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Research Article

Anti-Mitotic Properties Of Efavirenz On Cal 27 Cell Line.

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Abstract

Introduction: Some studies have suggested that Efavirenz may have anti-proliferative effects on various cancer cell lines, potentially through mechanisms that involve cell cycle arrest and inhibition of cell division. The aim of this work is to investigate the potential effects of efavirenz on cancer cells due to its ability to interfere with cellular processes.

Cal 27 cells are human oral squamous cell carcinoma of the tongue, a cell line commonly used in cancer research. These cells have a limited specific information available.

Aims and objectives: To evaluate the anti-mitotic effects of efavirenz on CAL 27 cell lines, and to determine the toxic concentration on efavirenz on the cells.

Methods: Experimental groups were established with differing concentrations of the drugs, along with control groups. Cell viability was assessed 48 hours after treatment using a trypan blue assay, and the cells were harvested based on confluence criteria. Cell counting was done using an automated Luna-FL™ dual fluorescence cell counter. Data analysis was conducted using one-way Analysis of Variance (ANOVA) to compare drug efficacies, with a significance level set at 0.05 ($p \leq 0.05$).

Result : Our data analysis revealed that EFV treatments did not exhibit significant main effects on the viability of the cancer cell line ($F(6,20) = 0.7970$; $p = 0.5878$).

Discussion/Conclusion: Efavirenz is an antiretroviral drug commonly used to treat HIV-1 infection. Our results show anti-mitotic effects that suggest Efavirenz has potential as a therapeutic agent in the treatment of oral cancer, particularly in combination with other chemotherapeutic agents. However, further research is needed to enable us fully to understand its action mechanisms and grade its efficacy in this context.

Keywords: Cal 27 cells ; Efavirenz; anti-mitotic; oral squamous cell; anti-angiogenic; AIDS -defining cancers; anti proliferative; Apoptosis.

INTRODUCTION

CAL 27 cells were derived from epithelial tissue obtained pre-treatment from a Caucasian male with a tongue lesion in 1982[1] and these cells are characterized by their epithelial traits, including a polygonal shape and a highly granular cytoplasm. Immunocytochemical analysis of these cells confirmed strong positive staining with anti-keratin antibodies. Over 90% of oral cancers was discovered to be squamous cell carcinoma (SCC), and oral cancers account for 3% of total cancer incidence in the USA[2,3]. Though incidence and mortality patterns do differ geographically, factors like smoking, alcohol, DNA oncogenic viruses, and habits such as betel nut consumption drives the incidence, prevalence and mortality[4] . The incidence was said to have decreased in some regions, but certain low-income countries and female populations have seen an increase[5]. CAL 27 cells has been identified as squamous cell cancer of the tongue, and it is one of the most commonly used cell lines in oral squamous cell carcinoma (OSCC) research[6].

HIV cancers

HIV remains a global health hazard and a particular health concern in South Africa[7], up to 90% of South African patients with Kaposi sarcoma (KS) were black South Africans[8]. Cancer,

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a prevalent disease worldwide, further complicates HIV infectivity and its management, diminishing life quality and contributing to premature mortality [9].

About 80% of cancer diagnoses occur at advanced stages in sub-Saharan Africa, increasing the disease burden, especially in these regions. This is mainly due to exposure to major risk factors, limited awareness of early symptoms, and insufficient diagnostic facilities and access to care [10].

Individuals living with HIV face a higher risk of certain cancers compared to HIV-negative individuals of the same age[11]. Those with AIDS are significantly more susceptible to specific cancers [AIDS -defining cancers], including Kaposi sarcoma (over 500 times), non-Hodgkin lymphoma (20-70 times), cervical cancer (5 times), lung cancer (3 times), Hodgkin lymphoma (10 times), and anal cancer (25 times).

General Cancer management

Conventional cancer therapy typically involves using drugs and/or radiation to target and kill cancer cells, which replicate faster than normal cells. Surgery can help reduce tumor size, but it becomes ineffective once cancer cells metastasize. Radiation therapy is useful only if the cancer is localized [12], necessitating alternative treatment methods. The present invention focuses on enhancing the immune response by removing inhibitors of immune mediators. This approach can be combined with anti-angiogenic compounds, cytokines, compounds that induce a procoagulant state, chemotherapeutics, and/or radiation [13, 14].

Cell cycle

According to the National Human Genome Research Institute[15], the cell cycle is the process by which cells replicate. It consists of different stages: G1, S, G2, and M. In the G1 stage, the cell prepares to divide. Then, it enters the S phase, where it copies all the DNA, with S standing for DNA synthesis. Next, the cell transitions to the G2 stage, where it organizes and starts to condense the genetic material, preparing for division. The final stage is M (mitosis). During mitosis, the cell splits the two copies of genetic material into two daughter cells. Once the M phase is complete, cell division occurs, resulting in two new cells, and the cycle begins again.

Cancerous change in cells

According to Cancer Research UK [16], unlike normal cells that can reproduce when needed, adhere to their correct locations in the body, and undergo apoptosis as controlled by the body, cancer cells lose these regulatory abilities and behave oppositely. Due to their inability to remain in place and function properly, cancer cells can break away and continue to grow elsewhere, a process known as metastasis. Additionally, the molecules that influence cell repair, such as the protein p53, are often faulty in cancer cells. This fault is partly due to the rapid division of cancer cells, which leaves little room for cellular maturity before division. The defective p53 protein exacerbates the cells' inability to repair themselves or undergo apoptosis, making them resistant to treatment.

Efavirenz

Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) commonly used in HIV treatment. It is a key component of first-line Highly Active Antiretroviral Therapy (HAART) for managing HIV-1 infection in both adults and children [17-19]. EFV suppresses viral DNA replication by binding to and blocking the activity of the HIV reverse transcriptase enzyme [20].

The anti-mitotic effects of EFV suggest its potential as a therapeutic agent in treating oral cancer, especially when combined with other chemotherapeutic agents. However, further research is needed to fully understand its mechanisms and efficacy in this context. While EFV is primarily used for its antiviral effects against HIV, some studies have also investigated its potential impact on cancer cells due to its ability to interfere with cellular processes [21]. Recent studies have explored its anti-cancer properties, including anti-mitotic effects on various cell types, such as CAL 27 cells, a type of oral squamous cell carcinoma commonly used in cancer research [1,6].

Beyond its role in HAART, EFV is significant in improving outcomes in TB-HIV co-infection among children [22]. EFV has also emerged as a crucial element in cancer management, particularly in individuals living with HIV [23]. Notably, EFV has been repurposed and shown effectiveness against various cancers, including prostate, pancreatic, and triple-negative breast cancer (TNBC), EFV exerts its anticancer effects on TNBC through the modulation of the fatty acid metabolism pathway [24]. Additionally, EFV induces apoptosis in Human Squamous Cell Carcinoma from Uterine Cervix (HCS-2) cells [23]. Regarding the anti-mitotic properties of EFV on CAL 27 cells specifically, there is limited information available as of my last update in September 2021. However, some studies have suggested that EFV may have antiproliferative effects on various cancer cell lines, potentially through mechanisms involving cell cycle arrest and inhibition of cell division [23].

Effect of Efavirenz on Cal 27

Beyond its role in HAART, EFV has shown importance in improving outcomes for TB-HIV co-infection in children [22]. EFV has also become crucial in cancer management, particularly for individuals living with HIV [23]. Notably, EFV has been repurposed and proven effective against various cancers, including prostate, pancreatic, and triple-negative breast cancer (TNBC). According to Fong [24], EFV exerts its anticancer effects on TNBC by modulating the fatty acid metabolism

pathway. In addition, EFV induces apoptosis in Human Squamous Cell Carcinoma from Uterine Cervix (HCS-2) cells [23]. Research has shown that Efavirenz can inhibit the growth and proliferation of Cal 27 cells by:

- Arresting the cell cycle at the G1/S phase [23].
- Inhibiting the expression of cell cycle regulatory proteins (e.g., cyclin D1, CDK4)[23].
- Inducing apoptosis (programmed cell death)[21].

METHODS

The primary aim of the study was to address the question of whether EFV have the ability to inhibit mitosis in the CAL-27 cell line.

The experimental groups were set up as follows:

Test Group 1: CAL-27 cells were treated with EFV at a concentration of 4µg/mL each.

Test Group 2: CAL-27 cells were treated with EFV at a concentration of 2µg/mL each.

Test Group 3: CAL-27 cells were treated with EFV at a concentration of 1µg/mL each.

Test Group 4: CAL-27 cells were treated with EFV at a concentration of 0.5µg/mL each.

Test Group 5: CAL-27 cells were treated with EFV at a concentration of 0.25µg/mL each.

There were two control groups:

Control Group 1: This group served as the solvent control, no any active substances was used in this group, it was treated with an equal volume of physiological saline.

Control Group 2: This group was left untreated and was not subjected to any experimental intervention, normal growth processes allowed to occur in this cells.

To establish these groups, a stock solution of EFV at a concentration of 10 µg/mL was prepared. Serial dilutions were then performed using physiological saline, and the resulting solutions were added to separate culture flasks containing CAL-27 cells. This setup included five test groups and two control groups, enabling a thorough evaluation of EFV's impact on inhibiting mitosis in the CAL-27 cell line.

Penstrep Treatment

To promote cell health and growth, each culture medium received 2-3 drops of penicillin-streptomycin solution containing penicillin (50 U/ml) and streptomycin (50 µg/ml). After this addition, the cell cultures were incubated in a controlled environment with 5% CO2 at 37°C. Daily observations were conducted over a period of 10 days using an inverted microscope. On the 10th day, the cell cultures were harvested for final assessment.

Cell Dissociation with Trypsin

Cell dissociation with trypsin was initiated once the cell growth reached 90% confluence within the culture flask. This process ensured the controlled separation of cells from the culture substrate, facilitating subsequent manipulation and analysis.

Mycoplasma Contamination Test

To maintain the integrity of the cell lines, a mycoplasma contamination test was conducted using Hoechst dye from Sigma-Aldrich, St. Louis, USA, on both day 5 and day 10 of the experiment. This procedure assessed the presence of mycoplasma, a type of bacterial contamination, thereby ensuring the reliability and accuracy of the experimental results.

Sample size determination

In cell biology studies, manufacturers provide specifications for plates, dishes, and flasks used in cell culture, including recommended seeding densities specific to each vessel type. This differentiation is crucial because the capacity of reaching confluence varies depending on the cell type cultured. The volume of a flask is particularly influential in determining the number of cells it can accommodate, shaping sample size considerations.

According to Lazic *et al.*[25], a flask achieving at least 90% confluence is considered to have sufficient sample size. Each experimental group and subgroup in this study consisted of three flasks, and the experiment was repeated three times to ensure result precision. Moreover, as outlined by Pollard *et al.*[26], increasing the sample size through repeated growth in cell culture experiments brings the mean results closer to the true population mean, thereby enhancing statistical significance.

In this descriptive analytical study, achieving statistical significance involves cloning experimental cells from the same cell group, a practice followed consistently. Each experimental group was also replicated in triplicates, with all groups reaching a minimum of 90% confluency.

Both Lazic [27] and Lazic *et al.* [25] agree that determining sample size in cell culture need not be overly complex, given that treatments are uniformly applied to all cells within a well or flask. This approach considers interactions between cells, such as cell-to-cell connections, release of signaling molecules, and competition for nutrients in the culture media, which collectively influence experimental outcomes.

Cell viability – using Trypan Blue

A trypan blue assay was performed 48 hours after treatment to assess the viability of CAL27 cells.

Exclusion criteria

At harvest, any cell with confluence of less than 90% was eliminated from the study.

Data collection and statistical analysis

To determine the concentration of cells per milliliter (ml) in my culture media, I followed the method outlined by Srivastava *et al.*[28], which utilizes hemocytometers and trypan blue staining. First, I took a 10 µL sample from the cell culture and mixed it with 90 µL of trypan blue solution in an autoclaved microcentrifuge tube, ensuring thorough mixing. Then, I transferred a 10 µL aliquot of this mixture onto an automated hemocytometer. By counting the cells in all the squares of the hemocytometer, I calculated an average cell count. To convert this average count into cells per milliliter, I multiplied it by the dilution factor, which in this case is 10. This calculation provides the number of cells in millions per milliliter of the cell culture, offering a valuable metric for assessing cell concentration in the experimental setup.

Counting of cells

As part of the experimental process, a portion of freshly prepared cell suspension was extracted from each experimental group. To assess cell viability, trypan blue stain was used. This stain distinguishes live cells by selectively staining dead cells.

Live cells have intact cell membranes that prevent trypan blue from entering the cytoplasm. Conversely, dead cells have compromised membranes, allowing trypan blue to penetrate and stain the cytoplasm. For quantifying live cells, an automated cell counting method was employed using the Luna-FL™ dual fluorescence cell counter from Logos Biosystems, USA. This device combines the principles of traditional counting chambers or hemocytometers with fluorescent staining for enhanced accuracy. The cell suspension was loaded into a Neubauer cell-counting chamber, which was then inserted into the automated cell counter. Relevant settings were adjusted according to experimental requirements, and the automated counting procedure was initiated. This automated approach streamlined cell counting, delivering reliable results through the integration of fluorescent staining and advanced technology.

Data analysis

Cell count method: Results obtained was analyzed using the one-way Analysis of Variance (ANOVA) and obtained values was used to compare the efficacies of the drugs, at 0.05 significance level ($p \leq 0.05$).

Statistical Analysis

One-way Analysis of Variance (ANOVA) was used to compare the efficacies of the drugs, at 0.05 significance level ($p \leq 0.05$).

RESULTS

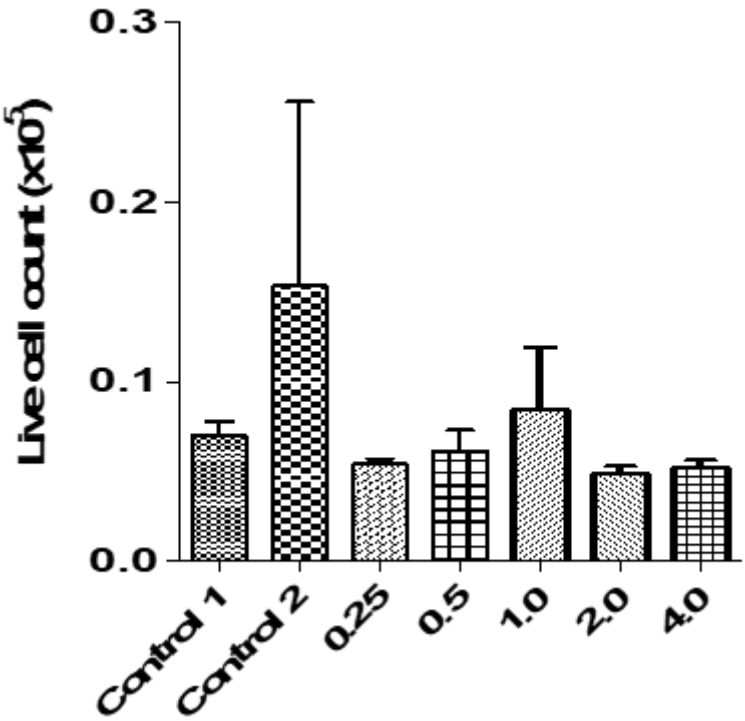
The results presented in the table below demonstrate the impact of EFV on CAL-27 cell lines. These findings were recorded at a wavelength of 595.0 nm. Ultraviolet (UV) radiation is known to induce squamous cell carcinoma by causing DNA damage, with the effects varying depending on the wavelength [29]. Previous studies have indicated that cell viability increases with longer wavelengths[30].

Table 1. Results

Treatment/ Dosages(µg/ml)	Description of Group	Flask 1	Flask 2	Flask 3	Average [of the flasks]
4.0	Test group 1	0.044	0.059	0.053	0.052
2.0	Test 2	0.049	0.042	0.056	0.049
1.0	Test 3	0.154	0.052	0.047	0.084
0.5		0.084	0.055	0.046	0.062
0.25		0.06	0.05	0.052	0.054
DMSO	Negative control [Control 1]	0.084	0.058	0.069	0.070
DMEM	Positive Control [Control 2]	0.358	0.049	0.054	0.154

Our data analysis revealed that EFV treatments did not exhibit significant main effects on the viability of the cancer cell line ($F(6,20) = 0.7970$; $p = 0.5878$, **Fig. 1**).

Figure 1. Different conc. Of EFV in in µg/ml.s.



The effects of Efavirenz on cell Division. The graph shows the average number of live Cal-27 cancer cells following incubation with DMEM, DMSO and different concentrations of EFV in µg/ml. Data shown represents mean ± SEM; n=3 per group. *P<0.05 compared to control, #P<0.05 compared to other treatment groups; One-way ANOVA, followed by Bonferroni post hoc comparison test.

DISCUSSION

The analysis indicates that the p-value for the effect of efavirenz on CAL 27 cells exceeds 0.05, suggesting that the null hypothesis holds true, indicating no significant effects of EFV on the CAL 27 cell lines. Squamous cell carcinoma can present with various growth patterns, including exophytic, ulcerative, and infiltrative types. Infiltrative and ulcerative types are most common due to loose tissue planes around intrinsic tongue musculature, facilitating cancer cell spread and symptom development as tumor size affects tongue mobility [31]. Angiogenesis plays a crucial role in the progression of most solid tumors [32]. Studies have also identified a correlation between increased vascularization and the development of oral carcinoma, underscoring the pivotal role of angiogenesis in its progression [33].

Research has consistently linked high intratumoral microvessel density (MVD) with poorer prognosis in oral carcinoma, associated with larger tumor size, higher relapse rates, and node metastases[34,35]. Additionally, another study found that the extent of neoangiogenesis correlates strongly with histological grade of differentiation and the presence of locoregional metastases in carcinoma [36]. Despite evidence of EFV's antiangiogenic effects [20], its impact on antiangiogenesis in CAL 27 cells is not significant, suggesting that tumor staging may influence EFV's antiangiogenic effects and warranting further investigation to verify this hypothesis. There are conflicting findings regarding EFV's cytotoxic effects on cancer cells. While some studies suggest EFV may have antitumor effects, others find no significant impact on specific cancer cell lines. Notably, EFV has shown effects on breast cancer stem cells, which are implicated in cancer metastasis and resistance to conventional therapies [37].

Although some studies report cytotoxic effects of EFV on cancer cell lines and animal models, our study indicates no significant effects on the CAL 27 cell line. It is essential to consider that study outcomes can vary due to factors such as specific cancer cell types studied, EFV dosage and duration of treatment, and experimental conditions.

CONCLUSION

The prevalence of squamous cell carcinoma underscores the importance of angiogenesis in its progression. Specifically, intratumoral microvessel density (MVD) correlates with prognosis, where higher MVD is linked to larger tumor size, increased rates of relapse, and node metastases. Efavirenz (EFV), known for its potential antiangiogenic effects in previous studies, did not demonstrate a significant impact on antiangiogenesis in CAL 27 oral carcinoma cells in this

study. Further research is required to explore whether tumor staging influences EFV's antiangiogenic effects in this context, fully assessing its potential as a cancer treatment. It's crucial to recognize that these findings may not generalize to other cancer cell lines or in vivo models. Overall, this passage emphasizes the intricate nature of oral carcinoma and the necessity for continued research to comprehend its underlying mechanisms and explore potential treatments.

Purpose of the study

To evaluate the anti-mitotic effects of Efavirenz on Cal 27 cell line, and to determine the toxic concentration of efavirenz on the cells.

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Conflicts Of Intrest

The authors declare that the research was conducted without any commercial or financial relationships that could be interpreted as potential conflicts of interest.

A Consent Confirming The Approval By All The Co-Authors To Submit The Manuscript

The authors all consent to submitting this manuscript for review and publication.

Confirmation That The Content Of The Manuscript Has Not Been Published Or Submitted For Publication Elsewhere

The authors declare that this manuscript has not been published, or submitted for publication elsewhere, neither is it under consideration for publication by any other journal.

Declaration

The authors declare that the research was self-funded and there is no financial interests or incentives in products or services from anyone or group.

Ethics

Ethics approval was obtained from the Research ethics committee [SREC] of the Sefako Makgatho Health Science University.

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