An In Vitro Study of the Effects of Emtricitabine, Tenofovir Disoproxil Fumarate and Efavirenz on a Breast Cancer Cell Line, MCF-7

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ABSTRACT

Non-AIDS Defining Cancers including breast cancer are becoming prevalent in people infected with HIV/AIDS during the highly active antiretroviral therapy (HAART) era. The possible role of HAART in the expression or prognosis of these cancers is of interest because it has been previously shown to significantly reduce the incidence of AIDS-defining malignancies. This study was aimed at examining the effects of Emtricitabine (FTC), Tenofovir Disoproxil fumarate (TDF) and Efavirenz (EFV), agents which are commonly used as part of HAART on a breast cancer cell line, MCF-7 with regard to cell function. The MCF-7 cell line was cultured and treated with the individual drugs and their combination at concentrations corresponding to steady-state peak plasma levels (Cmax) and also ¼ X, ½ X, 2X and 10X Cmax. Control cells were incubated in Media without the drug compounds. A Neutral Red cytotoxicity assay was conducted. Immunocytochemistry was performed for localisation of Bax and bcl-2 in order to examine how the drugs affect the mitochondrial pathway of apoptosis, and plasma membrane morphology was studied under the scanning electron microscope. An increase in cell viability was observed following the 24 and 48 hour treatment with all the drugs except for EFV at 10X Cmax whereby cell viability was significantly reduced (P<0.001). Bax and bcl-2 were co-localised in the perinuclear region following treatment with FTC and TDF, whereas the cytoplasmic expression of Bax was up-regulated and that of bcl-2 was down-regulated following EFV treatment. In contrast no co-localisation was observed between Bax and bcl-2 following the drug combination however plasma membrane integrity was greatly altered in treated cells in comparison to controls. In conclusion only EFV had cytotoxic effects on the cells however Bax and bcl-2 co-localisation together with the plasma membrane displayed morphological features which suggest cell death via apoptosis and/or apoptotic necrosis. 

KEYWORDS: Toxicity, Bax, Bcl-2, Scanning electron microscopy, plasma membrane integrity.

INTRODUCTION

HAART consists of drugs belonging to the family of nucleotide/nucleoside reverse transcriptase inhibitors (NRTI), non-nucleotide/nucleoside reverse transcriptase inhibitors (NNRTI) and/or protease inhibitors (PI) [1]. The use of HAART has proved to be effective in reducing HIV progression into AIDS and has therefore reduced AIDS-related mortality [2]. The long term effects resulting from prolonged survival due to this therapy are still under investigation however altered cause of cancer risk is of particular interest as AIDS has been previously associated with AIDS defining malignancies which were a major cause of mortality amongst AIDS patients [3]. HAART was shown to have a significant role in reducing the risk of some AIDS associated cancers, but it is suggested that this treatment has the opposite effect on Non-AIDS Defining Cancers [4]. Recent studies have shown that Non-AIDS Defining Cancers are becoming prevalent in people infected with HIV/AIDS during the HAART era [5].

Breast cancer is amongst Non-AIDS defining cancers and it is the most commonly diagnosed cancer amongst women globally [6]. Since the initiation of HAART the number of women living with HIV has also increased, therefore studies aimed at investigating the association between breast cancer diagnosis and HIV infection during the HAART era are increasing [6]. Breast cancer risk increases with age and previously HIV infected women would have died of AIDS sooner, therefore they were in so doing not seen to be at high risk for diagnoses with breast cancer [6]. HAART however prolongs life expectancy and therefore increases breast cancer risk [6].

The number of people living with HIV and on prescribed antiretroviral therapy has increased since the onset of HAART and subsequently there has been a demand for simple and effective antiretroviral regimens consisting of fixed dose once-daily combinations of antiretroviral drugs [6]. The first example of a fixed-dose combination is Atripla™ and it contains all the necessary elements of an antiretroviral regimen [6]. Atripla is manufactured as a single pill containing agents which were previously approved by the US Food and Drug Administration (FDA) as antiretroviral medications including Emtricitabine (FTC), Tenofovir Disoproxil Fumarate (TDF) and Efavirenz (EFV) [6,8].

Emtricitabine belongs to the new class of NRTT’s known as oxathioline-cytosine analogues; it has a high potency against HIV by selectively blocking its replication [1,9]. Tenofovir disoproxil fumarate is a NRTI and it...
undergoes monophosphorylation during bio-activation into Tenofovir, it is an analogue of adenine 5’monophosphate and it has been clinically prescribed for HIV infected patients who are new to antiretroviral therapy and has been shown to have the least in-vitro toxicity in comparison to other NRTIs \cite{8,9,10}. Efavirenz was the third NNRTI to be approved by the FDA and has been available for the longest period \cite{8}. In comparison to other accepted NNRTI, efavirenz has a higher resistance and it requires multiple mutations in reverse transcription in order to produce elevated levels of drug resistance \cite{11}.

The current study investigated the effects of three antiretroviral drugs commonly used as part of HAART on a breast cancer cell line, MCF-7, that is not infected with HIV. The outcome of the study is expected to provide a basis for understanding how the ARV’s affect cell function in breast cancer cells and it might also be used to compare with other studies where the breast cancer cells are also infected with HIV.

The aim of this study was to examine the effects of FTC, TDF and EFV on a breast cancer cell line, MCF-7 with regard to cell function. In vitro studies were conducted in order to investigate whether the antiretroviral drugs induced toxicity and cell death in cancer cells. To investigate toxicity the neutral red toxicology assay was conducted and to investigate cell death key cellular markers of the BCl-2 family which regulate programmed cell death otherwise known as apoptosis bcl-2 and Bax were localised and additionally the apical plasma membrane integrity was examined using scanning electron microscopy in order to further investigate the effects of the ARV drugs used in a breast cancer cell line.

\section*{MATERIALS AND METHODS}

\subsection*{Materials}

Cell culture supplies were purchased from BD Biosciences (Johannesburg, South Africa). Dulbecco’s modified eagle’s medium (DMEM) containing phenol red was purchased from Lonza (Whitehead Scientific PTY LTD, Cape Town, South Africa). The FTC, TDF and EFV were purchased from Toronto Research Chemicals Inc (Canada, USA). The neutral red kit, Dapi, FluoroGuard and HMDS were purchased from Sigma-Aldrich PTY LTD (South Africa). The primary antibodies and normal goat serum were purchased from Dako (South Africa). The secondary antibodies were purchased from Rockland (Biocom, South Africa).

\subsection*{Cell culture}

MCF 7 cells were routinely grown in 25cm$^2$ BD Falcon™ tissue culture flasks as a monolayer. The cells were maintained in a humidified 5% CO$_2$ environment at a temperature of 37°C in Dulbecco’s modified eagle’s medium (DMEM) containing phenol red. The medium was supplemented with 10% fetal calf serum and 0.04% Penicillin. When the cells had reached approximately 70-80% confluency they were harvested with 5% trypsin in EDTA.

\subsection*{Dissolving compounds}

The mean steady-state peak plasma concentrations (C$_{max}$)for the drugs are as follows; FTC 7.3µM \cite{12} TDF 1.2µM \cite{13} and EFV 12.4µM \cite{14}. To obtain these concentrations initial drugs volumes of 3.6mg FTC, 0.6mg TDF and 8mg EFV were dissolved in 1ml DMSO (FTC and TDF) and 1ml Methanol (EFV only), diluents for each drug were indicated in the Toronto Research Chemicals Inc. Certificate of Analysis. The initial stock solution of each drug was diluted with DMEM to obtain C$_{max}$ concentrations. Additionally various drug concentrations corresponding to the Cmax were also obtained ; ½ C$_{max}$, ¼ C$_{max}$, 2X C$_{max}$ and 10X C$_{max}$ in order to test a range of concentrations for the Neutral Red toxicity assay.

The maximum concentration of diluent (either DMSO or Methanol) in the final stock solution was 0.5% (v/v) as recommended by the National Toxicology Programme (NTP) Interagency Centre for the Evaluation of Alternative Toxicological Methods \cite{15}. The drug combination ratio used was 1FTC: 1.5TDF: 3EFV (based on oral doses) \cite{16} and solutions corresponding to various combination ratios of the drugs were made up using the 1X C$_{max}$ solutions for each drug. Only the original C$_{max}$ concentration was used in Bax/bcl-2 localisation and Scanning electron microscopy and the treatment duration of 24 hours because it is seen to be more physiologically relevant.

\subsection*{Neutral red assay}

Cell seeding and treatment; A 96-well Falcon tissue culture plate was seeded with 10000cells/well and the plates were incubated until they achieved 60-70% confluency. The cells were then treated with medium containing specified concentrations of the drugs and combination ratios based on the Cmax concentrations for 24hrs and 48hrs. The negative controls consisted of untreated cells, in medium alone, and the diluent controls were cells treated with the highest concentration (0.5%) of drug solvents used (DMSO or Methanol).

Neutral red assay; the assay was conducted as previously described by Borenfreund and Puerner, 1985\cite{17}. Statistical analysis; statistical analysis for the data obtained was performed using JMP software (JMP® 10). The data was assessed for homogenous variances using an F-test and it was found that the variances were significantly different in all cases. The data was then transformed using log$_e$ before undergoing further statistical analysis \cite{18}. A one-way ANOVA was used in order to evaluate whether the drugs had significantly different effects on the cells at various concentrations. Multiple comparisons of the means were conducted using the
Tukey-Kramer Post Hoc test. The tests used a confidence level of 5% and p<0.05 was considered to be statistically significant.

**Immunocytochemistry for Bax and bcl-2 proteins**

**Cell seeding and treatment;** 10000 cell/ml MCF 7 cells were seeded and grown onto glass coverslips for 48 hrs or until they were confluent. The cells were treated with media containing the Cmax concentration of each drug and the drug combination; they were then incubated for 24 hours. The media was removed and cells were fixed in 4% formaldehyde in PBS for 15 mins at room temperature. Cells were then rinsed three times in 1%BSA/PBS and permeabilised with methanol for 10 mins at 4°C.

**Antibody labelling;** A range of dilutions were tested prior to antibody labelling and the optimal dilution for each antibody was chosen. Cells were blocked with 1XPBS/5% normal serum/0.3% Triton X-100 blocking buffer for 1 hr and then incubated overnight with polyclonal rabbit anti-human Bax at a dilution of 1:2500 and monoclonal mouse anti-human bcl-2 at a dilution of 1:100 primary antibodies at 4°C. Following the overnight incubation the primary antibodies were removed and cells washed three times with 1%BSA/PBS for 10 mins each. Cells were treated with secondary antibodies for 2 hrs; Rhodamine conjugated goat anti-rabbit, at a dilution of 1:300, for Bax localisation and FITC conjugated rabbit anti-mouse IgG, at a dilution of 1:400, for bcl-2 localisation. The cells were washed three times with 1%BSA/PBS. The nucleus was counterstained with Dapi at a dilution of 1:1500. Cells were rinsed three times with 1%BSA/PBS for 10 mins each and mounted onto glass slides with FluoroGuard.

**Imaging;** Image acquisition was done using a Zeiss Laser Scanning Confocal Microscope 780. The resultant colour between the FITC and Rhodamine conjugated secondary antibodies which is yellow, was used as evidence for bcl-2 and Bax co-localisation. The cellular compartment in which they were found was also noted.

**Scanning electron microscopy**

**Cell seeding and treatment;** 10000cell/ml were seeded and grown onto 22mm by 22mm glass coverslips until they were confluent. The cells were treated with media containing Cmax concentrations for each drug and 1X combination ratio and incubated for 24 hrs.

**Fixation and dehydration;** The cells were then fixed in 2.5% glutaraldehyde in 0.1M sodium phosphate at pH 7.4 for 30 mins, at room temperature. They were post-fixed in a drop of osmium tetroxide for 15 mins, at room temperature. The cells were rinsed in 0.1M phosphate buffer and dehydrated in 50%, 70%, 80% and 95% alcohol solutions for 5 mins each and finally dehydrated in 100% alcohol for 10 mins. The cells were incubated in Hexamethyldisilazane for 1 min and left to dry in a dessicator containing silica gel overnight.

**Coating and imaging;** The coverslips were mounted onto stubs and coated with carbon and gold-palladium using EMITECH carbon coater and EMITECH sputter coater. The cells were examined under the FIE ESES Quanta 400F in order to study morphological changes that occurred as a result of treatment specifically changes in the plasma membrane integrity.

**RESULTS**

**Cell viability as determined by the neutral red assay**

At the end of the 24 and 48 hrs drug treatment, the absorbances for the specified concentrations were read. The data obtained did not have equal variances, therefore it was transformed using loge in order to estimate a normal distribution. The data was rechecked using an F-test and was found to have normal distribution, therefore it was transformed using loge in order to estimate a normal distribution. The data was rechecked using an F-test and was found to have homogenous variances, therefore a one-way analysis of variances was used to analyse the data. The results showed that there was a significant difference in the sample means (p<0.0001), suggesting that the absorbance obtained for at least one of the drugs was different (Table 1).

The Tukey-Kramer post hoc test was used in order to determine which means were significantly different. When all the drugs were compared at the same Cmax during the same treatment period using the One way-anova test, 24 or 48 hours, it was found that for most drug concentrations there was no significant difference observed between the absorbances; however there was a significant difference observed between the 24 and 48 hours treatments for all the drugs (Table 1).

The Tukey-Kramer post hoc test showed that the absorbance resulting from treated cells was significantly different from controls, with the exceptions of FTC following the 24 hrs treatment and drug combination following a 48 hrs treatment at ⅛Cmax. Treatment with the drug combination after 48 hrs at ⅛ Cmax and 48 hrs at 2X Cmax, FTC, TDF and the drug combination after 24 hrs and 48 hrs at 10X Cmax were not significantly different to the control at either 24 or 48 hrs (Table 2). However EFV treatment after 24 and 48 hrs at 10X Cmax was significantly different from the control and other drug treatments (Table 3); the graphs indicate that at this concentration cell viability for EFV treated cells was significantly reduced in comparison to the control and other drug treatments (Fig1: a-d).

In summary there was a significant difference observed between the same drug at 24 and 48 hrs post treatment, however there was no significant difference observed within different drugs at the same Cmax concentration also the drugs generally increased cell viability at both 24 and 48 hours post treatment in
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comparison to the controls, except for EFV at the 10X Cmax concentration whereby it was shown to be toxic to the cells.

Table 1: A summary of a one-way analysis of variance (ANOVA) for the drug treatments

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Table 2: Results of the Tukey-Kramer Post Hoc test for the different drug treatments at the 5% level of significance. Underlines join groups that are not significantly different whereas significantly different groups are not underlined by the same line. The groups are arranged in descending order of the mean. It is clear from these results that essentially the 24 hour and 48 hour treatment group for drugs belonging to the NRTI were not significantly different from controls whereas EFV, NNRTI, was significantly different at the highest concentration.

Table 3: Key explaining the numbers used in table 2

<table>
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<th>Parameter</th>
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<tr>
<td>½ X Cmax</td>
<td>00; 1; 5; 7; 3; 4; 2; 6; 8; 0</td>
</tr>
<tr>
<td>1X Cmax</td>
<td>00; 7; 1; 5; 3; 4; 8; 2; 6; 0</td>
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<tr>
<td>2X Cmax</td>
<td>00; 7; 5; 3; 4; 2; 6; 8; 0</td>
</tr>
<tr>
<td>10X Cmax</td>
<td>5; 00; 7; 1; 3; 2; 4; 8; 0; 6</td>
</tr>
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</table>

Figure 1: The absorbance of the antiretroviral drugs at various concentrations and combination ratios corresponding to the Cmax (¼X to 10X Cmax) following 24 and 48 hour treatment duration; (a) FTC, (b) TDF, (c) EFV and (d) Drug combination. The results were analysed using a one-way ANOVA and a significant difference was observed between the drug treatment and the controls (P<0.0001).
Immunofluorescence staining viewed with confocal microscopy was utilized in order to identify the subcellular location of Bax and bcl-2 proteins and to determine whether they formed heterodimers during co-localising. In all the untreated cells (controls) cytoplasmic expression of bcl-2 was high whereas Bax expression in the cytoplasm was low, there was no co-localisation between the proteins (Fig2; a). Following treatment with FTC and TDF, Bax and bcl-2 were co-localised in the perinuclear region as indicated by the yellow coloration resulting from the red and green fluorescence of Bax and bcl-2 respectively (Fig2; b& c). Treatment with EFV resulted in a high cytoplasmic expression of Bax and down-regulation of bcl-2 in the cytoplasm (Fig2; d). Bcl-2 expression was found in the cytoplasm following treatment with the drug combination, although it was slightly reduced in comparison to controls and Bax expression was found in the nucleus, no co-localisation was found between Bax and bcl-2 proteins (Fig2; e).

Figure 2: (a) Untreated MCF-7 cells (control); nuclear staining with Dapi. Cytoplasmic expression of bcl-2 (FITC) and Bax (Rhodamine) expression in the nucleus [19]. The cytoplasm stained green due to bcl-2 over-expression which is common in cancer cells [20]. (10µM). (b- e) FTC and TDF treated MCF-7 cells (respectively); nuclear staining with Dapi, bcl-2 (FITC) expression in the cytoplasm and Bax (Rhodamine) expression in the cytoplasm. The yellow coloration resulted from overlap between FITC (green) and Rhodamine (red) fluorochromes, indicating co-localisation of bcl-2 and Bax in the cytoplasm, mainly around the nucleus, although Bax is also seen in the nucleus. (10µM). (d) EFV treated MCF-7 cells; nuclear staining with Dapi, reduced cytoplasmic expression of bcl-2 (FITC) and Bax (Rhodamine) over-expression in the cytoplasm. (10µM). (e) MCF-7 cells treated with the drug combination; nuclear staining with Dapi, little cytoplasmic staining of bcl-2 (FITC) and Bax (Rhodamine) expression in the nucleus and cytoplasm as suggested by the yellow fluorescence seen in the cytoplasm of the cells; a result of the overlap between FITC (green) and Rhodamine (red) fluorochromes and it indicates that there was co-localisation of bcl-2 and Bax. (10µM).
Figure 3: (a) Control MCF-7 cells. Note the polyhedral shape of the cells, extensive cell contacts, flat nucleus with nucleolus and a uniform layer of microvilli in the plasma membrane. (b) Micrograph depicts the uniformity and density of the mv in the PM at a higher magnification. (c) Emtricitabine (FTC) treated MCF-7 cells. The cells display a rounded morphology and the cell junctions are less prominent, the nucleus appears condensed. Presence of sparsely dispersed microvilli throughout the plasma membrane, blebbing is also prominent with large “square” blebs seen (d) Micrograph depicts FTC treated MCF7 cells at a higher magnification. (e) Tenofovir disoproxil fumarate (TDF) treated MCF-7 cells. The cells display a round to oval shape with a few cell junctions. Note the condensed nucleus, sparse microvilli and abundant blebs in the plasma membrane. (f) Micrograph depicts TDF treated cells at a higher magnification, blebs are also “square” but smaller when compared to those of FTC (g) Efavirenz (EFV) treated MCF-7 cells. Note the rounded shape, few cell junctions and a condensed nucleus. The sparse dispersed microvilli in the plasma membrane and many disrupted blebs. (h) Micrograph depicts EFV treated MCF-7 cells at a higher magnification, blebs are less prominent and disrupted in comparison to previous treatments (i) MCF-7 cells treated with the drug combination. Note the few membrane blebs secreting an electron lucent substance and a flat nucleus. (j) Micrograph illustrates a region which clearly depicts microvilli in the plasma membrane and the electron lucent substance at the same magnification as (i).
DISCUSSION

Cytotoxicity of the antiretroviral drugs was measured using the Neutral red uptake assay and cell viability was assessed as the endpoint to indicate drug toxicity. The neutral red assay measured cell viability based on the uptake and accumulation of the neutral red dye into lysosomes of viable cells [17, 21]. The results obtained from the neutral red cytotoxicity assay showed that the drugs had similar effects on cell viability at concentrations corresponding to the same Cmax and generally all drugs promoted cell viability. This effect is not favourable in cancer because by promoting viability in cancer cells it suggests that the drugs assist cancer cell growth and this would not be desirable for instance in a HIV positive patient who is on the ARV treatment and has cancer.

Interestingly EFV treatment at 10X Cmax following 24 and 48 hour treatment significantly reduced cell viability suggesting cytotoxic effects on the cells which were not observed with other drug treatments. FTC and TDF belong to the same class of antiretrovirals, NRTI, and they were not cytotoxic to the cells at the tested concentrations whereas EFV belongs to NNRTI and it was the drug group that significantly altered cell viability but only at the highest concentration tested, hence suggesting that it was more toxic than the other drugs, this finding is consistent with a previous study which also found that ARV drugs belonging to the NNRTI are the most cytotoxic and could be a possible candidate for cancer treatment and those belonging to the NRTI are the least cytotoxic [22].

Bcl-2 family proteins are key regulators of apoptosis and they have been shown to act through the mitochondrial pathway, by promoting or inhibiting the release of apoptogenic factors such as cytochrome c [23]. The relative expression ratios of pro-apoptotic and anti-apoptotic members of this family determine the ultimate susceptibility of cells to different apoptotic stimuli [24].

This study showed cytoplasmic co-localisation of Bax and Bcl-2 in cells treated with FTC and TDF, an increased cytoplasmic expression of Bax coupled with a decreased bcl-2 expression in EFV treated cells and the drug combination resulted in expression of both the proteins however no co-localisation was found. Bax and bcl-2 heterodimerisation is a well known phenomenon during apoptosis [23] and the observation of co-localisation between these two proteins suggest that the cells were undergoing apoptosis; however this is in contrast with findings from the Neutral red cytotoxicity assay which suggests that the drugs promoted cell viability at the tested concentrations. In contrast Bax over expression observed in EFV treated cells together with the morphological effects of this drug on the cells suggests that there was cell death induction however the lack of clear co-localisation between Bax and Bcl-2 suggests that the mitochondrial pathway was not a major player in facilitating cell death in those cells.

The execution phase is the end point of the extrinsic and intrinsic apoptotic pathway [25]. Initiation of the execution pathway results in the classic morphological features of apoptotic cells; loss of cell to cell contacts, shrinkage, nuclear material condensation, DNA fragmentation, cytoskeletal and protein degradation, cross-linking of protein, blebbing of the plasma membrane, formation of apoptotic bodies, ligand expression for phagocytic cell receptors and ultimately phagocytosis of affected cells by phagocytic cells [26].

Studies have shown that most epithelial cell types including MCF 7 cells undergo these morphological changes during apoptosis [26]. A study of the plasma membrane integrity revealed characteristics of apoptotic cell morphology in treated cells and this suggests the presence of altered cell function through induction of cell death.

Treated cells were observed to be losing contact with each other in comparison to controls, and they were acquiring a more rounded morphology as opposed to the polyhedral shape of control MCF 7 cells. These features are one of the early phase markers of apoptosis that a cell undergoes following disturbance to its normal cell cycle progression [28, 29]. The rounded morphology suggests that the cells were retracting from the substratum and they also appeared to have a reduced cell volume, indicative of pyknosis, which is another classic feature of cells undergoing apoptosis [30].

The treated cells had nuclei expressing characteristics of a cell undergoing nuclear condensation as indicated by bulging out from below the plasma membrane thus suggesting that the cells underwent chromatin destruction. The absence of this morphological feature in all controls (negative and diluent) suggests that it was induced by the drug treatment. This observation was consistent with previous findings in that chromatin condensation has been shown to form part of the initial morphological changes observed in most cell types undergoing apoptosis [20, 29]. The fate of the disrupted nucleus is the release of nuclear contents into the cytoplasm, however remaining within the intact plasma membrane of the apoptotic cell [20, 29, 30].

Surface cytoplasmic projections, often identified as microvilli, have been observed in a majority of mammalian cells in vitro although their density and distribution varies in different cell types [31]. It has been shown that the cytoplasmic processes differ in distribution, abundance and length under certain conditions of culture [31]. Microvilli have been reportedly lost during the early stages of apoptosis [29, 31] similarly the present study also showed a relatively reduced number of the microvilli on the cell surface of treated cells suggesting that the drugs had an adverse effect on the normal functioning of the cells. The presence of the microvilli on the surface of cells is likely to reflect its specialised role in absorption of nutrients and secretion of synthesized substances [31] and the loss of these surface structures suggest that the cells are losing their physiological ability.
to absorb nutrients from the extracellular matrix and perhaps signals that the cells are becoming inactive due to disturbed cell function.

The present study found an excess bleb expression in drug treated cells as opposed to untreated cells and a majority of the blebs were observed in cells treated with FTC and TDF. The cells were intact and contained within the plasma membrane and few of the blebs had pinched-off and were observed in the intercellular space. In contrast, cells treated with EFV displayed very distinct morphological features. The blebs were retracted and had released contents onto the plasma membrane and intercellular space suggesting that EFV had more severe effects on the cells and possibly induced cell death via a different mechanism to that of FTC and TDF.

Plasma membrane blebbing has been observed at a much later stage of apoptosis and its role is to aid the dispersal of nuclear and cytoplasmic contents in the form of apoptotic bodies \[^{[20]}\]. Blebs are described as spherical plasma membrane protrusions and their appearance in damaged cells signals cell death \[^{[32]}\]. Blebs have recently been identified as one of the indications of apoptosis which play a role in the initial stages of the execution phase \[^{[32]}\]. In addition blebs are characterised as prone to swelling and often after pinching off from the plasma membrane, burst \[^{[33]}\].

The mode of effects induced by the drug combination seemed to have very distinct morphological features, different to those seen in the single drug treatments. There appeared to be no drastic alteration observed in the plasma membrane morphology except for the large electron lucent secretion onto the plasma membrane and intercellular space. This unique feature shows that the drug combination had distinct effects on cell morphology which suggests that apoptotic-necrosis was induced due to the release of an electron lucent cytoplasm which is often characteristic of apoptotic-necrosis \[^{[33]}\]. In addition if the drugs tested act via different cell death pathways then that could also be a factor in the cell death mechanism employed and therefore cell morphology observed.

CONCLUSION

Cell cytotoxicity was only observed following EFV treatment at 10X Cmax however Bax and bcl-2 expression in all treated cells suggests that cell death was induced, possibly through the mitochondrial signalling pathway, and morphological features observed with the SEM in the plasma membrane revealed characteristics commonly found in apoptotic cells.

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patients is significantly associated with TDF and consists of ubiquitous mitochondrial CK. Antiviral Therapy 11: 1071-1080.


