Time Dependent Effect of 415 nm Blue Light and Piperine on A549 Lung Cancer Cells

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Abstract

The burden of cancer is increasing worldwide with lung cancer ranking the first in males. The main downside of the existing treatments is inability to differentiate between healthy and cancerous cells. As a result, destruction of healthy cells occurs in addition to the cancerous cells. So, the alternatives that can affect only cancerous cells should be explored. These studies included one such phytochemical, piperine, in addition to 415nm blue light to increase the efficiency. We found that the IC50 of piperine was 34µg from the dose response analysis and further it was used for blue light treatment. These findings demonstrated that blue light radiation could enhance piperine induced cell death of lung cancer cells, suggesting light radiation may be an efficient enhancer of piperine in the management of lung cancer.

Keywords: *Cancer, Piperine, Cytotoxicity, MTT, cell viability*

1. Introduction

Cancer is a disease that causes pathophysiological changes in the natural process of cell division. It has become a major health issue that kills a lot of people every year all over the world [1, 2]. It is anticipated that 1.46 million cancer cases in India in 2022 will rise to 1.57 million cases in 2025. There were 1,03,371 instances of lung cancer in 2022, and it ranked among the top five major causes for both men and women [3].

Lung cancer is classified into non-small-cell lung carcinoma (NSCLC), small-cell lung carcinoma (SCLC), mesothelioma, sarcoma, and carcinoid based on the histological evaluation. There are additional varieties of lung cancer, but they are uncommon. SCLC and NSCLC account for approximately 90% of all cases of lung cancer [4].The primary risk factor for lung cancer is tobacco use, accounting for 80% of deaths from the disease.

Genetics and exposure to environmental risk factors are the next most common causes of lung cancer-related deaths [5, 6]. The cornerstone of the current treatment regimens has been traditional methods including chemotherapy, radiation, and surgical resection, either alone or in combination [7].However, there are several reports of significant side effects while using these conventional therapy techniques. Furthermore, radiotherapy clearly damages the surrounding healthy cells, thus it shouldn't be the first choice for patients whose pulmonary systems are already badly weakened because it could cause the lungs to stop functioning [8]. Recent times have also seen the use of radiation in conjunction with chemotherapy and/or surgery. Chemotherapeutic drugs are non-selective, which means that they often cause unwanted side effects in the surrounding normal cells. This is one of the main causes of the high death rate among patients with tumours [9]. Therefore, it is obvious that ongoing research is required to enhance efficiency and reduce the cost of cancer treatment by developing safer and more effective chemoprevention and treatment methods. Natural phytochemical substances are being used in cancer chemoprevention, a new method to stop, slow down, or possibly cure cancer[10].It has been established that certain plant bioactive substances, including as resveratrol, piperine, genistein, curcumin, and tea polyphenols, have anticancer properties [10, 11].

Because of its various modes of action, piperine is one of these natural chemicals that has garnered a lot of attention. Piperine, also known by its chemical name 1-[5-[1,3-benzodioxol-5-yl]-1-oxo-2,4-pentadienyl] piperidine, is an alkaloid molecule that contains nitrogen and has a rich historical background. It was initially isolated from the dried fruit extract of pepper by the Danish chemist Hans Christian Orsted in 1820. It was then observed to be a yellow crystalline solid with a molecular weight of 285.33 g/mol and a melting point of 128–130 °C[12]. Conjugated aliphatic chains, which act as a link between the piperidine and 5-(3,4-methylenedioxyphenyl) moiety, make up the chemical structure of piperine [13].Because of piperine's special ability to target diseased cells while sparing healthy ones, it presents a strong candidate for cancer therapy and solves the problem of separating cancerous from healthy cells, which is a common problem with traditional treatments [14, 15].

Visible light (400–780 nm), which represents more than 50% of the solar spectrum, is generally considered non-toxic to cells. In cancer photochemotherapy, which includes photodynamic therapy (PDT) and photoactivated chemotherapy (PACT), intense visible light is used to induce a drug response in cancer cells, whereas a minimal chemotherapeutic response occurs in the dark [16]. The goal of this study was to check the effect of piperine on lung cancer cells in presence of the 415m blue light.

2. Materials and Methods

Piperine (Sigma Aldrich), Fetal bovine serum (FBS; Gibco), Dulbecco's modified Eagle medium (DMEM) (Sigma D7777), penicillin/streptomycin (Sigma, P4333), amphotericin B (Sigma, A2942), trypan blue (Sigma Aldrich, T8154), phosphate-buffered saline (PBS), Hank’s balanced salt solution (HBSS) (Invitrogen, 10–543 F), Opti-MEM (Gibco) and TrypLE Express were used for the present study.

Preparation of piperine

Piperine ($C_{17}H_{19}NO_3$, M.W. 285.34, 97% purity; Sigma Aldrich (P49007)) was used without further purification. A stock solution (500µg/ml) was prepared in dimethyl sulfoxide (DMSO) and stored in the dark at –20°C.

Cell culture

A549 lung cancer cells used in this study were purchased from ATCC (ATCC® CCL-185). A549 cells were maintained and cultured in DMEM medium complemented with 10% FBS, 0.5% penicillin/streptomycin, and 0.5% amphotericin B and incubated at 37°C in 5% CO_2 and 85% humidity. When the cells in the culture dishes were 80% confluent, the spent media were discarded, cells washed twice with HBSS and detached with TrypLE Express at a ratio of 1 ml/25 cm^2. For the biochemical analysis, approximately 2 X 10^5 cells were seeded in culture plates of diameter 3.4 cm^2 with 3 mL complete medium and incubated for 4 h to attach.

Dose response of Piperine in A549 cells

A stock solution of piperine(500µg/ml) was prepared in DMSO and stored at -20°C. Further dilutions were made in culture media to obtain the desired concentrations as per the experimental design. For dose-dependent analysis, A549 cells were administered with increasing doses of piperine (1, 5, 10, 20, 40, 80, 160, and 320 µg/ml) for 24 h.

Analysis of cytotoxicity by MTT Assay

Human lung cancer cell line A549 was procured from the National Centre for Cell Science (NCCS), Pune (India). The cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Gibco) and antibiotic penicillin/streptomycin (Sigma, P4333). The cell line was maintained at 37°C in a humidified atmosphere of 5% CO_2.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cell viability. Adhered cells were trypsinized and seeded in 96-well plates after detaching from the surface. After allowing the cells to adhere for 24 h, they were treated in triplicate with the relevant drug concentrations for another 24 h. Each well was filled with 0.5 mg/ml MTT and covered with aluminium foil. For 4 h, plates were incubated at 37°C. Following the incubation period, the culture medium was withdrawn from each well and dimethyl sulfoxide (DMSO) was applied to dissolve the blue-purple formazan crystals. At 570 nm, the absorbance of the blank, control, and treatment wells was measured on a microplate reader.

Effect of blue light on A549 cancer cells

The photocytotoxicity of blue light irradiation was assessed in human lung cancer cell lines according to the following method. Cells from the general culturing conditions were detached by trypsinization, DMEM complete was added for trypsin deactivation, and cells were pelleted by centrifugation. The cell pellet was re-suspended in Opti-MEM (without phenol red) supplemented with 2.4% v/v FCS, 0.2% v/v P/S, and 1.0% v/v GM (Opti-MEM complete). Cells were stained using a 1:1 ratio of cell suspension: trypan blue, counted using a BioRad TC10 automated cell counter, and diluted to the appropriate seeding density. All cells were seeded at 0 h, irradiated at 48 h, and assayed at 96 h. After seeding, cells were incubated in the dark for 24 h under a humidified, 37 °C atmosphere containing 7.0% CO_2. After 24h incubation, 100µL of Opti-MEM complete was added as a mock photochemotherapeutic drug treatment; the cells were incubated for an additional 24 h.

To compare experimental growth curves after blue light irradiation to that in the dark, cells were seeded at t = 0 and an additional 100µL of media was added at t = 24 h (mock treatment). At t = 48 h, cells were irradiated or left in the dark as control. The cell viabilities were determined using trypan blue assay.

Analysis of cellular metabolism by ATP proliferation assay

Luminescent ATP Detection Assay Kit (Abcam, ab113849, India) was used to detect the total amount of cellular ATP. The ATP assay is based on the production of light caused by the reaction of ATP with added firefly's luciferase and luciferin. The emitted light is proportional to the ATP concentration inside the cell. Briefly, the grown cells are treated with piperine and incubated at RT with detergent followed by incubation with substrate at RT for 5min and measuring the luminescence with luminometer.

Statistical analysis

Lung cancer cells, from passages 16–20, were employed for the present study. All the experiments were carried out at least 3 times (n = 3) and the results were statistically analysed by SigmaPlot version 13 using the student's paired t-test. Values are represented as mean± standard error (SE) at a statistical significance of $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

3. Results

The light inverted microscopy investigations showed the noticeable A549 cells death by piperine and as shown in Figure 1. The morphology of cells after the piperine treatments showed properties of apoptosis. Compared with control cells, the morphology of treated cells after the 24 h incubation period changed significantly. The number of dead floating cells and the cell debris were more after the piperine treatment. The apoptotic features such as membrane damage, change is shape of the cells and detached from the culture plate were observed in the treated cells.

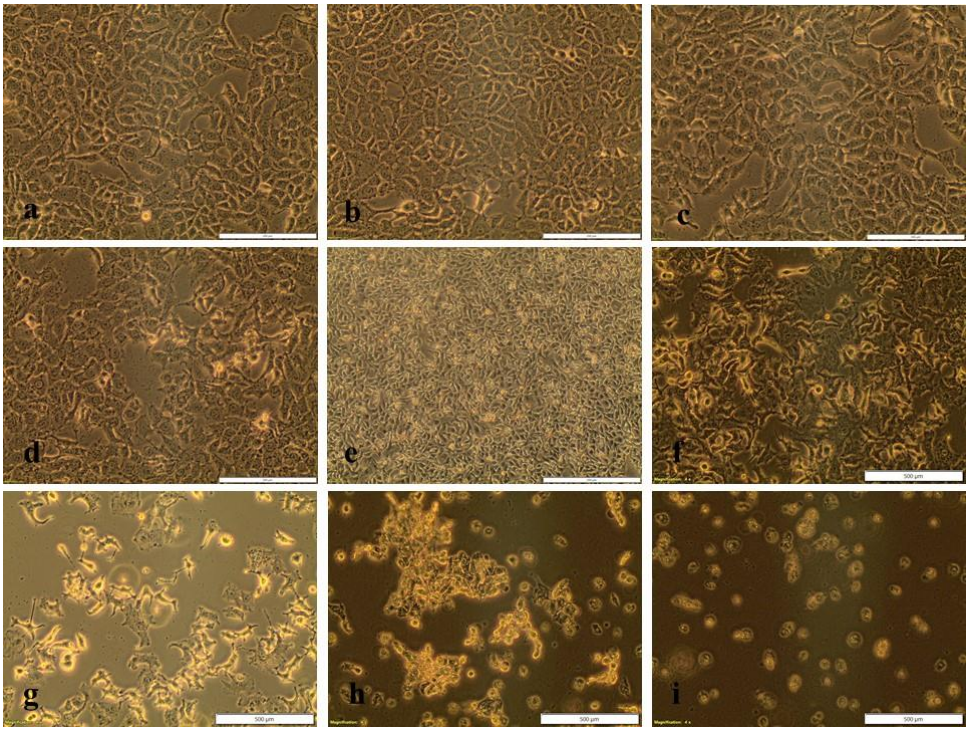

Figure 1: Effect of piperine treatment on morphological changes

Analysis of cytotoxicity by MTT Assay

In the present study, we used MTT cell viability assay to evaluate the antiproliferative potential of piperine at different concentration. We determined that piperine exerted a remarkable reduction in the number of viable cells in a dose-dependent manner (

Figure **2**). Notably, A549 cells were sensitive to piperine with IC_{50} value of 34µg). Based on these results, piperine at concentration of 34µg was employed for further assays [17].

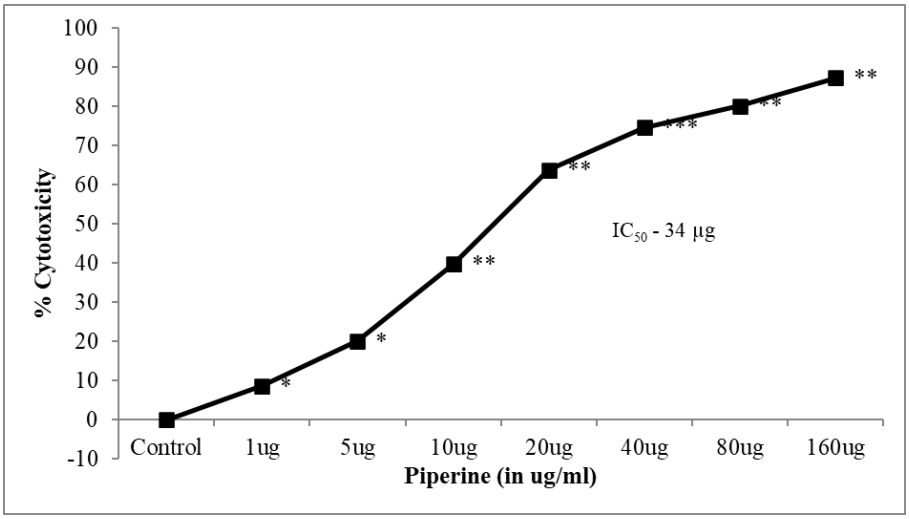

Figure 2: Cellular viability analysis by MTT assay

Effect of blue light on A549 cancer cells

We determined the effect of blue light radiation on cytotoxicity of the A549 cells using trypan blue assay [18]. It is based on the principle that viable cells have an intact cell membrane, which blocks the TB dye from entering the cells. Cells with compromised membrane integrity are stained blue. This assay represents a quick and inexpensive method to assess cell death, but it can also reflect cytotoxicity, resulting from damage to cell membrane integrity [19].

The cells were exposed to blue light at different times (0, 5, 15, 30, and 60 mins). Figure 3 shows the results of the cytotoxic effect as assessed 24 h after light radiation. Light dose response curves were observed. The results showed that cytotoxicity by blue light radiation was greatly affected by irradiation time, and the killing levels of A549 cells were proportional to light radiation times (**Figure 1**Figure 3). These demonstrated that blue light radiation could effectively enhance cell death of the A549 cells.

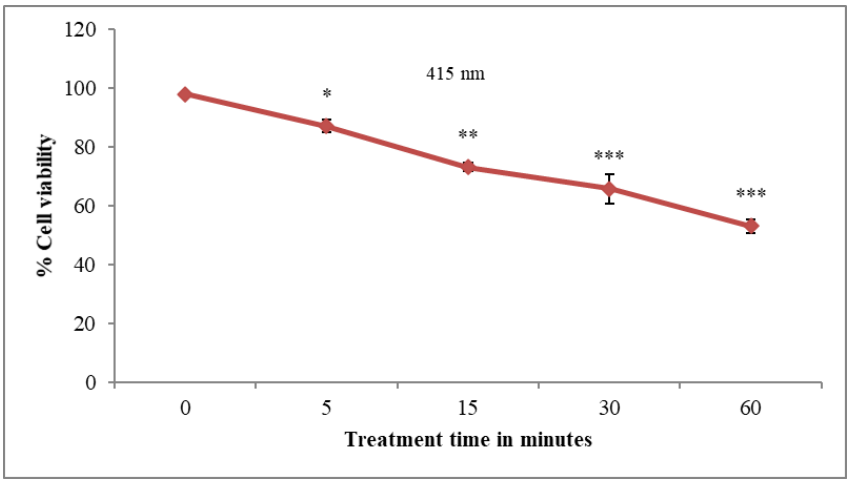

Figure 3: Effect of blue light (415nm) on cell viability

Analysis of cellular metabolism by ATP proliferation assay

The ATP assay is a sensitive method for measurement of cell viability because ATP is generated by cellular respiration and used as energy source in multiple processes in cells. Cells with damaged membrane lose the ability to synthesize ATP and endogenous ATPases rapidly deplete any remaining ATP from the cytoplasm.

We determined the effect of blue light radiation on piperine induced cytotoxicity of the A549 cells using MTT assay. The cells were incubated with piperine (IC_{50}, 34µg) for 4 h and then exposed to blue light at different times (0, 5, 15, 30, and 60 mins). Figure 4 shows the results of the cytotoxic effect as assessed 24 h after light radiation. Light dose response curves were observed. The results showed that piperine induced cytotoxicity of the A549 cells was significantly enhanced by blue light radiation. These demonstrated that blue light radiation could effectively enhance piperine induced cell death of the A549 cells.

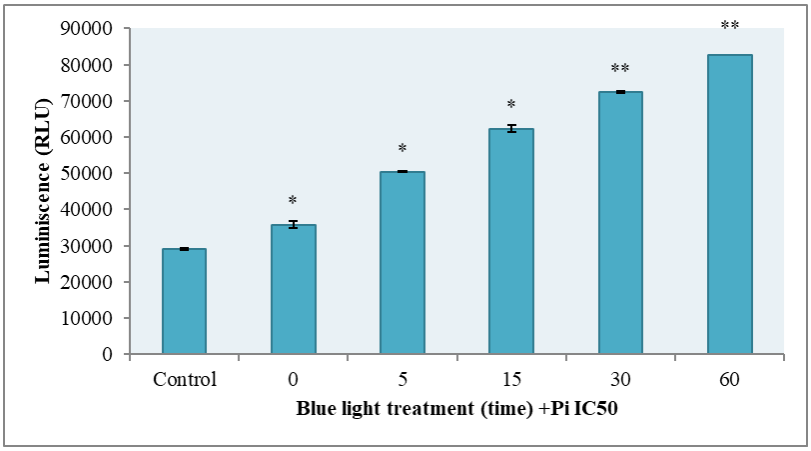

Figure 4: Effect of blue light (415nm) with piperine (IC50, 34µg) on A549 cells

4. Discussion

Recent developments in cancer treatment includes use of blue light as it is nontoxic healthy cells. Typically, light-induced drug activation occurs via generation of lethal oxidative stress (in PDT), or release of a caged compound that becomes cytotoxic (in PACT). Several considerations must be taken into account when testing photopharmaceuticals in vitro, such as cell type, light sources, cell environment, and cell counting assays. In addition, several of the endogenous photosensitizers (flavins, porphyrins, bilirubin, and melanin) that mediate oxidative damage by UVA irradiation, also strongly absorb high-energy visible light (HEVL, 400–500 nm). Consequently, cytotoxic effects of visible light may occur even in the absence of any photopharmacologically active compound [16].

Natural products especially the herbal products induce the cancer cell death by induction of apoptosis, alteration of cell cycle and by modifying the various signalling pathways. The major problem with the currently available chemotherapeutic agents is the side-effects, which kill both cancerous and normal cells since they are not selective. In light of this, the use of plant derived agents and low-level laser beams to kill cancer cells is a more promising area of cancer research thereby reduces the adverse effects of cancer treatment [20].So, in this study, the effect of one such phytochemical, piperine, is analysed in combination with the radiation of 415nm blue light.

From dose response analysis of piperine on A549 clung cancer cell line, it became obvious that the increasing dose of piperine resulted in increased cytotoxicity. This investigation goes in line with the findings of Lin et al, that the piperine can inhibit cell proliferation and apoptosis in A549 [21]. Based on the dose response analysis of piperine, the IC_{50} value was found to be 34µgin A549 lung cancer cells from an MTT assay. So, for further studies on A549, cells were treated with 34µg.

In this study, we determined that the greatest cytotoxic effects of piperine occurred in A549 cells when the treatment is combined with radiation of 415nm blue light. Further the time for which the radiation of 415nm blue light given to the A549 lung cancer cells also affected the cytotoxicity. As the duration of irradiation increased, significant increase in cytotoxicity was observed from cell viability by trypan blue assay.

The mechanism of inhibition of cell proliferation and apoptosis by piperine can be due to expression of high level of p53 by piperine. p53, one of the tumour suppressor proteins, that plays a crucial role in inducing cell cycle arrest in the G2/M phase and can also induce apoptosis in cancer cells [22]. Thus, a high level of p53 expressed in the presence of piperine could induce cell cycle arrest in the G2/M phase in A549 cells. Studies have revealed that piperine-treated A549 cells can show an increased level of Bax proteins and a decreased level of Bcl-2 proteins leading to a high Bax/Bcl-2 ratio, which further helps in inducing apoptosis in A549 cells [21]. Several other important proteins involved in the action of p53-mediated apoptosis are members of the caspase family [23]. Piperine can induce the activation of caspase-9 and caspase-3 in A549 cells, and thus these two proteins can induce p-53-mediated apoptosis in A549 cells. Thus, these are some signalling pathways through which piperine can inhibit the cell growth, differentiation, and induce apoptosis of lung cancer cell.

In summary, the cancer incidence burden is continuing to increase in India. Among the top five cancers in males, lung cancer was found to be the highest. It was evident from the research undertaken that piperine is having greatest cytotoxic effects in A549 lung cancer cells. The cytotoxicity is even enhanced when the piperine is combined with the 415nm blue light. As both of these piperine and 415 blue light are as such nontoxic to normal cells, the treat offers a good advantage of only targeting the cancerous cells. So, it can be a good choice for the future cancer treatments.

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