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Vitexin an Apigenin Flavonoid Induces Anti-proliferative and Apoptotic Effect in Human Laryngeal Carcinoma Cell Line Hep-2

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ABSTRACT

Vitexin, an Apigenin Flavonoid found in Mung bean, Bamboo leaves was showed to possess various biomedical properties against wide variety of diseases. Cisplatin, a familiar chemotherapeutic drug, used as a treatment for different types of cancers. However, the combined effect of VIT and CIS on laryngeal carcinoma remains unknown. So, in present study the Cytotoxicity assay through MTT reveals that Cells exposed to VIT, CIS individually, and their co-treatment synergistically inhibited cell proliferation with IC50 values of 10 µM for VIT and 5µM for CIS. Further Anti-Cancer effect of Combinational treatment over DNA fragmentation and HRBC assay suggests that an extremely significant depletion of RBC cells with Fragmented DNA was observed compared to individual treated cells and control. In conclusion, the current study indicates that the treatment of VIT combined with CIS in Hep-2 cells exerts potential Anti-Proliferative and Anti-cancer effect by inducing Mitochondrial mediated apoptosis against laryngeal cancer.

Keywords: Anti-proliferative; Anti-Angiogenesis; Apoptosis; Cisplatin; Laryngeal cancer; Vitexin.

INTRODUCTION

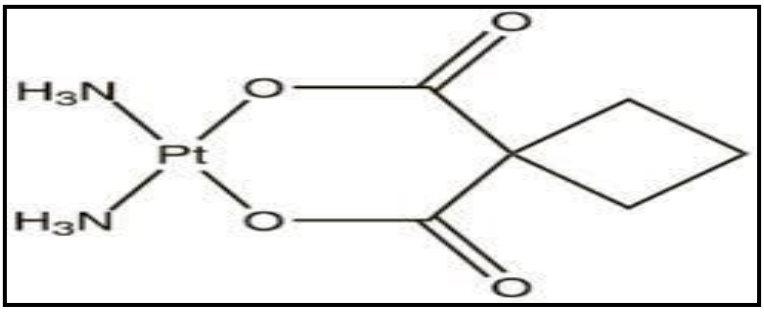
Laryngeal cancers represented as squamous-cell carcinomas, one of the most aggressive and prevalent tumors of the head and neck region, which systemically constitutes 1-3.5% of human malignant cancer cases worldwide, which may be considered to be a significant source of morbidity and mortality augmented in recent years, the epidemiological study states that one-third-of all head and neck cancers enormously occur in men and the eleventh-most prevalent type of cancer occurring wildly (**1**). Patients with a considerable history of smoking are more likely to have them identified, and more likely to develop malignancies in the other parts of the aerodigestive tract. They affect various laryngeal subsites, which may have an impact on how symptoms manifest themselves, how they spread, and how they are treated. While late-stage cancer has a poorer result, calls for multimodal therapy, and is less frequently larynx-preserving, the early-stage disease is highly curable with either surgical or radiation monotherapy, typically with larynx preservation (**2**).

Despite the progression in traditional, conventional, systemic treatment (Chemotherapy, cryoablation, Cisplatin) and diagnostic methods available for LCa, due to their drug resistance and toxicity effect, the limitation of cisplatin **(Figure 1A)** and related platinum-based analogs tend to show relapse with the cisplatin-resistant disease in the majority of patients, the overall existence rate of patients yet did not improve remarkably associated with the survival rate of wide cancer types patients (**3**). The above-mentioned drawbacks have provoked intensive research into cisplatin combination treatments, which are widely used clinically. So Currently, Combination therapies are becoming more familiar in the wide range of cancer treatments, including their potential to re-sensitize their resistant malignancies, potentiate their therapeutic effects, and reduce side effects without compromising their outcome (**4**).

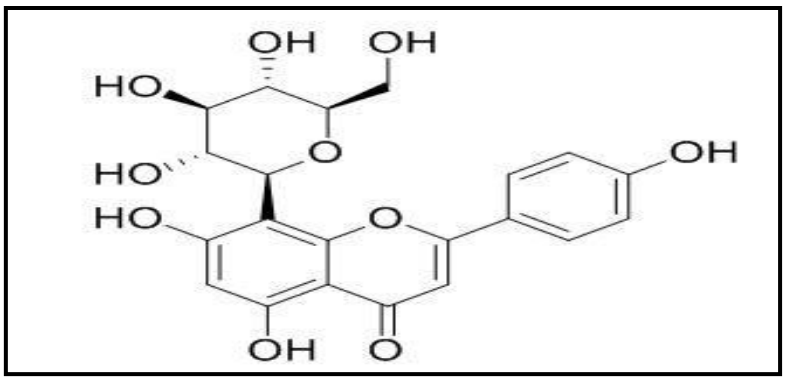
Therefore, in our present studies, herbal medicines or natural Phyto- therapeutic compounds are preferred, which are the major source for various complex diseases remedies from ancient times. Vitexin is one such apigenin Flavonoid found to possess in a glycosylated form significantly in various medicinal plants such as pearl millet, Mung bean, bamboo leaves, wheat leaves, passion flora, seeds of chaste berry, and fruits, tends to show a wide range of pharmacological properties including anti-cancer, anti-oxidant, anti-spasmodic, anti-convulsant, anti-tumor, anti-inflammatory, anti-angiogenic, anti-adipogenesis, anti-spasmodic, activities at various doses and

concentrations (**5**), and is considered as an option used either as a monotherapy or combined with conventional chemotherapeutic agents for various types of cancer to increase the sensitivity of cancer cells to drugs, In **(Figure 1 B)** VIT possesses seven hydroxyl groups with an O-Dihydroxyl structure in the A-ring proven to contribute effective bioactivities in a broad range of disorder (**6**).

Yet, the ultimate mechanisms of individual and Co-treatment of VIT and CIS on Laryngeal carcinoma remain indescribable and further studies were conducted for revealing the fundamental molecular mechanisms of laryngeal carcinogenesis necessary to enlighten the LCa pathogenesis in detail and develop more successful therapeutic modalities.



(A)



(B)

Figure 1: Chemical structure of (A) Cisplatin (B) Vitexin

MATERIALS AND METHODS

Chemicals

The Human laryngeal carcinoma (Hep -2) cell line was purchased from National Centre for Cell Sciences (NCCS), Pune. Vitexin and Cisplatin were procured from Sigma Aldrich. All other chemicals used were of high-quality grade.

Cell culture and maintenance

Human laryngeal carcinoma (Hep-2) cells were cultured in Minimal essential medium (MEM), with other supplements like a fetal bovine serum (FBS) (10%, v/v), 8,000 µg/ml penicillin, and 8,000 µg/ml streptomycin in a 25 cm2-culture flask and kept in a 5% CO2 incubator at 37°C with controlled humidified atmosphere and passaged for every 2 days, once the cells reached 90% confluency, they were trypsinized and it is utilized for further assays.

Anti-proliferative assay

Anti-proliferative effects of VIT and CIS individually and their combination treatment on cell viability were determined by MTT assay. Trypsinised cells ($_{2*10\^5}$) were treated with different concentrations (0 µM, 3.12µM, 6.25µM, 25µM, 50µM and 100 µM) of VIT, CIS (0µM,3.12µM, 6.25µM, 25µM, 50µM and 100 µM) individually and combinational dose for (1.25 µM VIT+1.25 µM CIS), (3.12 µM VIT+3.13 µM CIS), (6.25µM VIT+6.25 µM CIS), (12.5 µM VIT+12.5 µM CIS), (25µM VIT+25 µM CIS) and (50 µM VIT+50 µM CIS) for 24 h at 37ºC in a humidified chamber. After 24 h of incubation, 20 µl of MTT reagent was added to each well and incubated for 3 h in a 5% CO2 incubator. After 3 h, the purple-coloured formazan crystals were solubilized in 150 µl of DMSO and the optical density was measured in each well. For further experiments IC50 value of VIT (10 µM), CIS (5µM)

individually and a combination of ZER and CIS (5µM and 2.5 µM, respectively) was used for 48 h unless otherwise specified.

Combination index calculation

The Combination Index (CI) value was used to determine the drug interaction and CI value was analysed between VIT and CIS line through Chou and Talalay (1984) method using Compu-Syn software (1.0) in the Hep-2 cell line.

Lactate dehydrogenase (LDH)leakage assay

LDH activity was determined in the supernatant of cleaved cells using a commercial assay kit followed by the manufacturer's protocol (Invitrogen). The absorbance was measured at 490 nm using Microplate Reader (Abcam Instruments, Inc.)

Determination of ROS generation by DCFH-DA stain.

Intracellular ROS generation was assessed by 2, 7-Dichlorofluoroscin-diacetate (DCFH-DA) stain. Initially, cells were seeded at the density of 3 X 105 cells /well in 6-well plates and kept for overnight incubation at 37ºC in a CO2 incubator. After overnight incubation, cells were treated with VIT (10 µM), CIS (5 µM) individually, and a combination of VIT and CIS (5µM and 2.50µM, respectively) for 24 h, after incubation, treated and washed cells were stained with DCFH-DA dye at 37ºC for 30 min in a 5% CO2 incubator. The cells were then washed with PBS and the resulting fluorescence was identified at the wavelength 485/530 nm in the fluorescence plate reader.

DNA fragmentation assay.

Fragmented DNA in agarose gel electrophoresis was used as a biochemical marker for the measurement of apoptosis. The trypsinized Hep-2 cells were treated with (IC_{50}VIT, CIS, and a combination of VIT and CIS in the medium for 24 h. Following incubation, the cells were washed with 1X PBS, and pelleted again by centrifugation (5000rpm) for 5 min. The pellet was incubated with 300 µl of nuclear lysis buffer for 10 min at room temperature and centrifuged at 12000 rpm for 5 min. The resultant supernatant was incubated overnight with RNA s (0.3 mg/mL) at room temperature and with proteinase K (0.1 mg/mL) for 2 h at 37ºC. DNA was extracted by adding phenol/chloroform (1:1) and precipitated with 70 % ethanol for 2 h at 80ºC. The precipitated DNA was centrifuged at 12000 rpm at 4ºC for 5 min, and the pellet was air-dried for 30 min and then dissolved in Tris-EDTA buffer. The isolated DNA was quantified by a spectrophotometer and resolved over 1.5% agarose gel containing 0.4 µg/mL ethidium bromide. The bands obtained were visualized under an ultraviolet trans illuminator

Detection of RBC depletion through Hypotonicity-induced human red blood cell (HRBC) membrane stabilization method.

Anti-angiogenic effect of target drug were determined through measuring RBC depletion by HRBC assay, Once the cells reached confluency, they are treated with VIT, CIS individually or combination for 24 hrs, after incubation 1.0 ml of sample were collected from untreated and treated of different concentrations, (10– 100 ug/ml) in 1 ml of 0.2 M phosphate buffer and 0.5 mL of 10% HRBC suspension, 0.5 ml of 0.25 % hyposaline were incubated at 37° for 30 min and centrifuged at 3000 rpm for 20 min, resulted haemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm. Diclofenac was used as standard and a control was prepared by distilled water instead of hypo saline to produce 100% haemolysis. The percentage of HRBC haemolysis and membrane stabilization or protection was calculated by using the following formula:

% of Hemolysis = (Optical density of test sample / Optical density of control) X 100

% Protection = 100-(Optical density of test sample / Optical density of control) X 100]

Detection of caspase activity

The cells were seeded at a density of 3 X 105 cells / well in a 96-well plate and incubated for 24 h. After cell adherence, cells were treated with VIT (10 µM), CIS (5 µM) and their combination (5 µM of VIT + 2.5 µM of CIS) for 24 h. The resultant pellet was lysed by adding 25 µl of chilled lysis buffer and kept on ice for 10 min. After incubation, the protein was isolated from cells and estimated by Bradford's method. The obtained protein (100 µg) was added to 25 µL 2X reaction buffer containing 100 mM substrate (AC-DEVD-pNA) for caspase 3 and kept in an incubator at 37°C for 2 h in a 96-well plate. At the end of incubation, the production of cleaved p-nitroanilide from the tetrapeptide substrate DEVD-pNA was quantified in a microplate reader at the wavelength of 405 nm.

RESULTS AND DISCUSSION

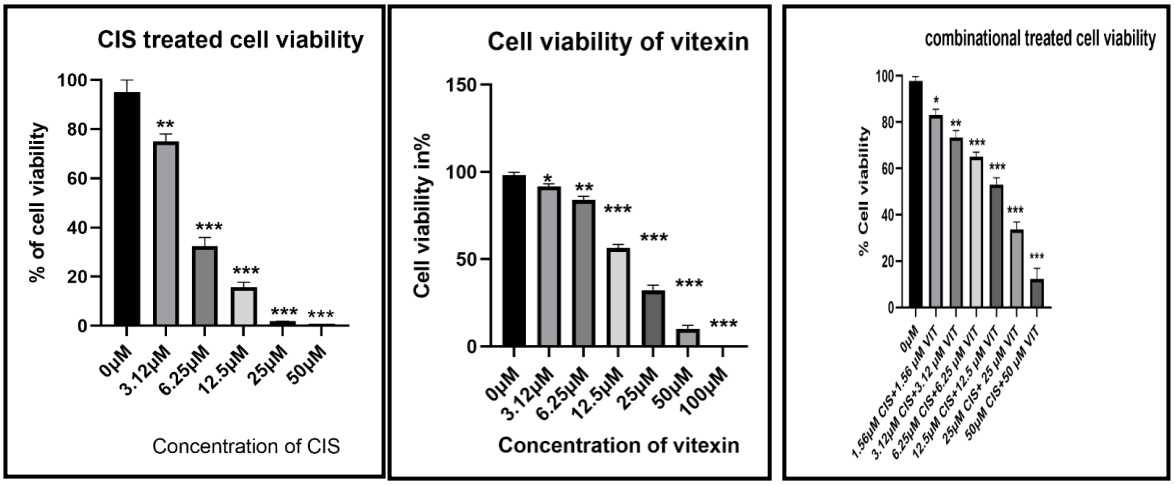


Figure 2 (A-C) Anti-proliferative effects of VIT, CIS individual and their combination treatment on cell viability of Hep-2cells.

VIT treatment: In VIT treated cells, A dose-dependent decrease in cell viability was observed. VIT at 3.12 µM dose, cell viability was marginally decreased ($p<0.012$) with 90% compared to the control. At 6.25, 12.5,25,50and 100 µM doses, VIT reduced the cell viability to 87%, 53%, 44 %, and 27 % ($p<0.001$), respectively with compared to control. (**fig.2 A**).

CIS treatment: As predicted, CIS inhibited the dose-dependent cell viability. At 3.12,6.25,12.5,25 and 50 µM doses of CIS, cell viability reduced to 70%, 31%, 53%, 20% and 0.95%, respectively ($p<0.001$) as compared with control (**fig.2B**). From the dose-response curve, growth inhibition was observed in cells undergone VIT or CIS treatment individually with the IC50 value at 10 µM for VIT and 5µM for CIS.

VIT + CIS combination treatment: Hep-2 cells were treated with CIS and VIT together as a combination therapy in a secure ratio (1:1) with a half-dose of each compound for 48 h. The combination treatment of VIT and CIS showed a dose-dependent decrease in cell viability was observed. At 1.25 µM dose, cell viability reduced marginally ($p<0.01$) to 70%, while 3.25and 6.25 µM doses caused a moderate decrease ($p<0.001$), and 25 and 50 µM doses further caused an extreme reduction in cell viability by 80% ($p<0.0001$). From the dose-response curve, the IC50 value was found to be at 5 µM of VIT+2.5 µM of CIS (**fig.2 C**)

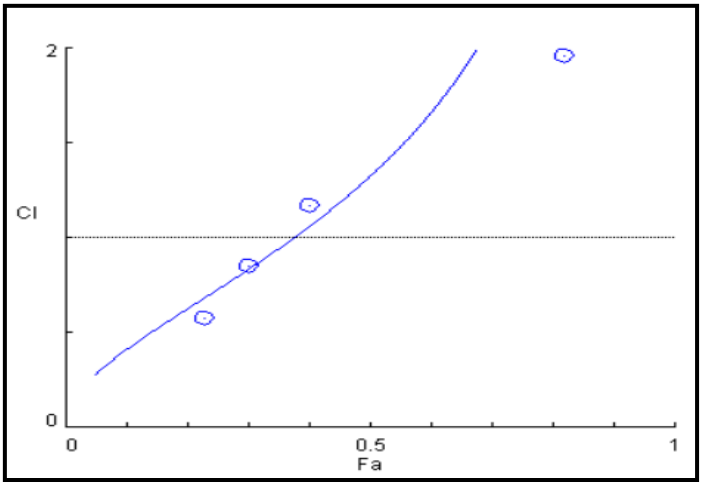


Figure 3: Combination Index Plot

Figure 3, Shows the calculated CI value by isobologram analysis using the Compu-syn software (1.0) and results revealed that the CI value of the combination dose was <1 at lower doses (3.12,6.25,12.5,25µM concentrations) which possesses a synergistic inhibitory effect on Hep-2 cells, whereas at higher concentration (50 and 100µM) the CI value found to be >1which indicates the antagonistic effect of our target drug vitexin towards cisplatin effect in laryngeal carcinoma.

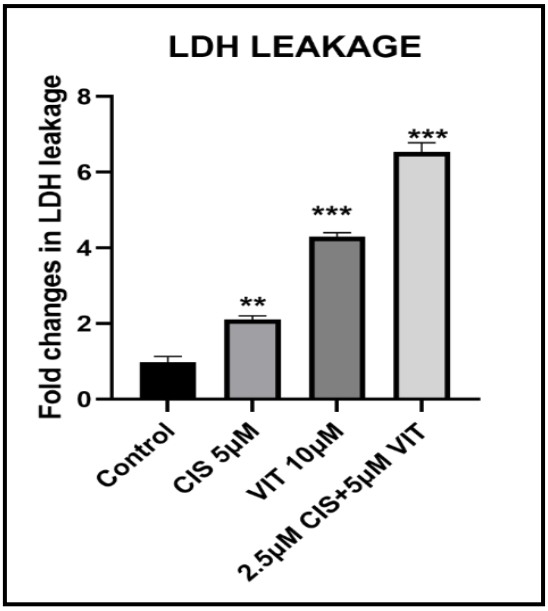


Figure 4. Assessment of LDH leakage from Hep-2 cells

When cells undergo necrosis or apoptosis, LDH a consistent marker of cytotoxicity is released at increased rate into the surrounding medium at dose and time dependent manner in the medium and in Hep-2 treated cells for 24 h (fig.4). LDH release in the medium was increased to four -fold (72%), in cells treated with 10 μM VIT, as associated to control. In cells with CIS treatment, LDH leakage was found to be reduced by two -fold ($p< 0.05$), whereas cells tested with both VIT and CIS together, LDH leakage was further augmented to seven -fold ($p<0.001$) as compared to control.

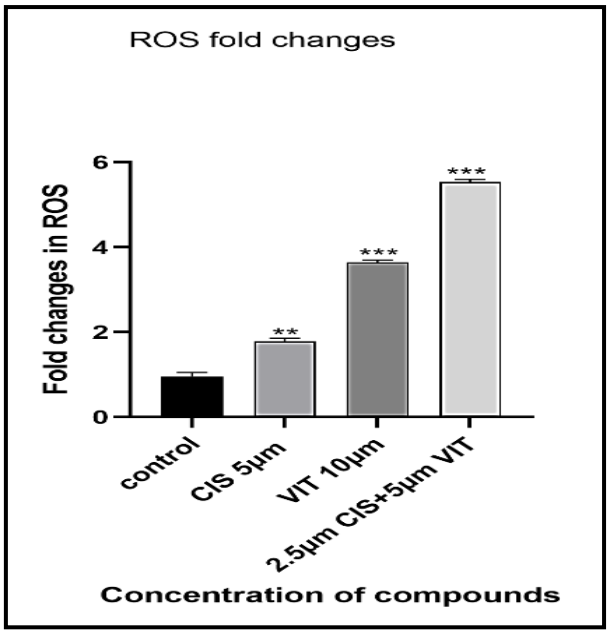


Figure 5: Assessment of intracellular ROS generation in Hep-2 cells

In the present study, Intracellular oxidation-reduction state was assessed by sensing ROS generation in VIT, CIS, and their combination treated cells by using the cell-permeant dye 2', 7-dichlorofluorescein diacetate (DCFH-DA) (**fig.5**). Cells exposure to VIT caused a significant increase in ROS generation by 3.5 -fold ($p<0.01$), while CIS treated cells evinced a moderate decrease in ROS by (1.8-fold) ($p<0.05$) compared to VIT alone treated cells, whereas in combination treated cells the ROS production was elevated to 5.8 -fold ($p<0.001$) as compared to control or individual treated cells. Thus, the additional surge in ROS level observed under combination treatment tends the cancer cells to reach their oxidative stress and hence a profound decline in cell viability was observed in the present study.

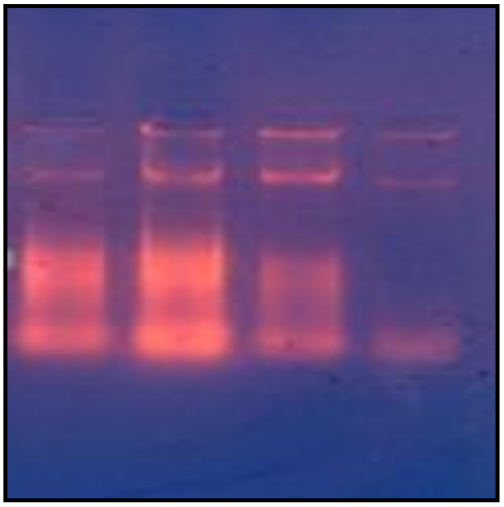


Figure 6: Assessment of DNA fragmentation in Hep-2 cells

Induction of apoptosis in Hep-2 treated cells was verified by DNA fragmentation analysis using gel electrophoresis technique. The DNA bands obtained from VIT or CIS or their combination treated cells produced ladder pattern as observed from lane 2 to 4 in **fig.6.** A ladder formation indicates that the DNA has undergone fragmentation and each fragment corresponded to a band in ladder. In combination treated cells cleaved Internucleosomal fragments found to be significant compared to individual treated cells or control.

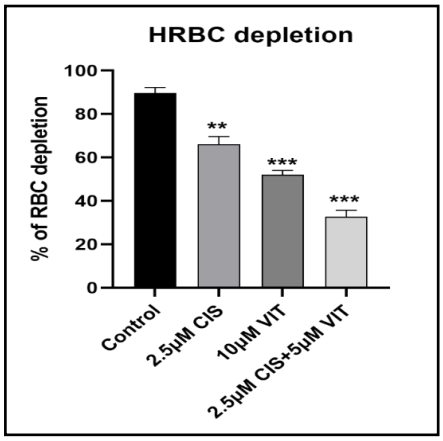


Figure7: Assessment of HRBC depletion in Hep-2 cells

In VIT treated cells the HRBC depletion found to be extremely reduced by 50% compared to CIS individual treatment and control, whereas in CIS alone treated cells the reduction in HRBC found to be moderately significant, and in combination VIT+CIS(5+2.5µM) treated cells the HRBC depletion found to be significantly diminished by 30% and shows effective Anti-angiogenic effect compared to VIT, CIS individual treated cells and control as per **Fig.7.**

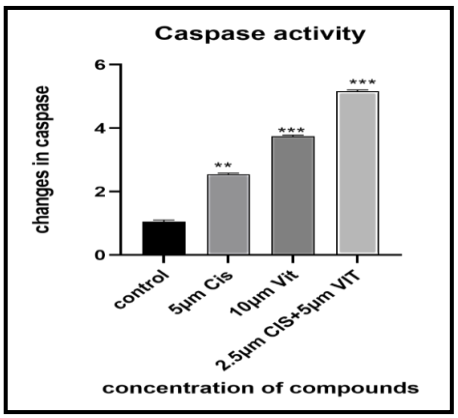


Figure 8: *Assessment of* Caspase-3 activities in Hep-2 cells

The caspase-3 activation was assessed in cancer cells to identify the apoptotic pathway. Anti-cancer effects of VIT, CIS individually, and their combination treatment on caspase-3 activities in Hep-2 cells are depicted in fig.8. In CIS treated cells the caspase-3 activity found to be increase by 2.5-fold and in VIT treated cells caspase-3 activity found to be further augmented to 3.7-fold respectively ($p<0.01$) compared to control. Whereas, in the Co-treated group, this elevation tends to be extremely significant by 5.8 fold($p<0.001$).

CONCLUSION

The fact that VIT increased the anticancer effectiveness of CIS on the majority of the studied parameters, it may be used in conjunction with chemotherapy to boost the effectiveness of chemotherapeutic agents like cisplatin to combat their harmful side effects and drug resistance. Further, in vivo research is needed to confirm the effectiveness of this new combination in suppressing multidrug resistance in specific cancer cells.

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