Riboflavin-mediated Photodynamic Therapy Induces Cytotoxic Effects in A549 Lung Cancer Cells

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Abstract. Lung cancer, a prevalent and deadly malignancy, arises from the uncontrolled growth of cells within the lungs, and it poses significant challenges in diagnosis and treatment. Photodynamic therapy (PDT) has promising therapeutic effects while causing minimal harm to healthy cells and tissues. This study explores the therapeutic potential and impact of riboflavin, a natural photosensitizer, and PDT against A549 lung cancer cells. The cells were treated with riboflavin at variable concentrations and irradiated using a laser of wavelength 470 nm at a fluency of 5 J/cm^2. Following a period of 24 hours post-irradiation, the A549 cells were analyzed using biochemical assays, namely adenosine triphosphate and lactate dehydrogenase assays. In addition, morphological analysis post-irradiation, reactive oxygen species detection, and mitochondrion localization studies were performed. Changes in cell viability and morphology were observed post-treatment, indicating the cytotoxic effects of riboflavin-mediated PDT on A549 lung cancer cells. These findings suggest that riboflavin-mediated PDT might have potential as an anticancer treatment against lung cancer.

1 Introduction

Cancer is a fatal disease that continues to claim the lives of its victims on a global scale. It has consistently continued to fall under the top 5 leading causes of death for the past two decades. Cancer affects both men and women, and it affects individuals of almost any age. According to the American Cancer Society, 618,120 cancer deaths and 2,041,910 new cancer cases are expected to occur in 2025 in the US [1]. Lung cancer is rated number one, with high mortality and incidence rates [2]. The most common causes of lung cancer include tobacco smoking, excessive alcohol consumption, unhealthy diet choices, and environmental pollutants [3]. While there are conventional treatments and other advanced therapy modalities, cancer continues to remain fatal and life-threatening. Most of the known treatments are associated with serious side effects, and some are not as effective in eradicating the disease in its entirety. Photodynamic therapy (PDT) has gained traction as a minimally invasive treatment option that uses photosensitizing agents, known as photosensitizers, and laser therapy. Photosensitizers are molecules that can accumulate in cells and, upon light exposure at a specific wavelength, can become excited to generate reactive oxygen species, leading to apoptosis or other cell death pathways [4]. Riboflavin, also known as Vitamin B2, is the photosensitizer of choice for this study due to its biocompatibility as a naturally-derived vitamin, and its ability to generate reactive oxygen species upon light activation in the blue region of the visible spectrum [5]. Moreover, there are very limited research studies of its use in lung cancer. Therefore, this study focuses on elucidating the potential cytotoxic effects of riboflavin with PDT on lung A549 cancer cells.

2 Methodology

2.1 Cell Culture and Maintenance of Lung A549 Cancer Cells

The A549 lung cancer cell line obtained from the American Type Culture Collection (A549, ATCC® CCL-185) was cultured in Rosewell Park Memorial Institute 1640 (RPMI) culture medium. The medium was supplemented with 1% amphotericin B, 1% penicillin/streptomycin antibiotics, and 10% fetal bovine serum (FBS). The cells were kept in a 37 ˚C incubator with 85% humidity and 5% CO_2 to promote an environment that supports optimal cell growth. Cell growth was observed every 24 hours, with media change being made every second day. The A549 passage numbers used for this study were kept between 26 and 29.

2.2 Cell Treatment and Morphological Analysis

Following the optimal growth of the A549 lung cancer cells in a T75 plate, they were then seeded into a 3.5 cm culture dish, using a seeding density of 5 x 10^5 cells/ml. The cells were allowed to attach, and after 24 hours, they were subjected to treatment using various concentrations of riboflavin (20, 40, 80, 110, 160, 320 µM) in the absence of laser. Subsequently, cells were also treated with the same varying concentrations of riboflavin and a 470 nm laser therapy with a fluency of 5 J/cm^2. After 24 hours, cellular morphology was analyzed using an inverted light microscope (Wirsam, Olympus CKX41, Tokyo, Japan) to evaluate the cytotoxic effects of the treatment.

2.3 Cell Cytotoxicity Evaluation using Lactate Dehydrogenase (LDH) Assay

The cells were seeded into a 96-well cell culture-treated plate using a seeding density of 10 000 cells/well/100 µL and subjected to the treatment conditions as mentioned in section 2.2. After 24 hours of treatment, cell cytotoxicity was evaluated using the LDH Non-radioactive CytoTox 96 assay kit (Promega, G179A, USA). The experiment was performed by transferring 55 µL of the reconstituted LDH reagent into each well of the 96-well plate containing an equivalent volume (55 µL) of cell culture media. The solution was gently mixed for 5 minutes on a shaker and incubated for 30 minutes at room temperature, in the absence of light. The analysis was made by reading the absorbance at 490 nm using a microplate reader (PerkinElmer, VICTOR Nivo™, South Africa).

2.4 Cell Viability Assessment using Adenosine Triphosphate (ATP) Assay

The cells were seeded into a 96-well cell culture-treated plate using a seeding density of 10 000 cells/well/100 µL and subjected to the treatment conditions as mentioned in section 2.2 to determine the half maximal inhibitory concentration (IC_{50}). After 24 hours of treatment, cell viability was assessed using the ATP CellTiter-Glo® 3D reagent (Promega, G968A, USA). The experiment was performed by adding an 80 µL volume of the ATP reagent equal to the volume of the cell culture medium (80 µL) present in each well. The solution was vigorously mixed for 5 minutes to induce cell lysis. This was followed by an additional incubation step of 10 minutes at room temperature, in the absence of light. The cells were then transferred to an opaque white, flat-bottom 96-well plate, and the ATP luminescence was read using a microplate reader (PerkinElmer, VICTOR Nivo™, South Africa).

2.5 Determination of Reactive Oxygen Species Produced by A549 Cells

The amount of reactive oxygen species produced by A549 lung cancer cells before and after treatment were quantified using the 2',7'-dichlorofluorescin diacetate (DCFDA) cellular ROS assay kit (Abcam, ab113851, UK). The cells were seeded into a black, high binding, flat-bottom, 96-well microplate using a seeding density of 10 000 cells/well/100 µL and incubated for 24 hours to allow for adhesion of the cells to the plate. After incubation, the cell suspension was discarded, and the cells were washed twice using 1X phosphate-buffered saline (PBS). This was followed by staining with 20 µM DCFDA solution for 45 minutes at 37 ˚C, in the absence of light. Thereafter, the cells were washed once with 1X PBS and treated with the riboflavin IC_{50} concentration of 142.5 µM for 4 hours, followed by laser irradiation treatment at 470 nm, including appropriate controls. After 30 minutes, the fluorescence was read at Ex485/Em535 nm using the microplate reader (PerkinElmer, VICTOR Nivo™, South Africa).

2.6 Mitochondrial Localization of Riboflavin Photosensitizer in A549 Cells

The A549 lung cancer cells were seeded into a 3.5 cm cell suspension dish with sterile coverslips using a seeding density of 2 x 10^5 cells/ml and incubated at 37 ˚C with 85% humidity and 5% CO_2. The cells were allowed to attach, and after 24 hours, the cells were subjected to treatment at a concentration of 142.5 µM for 4 hours. After treatment, the cells were washed thrice with ice-cold 1X PBS to remove unabsorbed riboflavin photosensitizer.

The cells were then fixed using 1 mL of 4% paraformaldehyde at room temperature and incubated for 15 min. Post-incubation, the cells were washed thrice with PBS and permeabilized for 15 min using 0.5 % Triton X-100 in 1X PBS. The solution was discarded, and the cells were washed thrice with 1X PBS. A pre-warmed probe containing 100 nM mitochondrial tracker Mito Red CM x ROS (Invitrogen, M7512, US) was added to the petri dish and incubated with the cells for 30 min at 4 ˚C. After incubation, a thorough wash using PBS was performed, and the cells were counter-stained for 5 min at 37 ˚C using 300 nM of 4′,6-diamidino-2-phenylindole (DAPI) and rinsed thrice using 1X PBS. The treated coverslip was removed from the petri dish and mounted onto a microscope glass slide using fluoromount aqueous mounting medium. The slides were sealed with a clear nail polish and examined using widefield/confocal fluorescence microscope (Leica Mica Microscope, Johannesburg, South Africa).

2.7 Statistical Analysis of Experimental Data

All experiments in this study were conducted 3 times ($n = 3$). GraphPad Prism 5 software was employed as a tool for the analysis of the data acquired from each experiment. One-way analysis of variance (ANOVA) was carried out to determine the statistical significance of the mean values acquired from the treated groups, and these were compared to the mean values of the untreated A549 lung cancer cells (control group). Dunnett's test was conducted at a confidence interval of 95% and the mean values from each experimental group were plotted as mean ± standard error of the mean (SEM). The statistical significance was defined using p-values where $p < 0.05$*, $p < 0.01$**, and $p < 0.001$***.

3 Results and Discussion

3.1 Morphological Analysis

The A549 lung cancer cells were exposed to variable concentrations of riboflavin to study the dark-toxicity effects after 24 hours (Figure 1A). Alongside, the cells were exposed to the same variable concentrations and irradiated at 5 J/cm^2 using a 470 nm laser to study the synergistic effects of the photosensitizer and laser (Figure 1B). At 0 µM concentrations, there are no visible changes in the morphology when the cells were treated with the 470 nm laser at 5 J/cm^2 fluency, suggesting that PDT alone, with no photosensitizer, does not elicit any cytotoxic effects on the A549 cells. It is seen that increasing the concentration of riboflavin resulted in morphological changes, especially from 80 µM to 320 µM, in the presence and absence of laser. There are slightly enhanced effects when the photosensitizer is used in combination with laser irradiation (Figure 1B). Moreover, the effects of the treatment are marked by a reduction in cell number and loss of cell shape and integrity, suggesting that cell death is occurring at this stage of treatment.

3.2 LDH Analysis

Lactate dehydrogenase, abbreviated as LDH, is one of the key enzymes found in all tissues and most cells. This enzyme plays a role in DNA metabolism and gluconeogenesis [6]. When A549 cells are subjected to treatment, their membrane integrity becomes compromised, leading to a release of LDH into the extracellular space [7]. Sensitive assays, such as the LDH cytotoxicity assay, can detect levels of LDH from the culture medium due to treatment. The control groups in Figure 2A are seen at a 0 µM concentration of riboflavin. Two controls were employed in this study: control 1 (in blue) is A549 lung cancer cells without any drug, while control 2 (in green) is an indication of A549 cells treated with a laser and no photosensitizer. The levels of LDH for both control groups are lower than the LDH levels in the treated groups. Moreover, LDH levels in cells treated with riboflavin and laser resulted in a greater increasing trend of LDH release, compared to cells treated with riboflavin alone, suggesting its enhanced tumor killing effect.

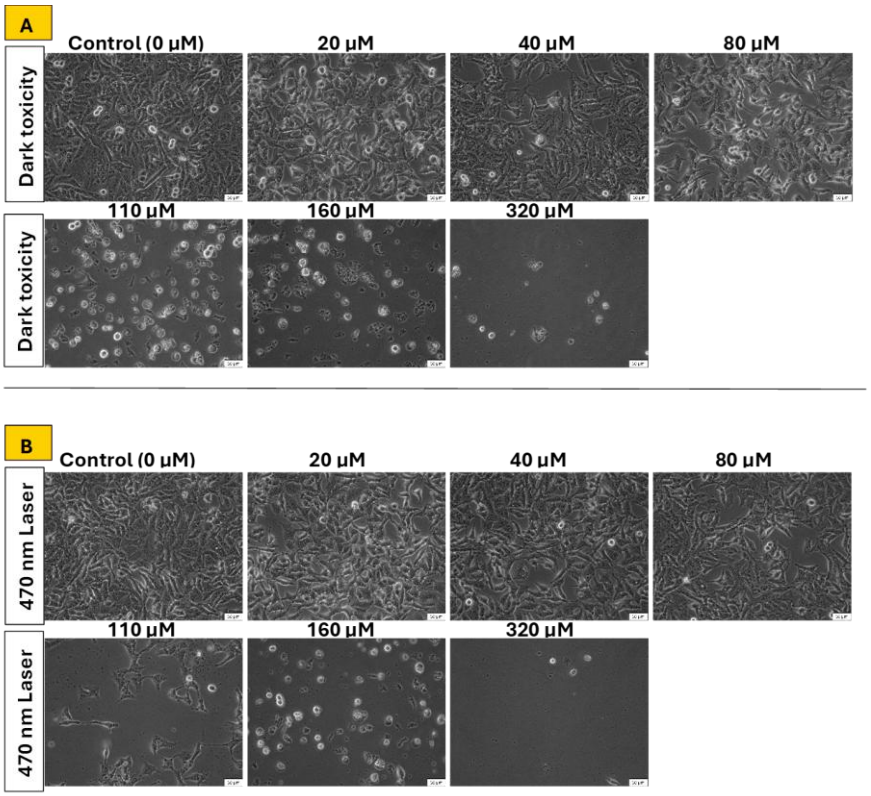

Figure 1: Morphological analysis of A549 lung cancer cells following a 24-hour treatment captured using a 40x objective lens. A) different riboflavin concentrations (0 – 320 µM) and no laser irradiation, and B) different concentrations of riboflavin (0 – 320 µM) with laser irradiation at 470 nm using a fluency of 5 J/cm^2.

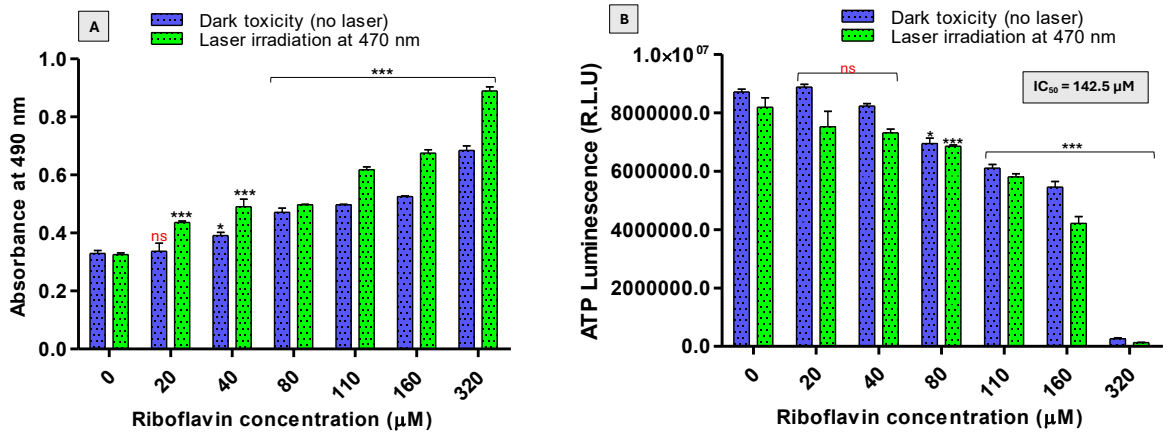

Figure 2: Biochemical assays indicating dark-toxicity and riboflavin-laser-treated groups in A549 lung cancer cells. (A) LDH cell cytotoxicity assessment 24 hours after treatment, (B) Dose-dependent cell viability ATP assay. At 0 µM riboflavin concentrations, two controls in both figures are depicted using blue and green, representing A549 lung cancer cells without laser treatment and with 470 nm laser treatment at 5 J/cm^2 irradiation, respectively. The data is represented as mean ± standard error of the mean for each group with n = 3. Statistical significance assessed using one-way ANOVA is defined using p-values where $p < 0.05$*, $p < 0.01$**, and $p < 0.001$***, while ns is an indication of a non-significant group compared to the control sample.

3.3 ATP Analysis

Adenosine triphosphate, also known as ATP, is an energy molecule that is found in all living cells, and it drives essential cellular processes [8]. Increasing levels of ATP from the cells are directly proportional to cell viability. Likewise, when cells experience cell death (due to treatment), the levels of ATP also drop. The ATP CellTiter-Glo® 3D reagent was used to quantify ATP levels in A549 lung cancer cells after treatment with riboflavin and laser. The half maximal inhibitory concentration (IC_{50}) was also determined using this assay and was found to be 142.5 µM (Figure 2B). The ATP levels in control groups (0 µM) in the absence and presence of laser therapy remained higher, compared to treated cells (Figure 2B). Also, there is no statistical significance in the riboflavin concentrations at 20 µM and 40 µM, compared to the controls, indicating that most cells are still viable at these treatment conditions. ATP levels begin to drop at 80 µM in both treatment groups, with a complete shutdown in cell viability seen at 320 µM. Both cells treated with riboflavin alone and those treated with riboflavin combined with laser therapy show significant changes in ATP levels as the concentration of the photosensitizer increases. However, a slightly greater decreasing trend in ATP levels is observed in cells treated with both the photosensitizer and laser therapy, suggesting that this combination enhances cytotoxic effects more than riboflavin alone.

3.4 Reactive Oxygen Species Analysis

The DCFDA assay is used to quantify the amount of reactive oxygen species produced by A549 lung cancer cells before and after treatment. DCFDA is a non-fluorescent dye that would enter the cells and, upon its oxidation by reactive oxygen species, will result in the formation of a fluorescent detectable compound DCF (2',7'-dichlorofluorescein) [9]. The resulting fluorescence is proportional to the levels of reactive oxygen species present. In the assay, a dimethyl sulfoxide (DMSO) control was included as it was the solvent utilized for dissolving riboflavin. The cells treated with both riboflavin and laser therapy resulted in a significant increase in reactive oxygen species production compared to non-treated cells (Figure 3). Also, there was no significant increase in reactive oxygen species production when cells were treated with riboflavin alone. Therefore, laser treatment with the photosensitizer increased the amount of reactive oxygen species, thus contributing to oxidative stress within the cell.

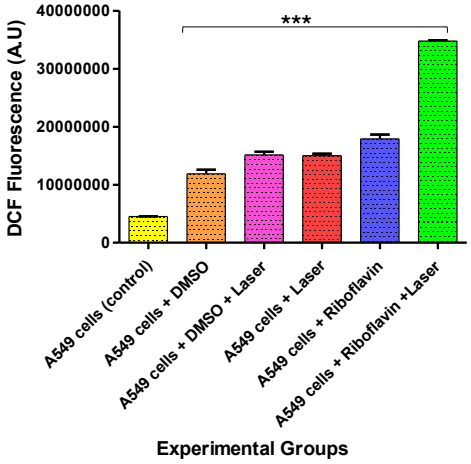

Figure 3: Cellular reactive oxygen species detection using DCFDA assay. The data is represented as mean ± standard error of the mean for each group at $n = 3$. Statistical significance assessed using one way ANOVA is depicted as *** $p < 0.001$.

3.5 Drug Co-localization

Drug co-localization study using Mito-tracker resulted in the generation of fluorescent images in Figure 4 that show the DAPI (a nucleus marker), mitochondrial marker, riboflavin, and the merged images (from left to right in Figure 4). The visible fluorescent signal in the merged image confirms the co-localization of riboflavin in the mitochondria. Mitochondria are known as an organelle that generates most of the cell's ATP. Since riboflavin co-localizes in the Mitochondrion, this correlates with the observed decrease in ATP levels following treatment, suggesting that riboflavin may directly impair mitochondrial function and thereby reduce cellular ATP production and cell survival.

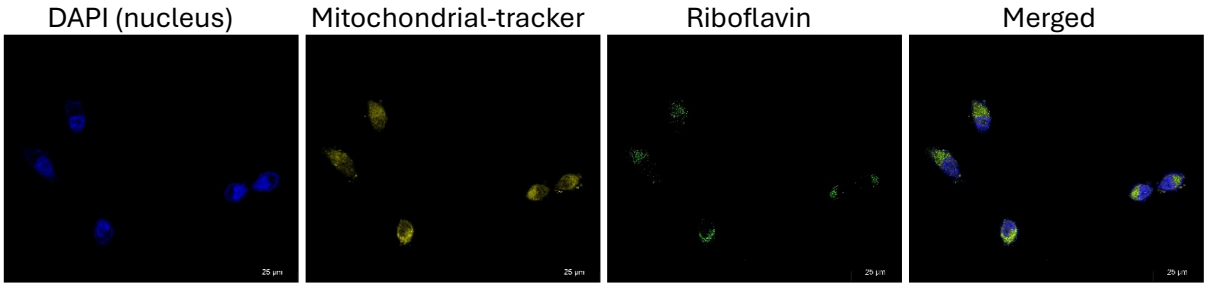

Figure 4: Intracellular (mitochondrial) localization of riboflavin in A549 lung cancer cells. The merged image shows the successful uptake of the photosensitizer (riboflavin) by the mitochondria.

4 Conclusion

Riboflavin is a naturally occurring vitamin that has gained attention in biomedical research as a photosensitizer for use in PDT [10]. In this study, the cytotoxic effects of riboflavin in the presence and absence of 470 nm laser therapy at 5 J/cm^2 against A549 lung cancer cells were demonstrated. In the control groups, no cell damage occurred. When cells were subjected to treatment using PDT and riboflavin at high concentrations, enhanced cytotoxic effects were observed on A549 lung cancer cells, as compared to treatment with riboflavin alone. The changes were exhibited by cells losing their shape, loss of structural integrity, and changes in morphology. Biochemical assay results correlated with the morphological analysis, and these were marked by an increase in LDH levels, a drop in ATP levels as cells lost their viability, and a substantial increase in reactive oxygen species levels was observed. In addition, riboflavin localizes in the mitochondrion, indicating this organelle as one of the targets of PDT-induced cytotoxicity. These findings suggest that riboflavin-mediated PDT might have potential as an anticancer treatment against lung cancer. While this study demonstrates potential, further research is required to enhance the efficacy of riboflavin at lower concentrations to minimize the need for the high doses used.

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