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Original Article

Synergistic anticancer activity of resveratrol with cisplatin and carboplatin in A549 lung adenocarcinoma cells

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Abstract: Background: This study looked at the efficacy of combining the phytochemical resveratrol with the anticancer drugs cisplatin and carboplatin on lung adenocarcinoma cell lines. Materials and Methods: We used MTT assay and generation of Reactive Oxygen Species levels using molecular fluorogenic probe 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) to investigate the effects of resveratrol in combination with cisplatin and carboplatin on the proliferation and viability of cells and levels of reactive oxygen species (ROS). Results: Resveratrol has an anti-proliferative effect on A549 lung cancer cells, inhibiting cell proliferation in a dose and time-dependent manner. Resveratrol in conjunction with cisplatin and carboplatin inhibited cell proliferation synergistically. The combination therapy of cisplatin and carboplatin with Resveratrol showed enhanced growth inhibition of lung cancer cells in *in-vitro* with IC50 values of 15.09 ± 0.71 μM and IC50 values of 21.72 ± 1.9 μM, respectively. The present investigation also revealed the significant dose-dependent ROS generation in A549 cells by cisplatin, carboplatin, and their combination with resveratrol. Carboplatin treatment in combination with Resveratrol induced a higher generation of ROS (3.4-fold) when compared to carboplatin treatment (2.4-fold) at the highest concentration. Conclusions: Our findings offered a basis for further research for assessing the potential of Resveratrol as a therapeutic agent to treat lung adenocarcinoma and whether it can be used as an adjuvant with drugs like cisplatin and carboplatin for improving their efficacies. However, the underlying processes of cell inhibition and cell death should be thoroughly investigated.

Keywords: Chemopreventive, lung cancer, natural products, resveratrol, cisplatin, carboplatin, therapeutic

Introduction

Lung cancer is a malignancy that is currently the top cause of cancer-related mortality globally. Lung cancer mortality now outnumbers those from colorectal, prostate, brain, and breast carcinomas combined. It is now the single most widespread cause of mortality due to cancer in males and the second most prevalent in women [1]. Non-small cell lung cancer (NSCLC) refers to several types of lung tumors, including adenocarcinoma, large cell carcinoma, and squamous cell carcinoma. Adenocarcinoma is among the most frequent type of lung cancer, that accounts for approximately fifty percent of all NSCLS [2]. Individuals with NSCLC have a poor prognosis along with a low 5-year overall survival (OS), which is roughly 17.4% [3]. The primary treatment options for lung cancer are radiotherapy, surgery, and chemotherapy. These treatments have substantial side effects and can cause discomfort [4]. Platinum-based chemotherapy continues to be the preferred course of care for most patients with advanced NSCLC. Cisplatin and carboplatin are the platinum compounds currently utilized to treat NSCLC. Cisplatin is a well-known chemotherapy agent, which has been utilized for treating a variety of tumors, including lung, bladder, ovarian, testicular, as well as head, and neck cancer. It works well over a wide range of tumors, including sarcoma, carcinomas, lymphomas, and germ cell tumors. Cisplatin chemotherapy is a powerful treatment, however, it has certain drawbacks related to drug resistance or multiple organ damage [5]. Cisplatin and carboplatin trigger a response involving mitochondrial reactive oxygen species (ROS) that contributes to its cancer cell-killing effects. This process is influenced by the mitochondrial

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redox balance and the cell's energy-producing activities [6]. However, there can be resistance to drugs and a variety of unwanted side effects, including gastrointestinal disorders, significant kidney issues, decreased immunity to infections, allergic responses, hemorrhage, and hearing loss, particularly in younger individuals. In addition, combination therapy of cisplatin along with different medications has been widely investigated to overcome resistance against drugs and minimize toxicity [7]. Carboplatin, a cisplatin derivative, has far less nonhematologic toxicity, though myelosuppression may be slightly more than that reported with cisplatin. New combination chemotherapy regimens using carboplatin might enhance longevity in small-cell lung cancer patients and, in certain cases, cure those who have limited conditions. Further research on carboplatin and other novel agents is required [8].

Natural products have been widely used for centuries to prevent a variety of ailments, including carcinomas [9, 10]. The renewed interest in phytochemicals derived from dietary plant products has offered a substitute for substances that are bioactive and may be utilized to prevent or treat various ailments [11, 12]. Phytoestrogens are demonstrated to impact several cellular signaling pathways while causing zero or minimum damage to normal cells [13]. Chemoprevention is the utilization of products for the prevention or delaying the development of cancer [14], and there is growing interest in natural substances as potential therapeutic and chemopreventive medicines for human beings. To increase the efficiency of cancer treatment, combined therapy is used, which includes combining drugs with polyphenols that synergically interact with classic chemotherapeutics [15]. Resveratrol (3,4',5-trihydroxy-trans-stilbene), a non-flavonoid polyphenol, is a phytoalexin found naturally in numerous plants, such as peanuts, berries, pines, and grapes [16]. Resveratrol's structure is stilbene-based, which exists in both cis- and trans-isoforms. The trans-isoform is the most well-studied chemical form [17]. Numerous biological effects of Resveratrol, such as being anti-viral, anti-inflammatory, anti-aging, and anti-cancer properties, have been demonstrated [18]. Resveratrol is gaining popularity because of its cancer-preventing and anti-cancer capabilities [19, 20]. Resveratrol has been found *in vitro* to have cytotoxic effects on a variety of human cancerous cells, such as lymphoid and myeloid cancer cells, as well as thyroid, breast, colon, cervix, stomach, skin, prostate, liver, ovary, and pancreatic carcinoma cells [21, 22]. A synergic impact occurs when the combined effect of the two treatments given simultaneously is greater than the overall effect of each drug administered individually [23]. As a result, novel approaches, such as combining natural products with chemotherapeutic drugs, are required to conquer resistance to cisplatin or to sensitize cancer cells to cisplatin. Several studies have indicated that combining natural substances with chemotherapeutic drugs could enhance their sensitivity and cytotoxicity [24].

In this study, the viability and ROS activity of cells in A549 lung adenocarcinoma cell lines were determined and we gained a better knowledge of resveratrol's harmful effect on cancer cells. The synergistic activity of the chemotherapeutic drugs, cisplatin and carboplatin with resveratrol was also studied using a cell viability assay. The IC50 (50% inhibitory concentration) of resveratrol was determined in NSCLC cell lines using cell viability assay. The generation of reactive oxygen species in living cells was determined using the molecular fluorogenic probe 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA).

Methodology

In-vitro A549 cell culture

A549 cells (human lung adenocarcinoma cell) were cultured in Dulbecco's Modified Eagles Medium (DMEM) in *in-vitro* conditions supplemented with 10% Fetal Bovine Serum (FBS), 3.7 g/L Sodium bicarbonate and 1% pen-strap antibiotic solution. The culture was maintained in a CO_2 incubator with a continuous supply of 5% CO_2 at a temperature of 37°C.

Cytotoxicity assay on A549 cells

After harvesting, 1×10^4 A549 cells/well were seeded on 96 well plates and incubated for 24 hours at 37°C with 5% CO_2. After 24 hours, cells were treated with varying concentrations of Resveratrol (starting from 100 μM concentrations), standard anticancer drugs, and in combination with cisplatin and carboplatin (at constant 5 μM concentration). The cells in the

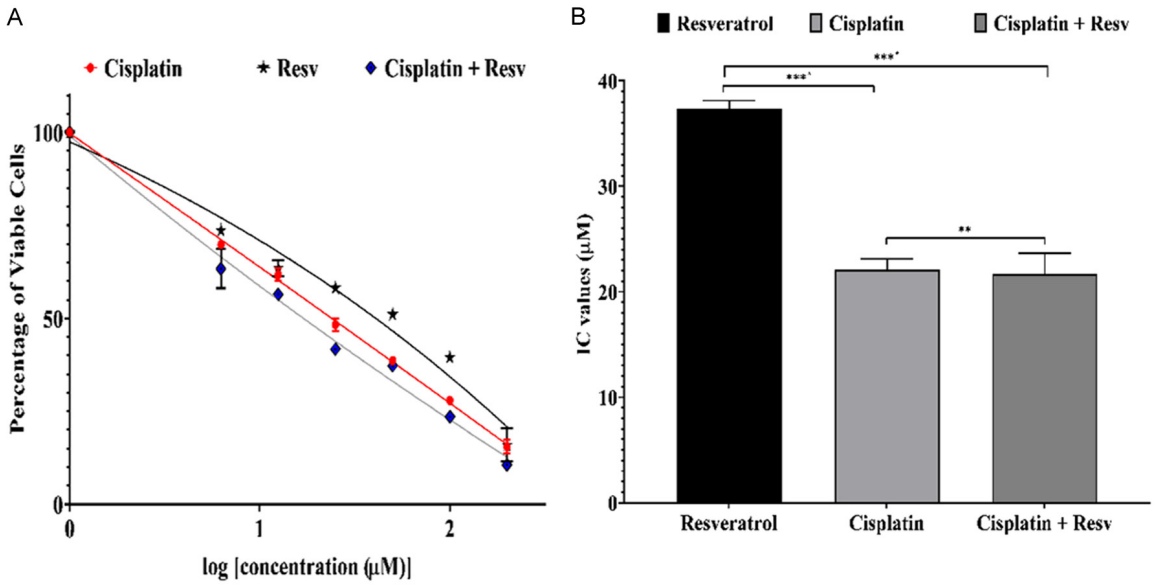

Figure 1. Cisplatin treatment on A549 lung adenocarcinoma cells in combination with Resveratrol: (A) Percentage of viable A549 cells on treatment with varying concentrations of Resveratrol (Resv); and (B) IC50 values of test compounds, and in combined with cisplatin. Significance levels with $P \leq 0.05$ was considered as statistical significance and represented as **, and ****.

well without any treatment were considered as positive controls. The plates were then incubated at 37°C for 48 hours inside the CO_2 incubator.

The MTT assay was performed by replacing the existing compound and medium in 96 well plates with 90 µl fresh incomplete DMEM with 10 µl of 5 mg/ml MTT solution prepared in PBS. The plates were then incubated at 37°C for 3 hours, and the formation of formazan crystals was solubilized in DMSO via incubation for 30 minutes. The plates were then read in a microplate reader at 570 nm.

Generation of reactive oxygen species using a molecular probe

A cell density of 1×10^4 cells/well was seeded on 96 well plates and incubated at 37°C, a day before the experiment. After 24 hours of incubation, adhered cells were washed gently with 100 µl 1× PBS. After washing, cells in wells were treated with 80 µl of H_2DCFDA (10 µM) and incubated for 45 minutes at 37°C. The wells were gently washed to remove excess H_2DCFDA with 1× PBS. The cells were then treated with compounds and drugs with varying concentrations (same concentrations as cytotoxicity) and incubated at 37°C for 6 hours. Cells incubated with 50 µM H_2O_2 served as a positive control. After incubation, the fluorescence was measured under the fluorometer at 485 nm excitation and 520 nm emission on a Synergy H1 hybrid (BioTek, USA) mode multiplate reader.

Statistical analysis

All the experiments were performed in triplicate. GraphPad Prism 8.0.1 was used for nonlinear regression analysis and One-way ANOVA analysis for statistical significance. $P \leq 0.05$ was considered as the level of statistical significance.

Results

Cytotoxicity assay on A549 cells

In the present study, the cytotoxic effect of the natural compound Resveratrol was investigated on A549 lung adenocarcinoma cells (**Figure 1A**). The investigations revealed higher cytotoxicity shown by cisplatin (IC50 = 22.12 ± 0.98 µM) followed by Resveratrol (IC50 = 35.05 ± 0.1 µM) (**Table 1**). The combination therapy with different concentrations of Resveratrol with standard drugs showed synergistic effects with enhanced anticancer efficacy (**Table 2**).

Table 1. Anticancer efficacy of Resveratrol and standard drugs on lung adenocarcinoma cells

Compounds Treatment	IC50 Values in μM (Mean ± S.E.M.)
Resveratrol	35.05 ± 0.1
Carboplatin	156.9 ± 0.2
Cisplatin	22.12 ± 0.98

The combination therapy of cisplatin with Resveratrol showed higher enhanced growth inhibition of lung cancer cells in *in-vitro* with an IC50 value of 15.09 ± 0.71 μM, which is lower in comparison to the compounds being used alone. The synergistic effect of Resveratrol with carboplatin showed a significantly enhanced anticancer effect with IC50 values of 21.72 ± 1.9 μM (**Figures 1B**, **2**).

Generation of reactive oxygen species using a molecular probe

Resveratrol can induce reactive oxygen species in lung cancer cells and inhibit cellular growth by apoptosis. In this study, the generation of ROS level was measured using molecular probe H_2DCFDA dye involving the production of fluorescence under a fluorometer shown in **Figure 3A**, **3B** and **Table 3**. The study revealed, that at the highest concentration (100 μM), ROS generation by Resveratrol was 2.9 times fold greater to the untreated cells as shown in **Table 4**.

Cisplatin and carboplatin are well known for their formation of ROS inducing apoptosis in many cancer cell lines [25, 26]. In our study, cisplatin generated 3-fold times the fluorescence intensity to the control, revealing that cisplatin kills lung adenocarcinoma cells by generating ROS. The positive control cells treated with 1 μM H_2O_2 for 2 hours, showed 6.4-fold times the ROS generation in comparison with untreated cells. Further, in our investigation, the killing of lung cancer cells by cisplatin and carboplatin drugs was examined in combination with resveratrol along with their comparative study. The present investigation revealed the significant dose-dependent ROS generation in A549 cells by cisplatin, carboplatin, and their combination with resveratrol (**Table 1**).

The compound resveratrol generated reactive oxygen species in A549 lung adenocarcinoma cells significantly comparable to the standard anticancer drug cisplatin ($P = 0.0107$), suggesting the mechanism performed the killing of cancer cells by targeting mitochondrial redox homeostasis. However, the combination study of cisplatin treatment with resveratrol induced ROS generation to a lesser extent compared to resveratrol rather than a higher generation of ROS seen in cisplatin. Further, carboplatin treatment in combination with Resveratrol induced a higher generation of ROS (3.4-fold) when compared to carboplatin treatment (2.4-fold) at the highest concentration.

Table 2. Anticancer efficacy of natural compounds in combination with standard drugs on A549 lung adenocarcinoma cells

Combinations	Resveratrol
Cisplatin	15.09 ± 0.71
Carboplatin	21.72 ± 1.9

Discussion

In the present research, we looked at the synergistic effects of resveratrol along with cisplatin and carboplatin on A549 cells using cell viability assays and ROS activity. Our findings showed that resveratrol decreased the viability of A549 cells in a concentration-dependent manner. Furthermore, resveratrol acted synergistically with cisplatin and carboplatin on A549 cells. Resveratrol's anticancer effect is possibly based on its ability to promote apoptosis through autophagy.

Oxidative stress is a major cause of cancer [27]. Conventional chemotherapeutic medicines have been shown to induce oxidative stress in cancerous cells [28, 29]. ROS has the ability to react with DNA as well as chromatin proteins, causing various kinds of damage to DNA resulting in cell death [30, 31]. Excessive ROS in mitochondria can lead to damage of DNA and oxidative stress, resulting in depletion of energy, and stimulation of particular pathways that dictate cell fate [32-34]. Increased generation of ROS is a hallmark of cancer cells. ROS is a molecular mechanism triggered by hypoxia and it causes oxidative stress, which in turn causes tissue damage and apoptosis [35]. Generally, the levels of ROS in tumor cells are substantially greater than those in normal cells [36], hence oxidative stress generated by overproduction of ROS can efficiently kill tumor cells by

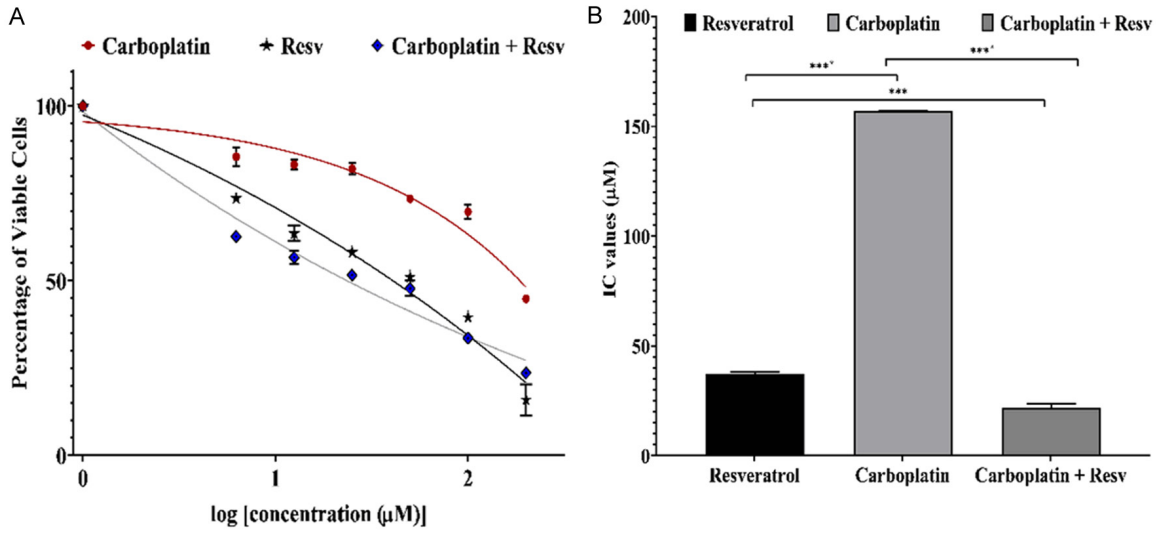

Figure 2. Carboplatin treatment on A549 lung adenocarcinoma cells in combination with Resveratrol (Resv): (A) Percentage of cell viability on treatment with different concentrations of Resveratrol; and (B) IC50 values of test compounds. $P \leq 0.05$ was considered for statistical significance and marked with ***, and ****.

raising it above the required level for cell death, as shown diagrammatically in **Figure 4**. In this sense, resveratrol and cisplatin's pro-oxidant-based induction of both necroptosis and apoptosis provides an additional benefit, for selective death of tumor cells and overcoming apoptotic resistance in lung adenocarcinoma (**Figure 4**). In a similar study conducted by Alayev et al., they found that resveratrol acts by inhibiting the mTOR pathway and by preventing activation of Akt in breast and human bladder cancer cell lines [37, 38]. In a different study conducted by Lee YJ et al., they found that the synergistic anti-proliferative impact of clofarabine along with resveratrol is associated with the suppression of Sp1 and Akt activities, which suggests that this combination might offer therapeutic benefit in treating malignant mesothelioma [39]. Akt helps in regulating many biological processes, like cell survival, growth, proliferation, and glycogen metabolism, and abnormal regulation of these activities is thought to be a hallmark of cancer [40]. Research conducted by Cocetta et al., found that Resveratrol and cisplatin together shows more effective inhibition of non-small lung cancer cell proliferation and induction of apoptosis than cisplatin treatment alone [18]. In a study conducted on ovarian adenocarcinoma SKOV-3 cells, it was observed that combination treatment of cisplatin with resveratrol can effectively reduce cell metabolic activity [41]. The lower generation of ROS by cisplatin and carboplatin when used synergistically with resveratrol can potentially be helpful in eliminating the side effects that occur due to these drugs. Resveratrol may improve the efficacy of conventional cancer therapy by additive or synergistic effects along with alleviating negative side effects. Additional research, particularly at the clinical level, is required to determine the prospective significance of Resveratrol as an adjuvant in chemotherapy for cancer.

These findings show that resveratrol could be an appealing supplementary component for chemotherapy for cancer, allowing for lower therapeutic doses of cisplatin and carboplatin while reducing their adverse effects. This can also be applied to all investigated anticancer medicines, whose cytotoxicity rises when combined with resveratrol.

Limitations and future perspectives

There are some limitations to the current investigation. To begin, this Resveratrol study was conducted only on A549 cells, and the mechanism by which it suppresses A549 cell growth was not investigated due to laboratory conditions. Our future research will focus on the effects of Resveratrol on different lung cancer cell lines. The mechanism by which Resveratrol along with cisplatin and carboplatin acts to

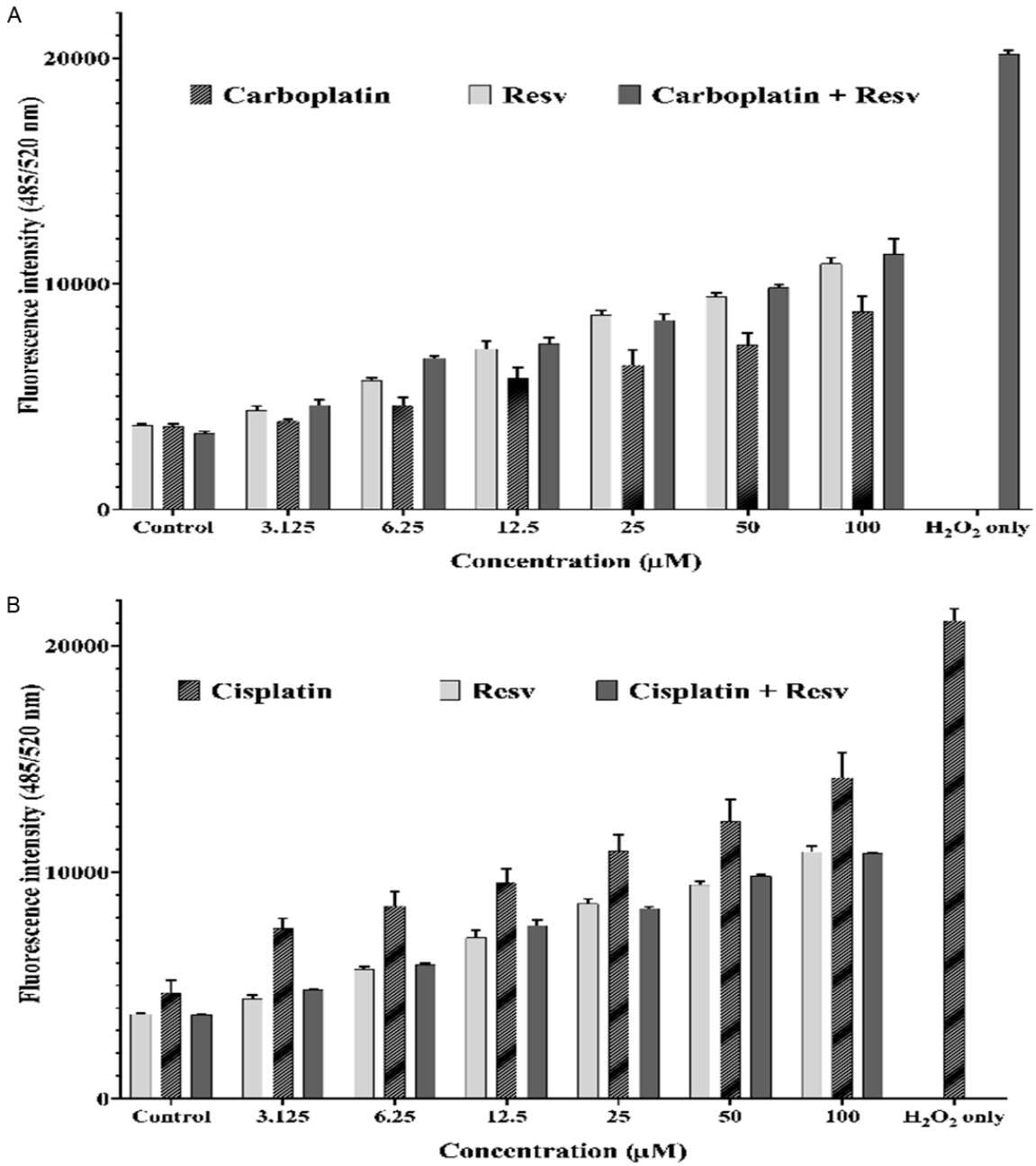

Figure 3. (A) Fluorescence intensity of reactive oxygen species after treatment with Resveratrol in combination with carboplatin; and (B) Fluorescence intensity of reactive oxygen species after treatment with Resveratrol in combination with cisplatin.

inhibit or kill cancer cells is not known which will also be explored in the future.

Conclusion

As the long-term objective of translational healthcare is to discover novel treatment options and enhance the overall health of the population, an increasing number of researchers are focused on natural product therapy in conjunction with chemotherapy. The performance of Resveratrol and the other data reported in the study opens new avenues for further investigation. Such research could lay the groundwork for the development of complex treatment techniques involving Resveratrol, a natural compound with several beneficial properties. Finally, our findings suggest

Table 3. Fluorescence intensity of DCFDA on treatment with different concentrations of compounds on A549 cell

Conc. (μM)	Resveratrol	Cisplatin	Cisplatin + Resv	Carboplatin	Carboplatin + Resv
Control	3735.8	4683.3	3706.5	3668.8	3361.5
3.125	4400.0	7532.5	4820.5	3917.5	4613.0
6.25	5711.0	8508.0	5932.0	4619.0	6709.0
12.5	7118.0	9561.3	7649.5	5825.3	7331.5
25	8610.3	10947.3	8403.0	6402.3	8380.0
50	9444.5	12231.8	9819.0	7306.0	9829.5
100	10905.3	14166.8	10821.5	8770.0	11318.5
H_2O_2 only	21102.8				

Table 4. Fold change in the intensity of DCFDA, the molecular probe in the generation of ROS

Conc. (μM)	Resveratrol	Cisplatin	Cisplatin + Resv	Carboplatin	Carboplatin + Resv
3.125	1.2	1.6	1.3	1.1	1.4
6.25	1.5	1.8	1.6	1.3	2.0
12.5	1.9	2.0	2.1	1.6	2.2
25	2.3	2.3	2.3	1.7	2.5
50	2.5	2.6	2.6	2.0	2.9
100	2.9	3.0	2.9	2.4	3.4
H_2O_2	6.4				

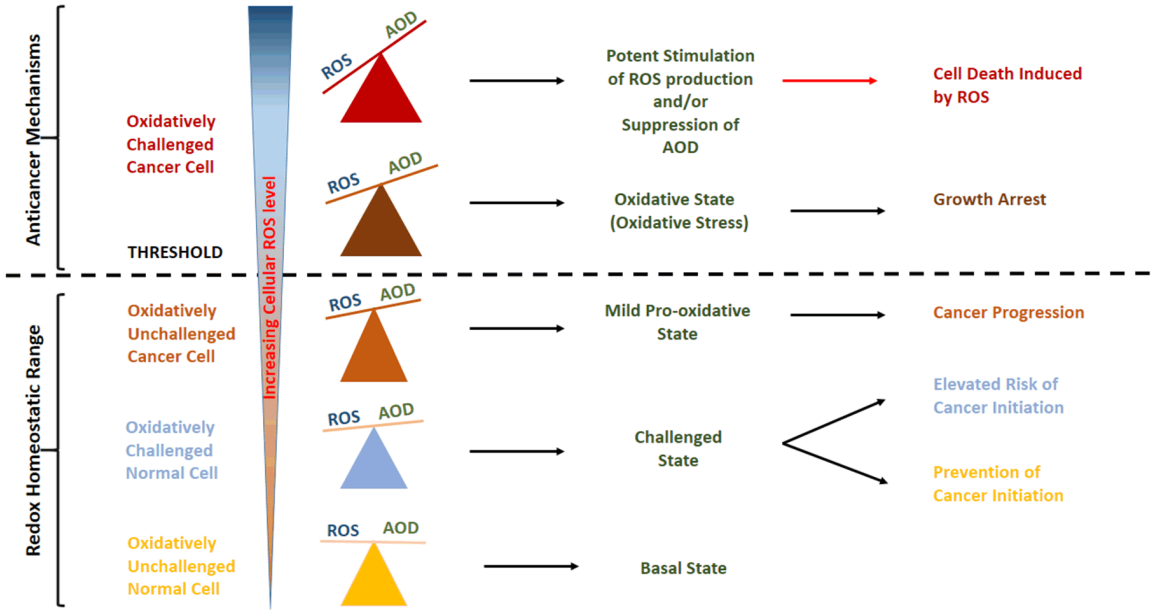

Figure 4. Reactive oxygen species (ROS) can cause cancer, growth arrest, and cytotoxicity. In normal cells, increased oxidative stress causes appropriate elevation of cellular antioxidant defence (AOD), preventing mutagenesis events and the onset of cancer formation. However, because AOD is not completely efficient, these "challenging states" are well-known risk factors for the development of cancer. Once established, progression of cancer appears to be accelerated by a modest pro-oxidative state caused by increased metabolism, ROS-producing cells, and so on. This state remains within the "redox homeostatic range" due to cancer cells' strongly increased AOD. However, due to increased AOD, cancer cells do not support additional increases in ROS levels and thereby cross the threshold into the condition of "oxidative stress". If ROS levels rise further (for example, because of chemotherapy), cancer cells can only avoid further harm by lowering ROS production through cell-cycle arrest to repair damage and prevent cell death. Nevertheless, if a ROS burst causes irreparable damage and/or there are insufficient components for repair mechanisms, cancer cells undergo programmed cell death or necrosis (cytotoxic effects of ROS).

that Resveratrol, in combination with cisplatin and carboplatin, produces synergistic cytotoxicity in lung adenocarcinoma cells via increasing ROS levels. ROS-induced damage to DNA is an upstream event that may be shared by apoptosis and necroptosis cell death mechanisms. Our findings offered a basis for further research for assessing the potential of Resveratrol as a therapeutic efficacy to treat lung adenocarcinoma and whether it can be used as an adjuvant with drugs like cisplatin and carboplatin for improving their efficacies. It may also be worth exploring as a treatment agent for the same. However, the underlying processes of cell inhibition and cell death should be thoroughly investigated.

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Disclosure of conflict of interest

The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

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