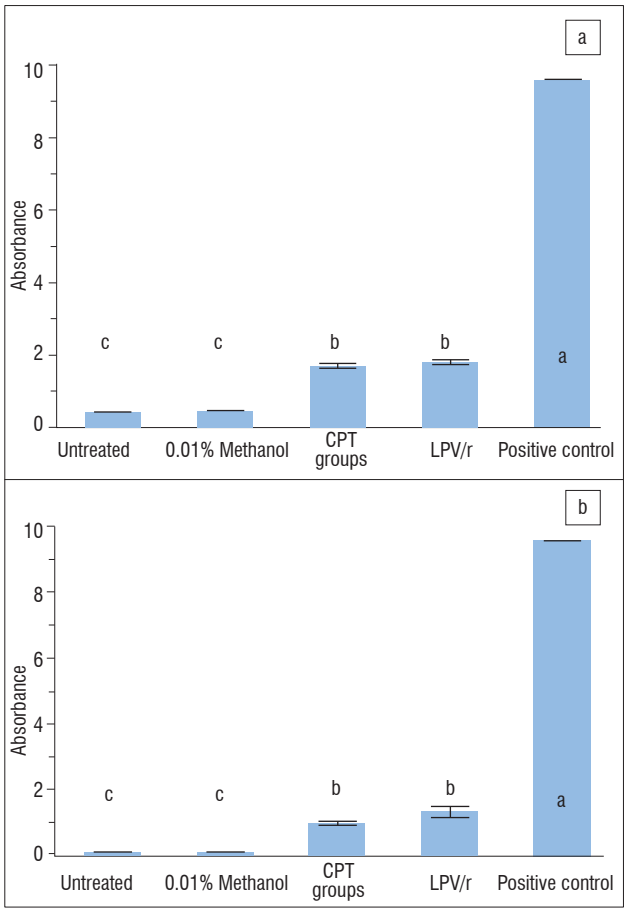

Figure 6: Effects of LPV/r on CPT mediated apoptosis in MCF-7 and MCF-10A cells evaluated by the Cell Death Detection ELISA. (a) MCF-7 and (b) MCF-10A cells were treated with methanol or LPV/r for 96 h and stimulated with CPT (1 μM and 30 μM, respectively) and apoptosis was assessed by quantifying internucleosomal DNA fragmentation. LPV/r treatment did not significantly ($p<0.05$) inhibit CPT induced apoptosis in both cell lines. Levels not connected by the same letter are significantly different from each other.

In the HAART era, it is a paradox that people living with HIV and AIDS are now at an increased risk for developing several specific non-AIDS-defining malignancies[4,49-53] including breast cancer.[12,52] Such that, despite the immune reconstitution induced by HAART, increased NADMs deaths have been reported amongst HIV patients.[54] It has also been reported that patients with NADMs often have more aggressive cancers and display more advanced stages of the disease[55,56], with breast cancer metastasising early and usually more poorly differentiated.[57] The effects of HAART on the risk for NADM have not been clearly established. Research evidence has been conflicting as to whether or which antiretroviral drugs or classes decrease, increase, or have no effect on the risk of developing NADM,[56,58] with no definite pattern emerging from such studies.

The speculation that some antiretroviral drugs or combinations may have an adverse effect on the risk of carcinogenesis among HIV patients under HAART led to this study. Our results show that these protease inhibitors do not alter the mRNA expression of apoptosis related *BAX* and *BCL-2,* neither do they alter the localisation of their proteins in the human breast cancer cell line MCF-7 and non-tumorigenic immortalised breast cell line MCF- 10A. The findings reported here also show that HIV protease inhibitors – LPV/r did not exhibit anti-apoptotic properties in MCF-7 and MCF-10A cells. If the ARVs initiate or promote cancer, these pathways are not likely to be involved, so the effects of these agents need to be investigated on other oncogenic pathways. Also, considering that this is an in-vitro study, it should be noted that the possibility of obtaining different results cannot be excluded when studied in an in vivo model, in which responses to pharmacological agents are much more complicated.

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Authors' contributions

G.A.A., K.E.T. and M.J.H. were involved with the conceptualisation of study and experimental design, standardisation and optimisation of all protocols, data acquisition, analysis and interpretation and manuscript write-up and final approval of the revised version. M.J.H was also involved with supervision of the project.

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