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## Preclinical report

# Induction of apoptosis by gallic acid in lung cancer cells

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**The apoptosis-inducing effect of gallic acid (3,4,5-trihydroxybenzoic acid) was investigated in four human lung cancer cell lines, SBC-3 (small cell carcinoma), EBC-1 (squamous cell carcinoma), A549 (adenocarcinoma) and SBC-3/CDDP (cisplatin-resistant subclone of SBC-3). Gallic acid induced apoptosis in a dose-dependent manner as evidenced by analyses of DNA fragmentation, changes in cell morphology and loss of viability. Fifty percent inhibitory concentration ($IC_{50}$) values of gallic acid on the cell viability of SBC-3, EBC-1 and A549 were around 10, 20 and 60 μg/ml, respectively. The $IC_{50}$ value for SBC-3/CDDP cells was almost the same as that of SBC-3, suggesting that susceptibility of cells to gallic acid-induced apoptosis is not altered by the acquisition of cisplatin resistance. The apoptotic process was effectively triggered by 30 min exposure to gallic acid. A caspase inhibitor and α-tocopherol effectively prevented the gallic acid-induced apoptosis, indicating the involvememt of caspase activation and oxidative processes during the course of apoptosis in gallic acid-treated cancer cells. These findings suggest the possible applicability of gallic acid in lung cancer therapy, especially to circumvent resistance to anti-cancer drugs. [© 1999 Lippincott Williams & Wilkins.]**

*Key words*: Apoptosis, gallic acid, lung cancer.

## Introduction

Apoptosis refers to physiological cell death, and plays an essential role during development and in maintaining tissue homeostasis.[1,2] Because the balance between mitosis and cell death determines the growth of cancer tissue, the induction of apoptosis in cancer cells is one of the major concerns in cancer therapy. A number of studies have explored the attractive idea that tumor cells could be eliminated by artificially triggering death through apoptosis.[3] Cell-mediated cytotoxicity involves induction of at least some features of apoptosis in the targets. The cell death that is induced by irradiation, hyperthermia and a range of cytotoxic drugs, including many used in cancer therapy, has been found to be apoptosis.[4–8]

Naturally occurring plant polyphenols have been found to have antimutagenic and anticarcinogenic activities.[9] The mechanisms by which plant polyphenols inhibit carcinogenesis are complicated, including anti-oxidative effects and inhibition of activities of proliferation-associated enzymes such as ornithine decarboxylase, DNA polymerase, ribonucleotide reductase, etc.[10–12] Some plant polyphenols have also been found to induce apoptosis in cancer cells. Inoue *et al.* first found that gallic acid (3,4,5-trihydroxybenzoic acid), a naturally occurring plant phenol obtained by the hydrolysis of tannins, had selective cytotoxicity against cancer cells and could induce apoptosis in some cancer cells such as HL60RG cells.[13,14] These reports indicate the possible usefulness of gallic acid in cancer prevention and treatment.

The effects of gallic acid on human lung cancer cells have not yet been investigated and the mechanisms by which gallic acid induces apoptosis in cancer cells are still not fully elucidated. In this paper, we demonstrate that gallic acid can induce apoptosis in human lung cancer cells and further investigation was carried out to characterize the mechanisms of gallic acid-induced apoptosis. Short-term exposure to gallic acid effectively induced apoptosis in these cancer cells. The susceptibility of cancer cells to gallic acid-induced apoptosis was not influenced by the acquisition of resistance to the cytotoxicity of cisplatin. Gallic acid is thus suggested as a candidate drug that can circumvent drug resistance in lung cancer therapy.

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## Materials and methods

### Cell cultures and drug treatment

A549 (human lung adenocarcinoma), EBC-1 (human lung squamous cell carcinoma) and SBC-3 (human lung small cell carcinoma) cell lines were obtained from the Japanese Cancer Research Resources Bank (Osaka, Japan). SBC-3/CDDP (cisplatin-resistant subclone of SBC-3) cells were kindly provided by Dr Katsuyuki Kiura (Second Department of Internal Medicine, Okayama University Medical School).[15] Cells were maintained at 37°C in an atmosphere of 95% air and 5% $CO_2$ in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum (Filton, Tasmania, Australia) and antibiotics (50 μg/ml of streptomycin and 50 U/ml of penicillin). Gallic acid was obtained from Nakalai Tesque (Kyoto, Japan). α-Tocopherol was obtained from Wako Pure Chemical Industries (Osaka, Japan), and cisplatin from Bristol-Myers Squibb (Park Avenue, NY). A caspase inhibitor, BOC-Asp-FMK, was obtained from Enzyme Systems Products (Livermore, CA). Stock solution was prepared in dimethylsulfoxide at 20 mM.

### Cytotoxicity assay

Cells were seeded at $2 \times 10^4$ cells /well in 96-multiwell plates and precultured for 24 h before treatment. After washing the cells with phosphate-buffered saline (PBS, pH 7.4), 100 μl of medium containing gallic acid at various concentrations was added. After 48 h of treatment, the relative viable cell number was evaluated using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay.[16] All the assays were carried out in triplicate. Each experiment was repeated at least 3 times.

### Morphological analysis

Cells were harvested, fixed with methanol and stained with DNA binding fluorochrome 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Boehringer, Mannheim, Germany). Fluorescent images were observed with an Olympus IX 70 microscope equipped with a filter set.

### Ultrastructure

For ultrastructural analysis, SBC-3 cells ($2 \times 10^6$ cells) treated with gallic acid for 12 h were fixed in 2% glutaraldehyde in a 0.1 M sodium cacodylate buffer (SCB) pH 7.5, on ice, for 1 h. Fixed samples were washed 3 times with cold SCB, post-fixed in 1% (w/v) $OsO_4$ in SCB for 1 h on ice, dehydrated in an ascending series of ethanol and then embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and viewed under a JEM-200CX electron microscope.

### Statistical methods

All values were expressed as mean ± SD. All data were analyzed by analysis of variance (ANOVA). Statistical significance was analyzed with a tailed Student's *t*-test.

## Results

### Induction of apoptosis by gallic acid in lung cancer cells

Figure 1 shows the effect of gallic acid on the cell viability of three kinds of lung cancer cell lines, A549, EBC-1 and SBC-3. MTT assay revealed a dose-dependent decrease in cell viability after 48 h treatment with gallic acid. SBC-3 cells were more sensitive to the

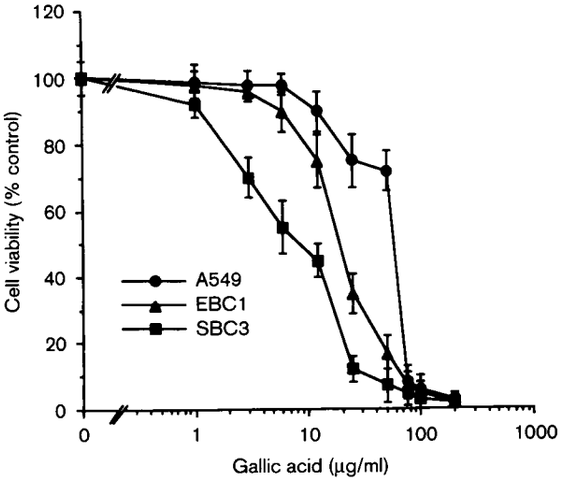


**Figure 1.** Dose-dependent effects of gallic acid on the cell viability of lung cancer cells. Lung cancer cell lines, adenocarcinoma (A549), squamous cell carcinoma (EBC-1) and small cell carcinoma (SBC-3), were cultured in the presence of varying concentrations of gallic acid for 48 h. Cell viability was assessed with MTT assay and the values were plotted as a percentage of non-treated control culture. Results are presented as the mean ± SD from triplicate wells. Similar results were obtained from three separate experiments.

cytocidal effect of gallic acid, with a 50% inhibitory concentration ($IC_{50}$) value of 10–15 μg/ml, compared to EBC-1 and A549 cells which had $IC_{50}$ values of around 20 and 60 μg/ml, respectively. These dead cells showed typical morphological changes due to apoptosis, such as cell shrinkage with condensed and fragmented nuclei (Figure 2A) and the appearance of many vacuoles without organelle disruption (Figure 2B, lower panel). Analysis of genome DNA by agarose gel electrophoresis showed a characteristic DNA fragmentation ladder pattern in the cancer cells treated with gallic acid (data not shown). Thus, loss of cell viability was suggested to be mainly due to the induction of apoptosis.

## Characterization of gallic acid-induced apoptosis in lung cancer cells

In order to characterize the apoptosis-inducing effect of gallic acid, several experiments were carried out as follows. First, cell survival was compared in cancer cells which were exposed to gallic acid for different lengths of time. As shown in Figure 3, the degree of cell survival 48 h after exposure to gallic acid for 0.5 or 1 h was at a similar level to that of the continuous 48 h exposure, indicating that 0.5 h exposure is enough to trigger the apoptotic processes in the lung cancer cells. The effect of gallic acid on cisplatin-resistant lung cancer cells was next investigated. Cytocidal effects of gallic acid and an anticancer drug cisplatin were compared in the parental SBC-3 cells and the cisplatin-resistant subclone SBC-3/CDDP cells. The experiment showed that the apoptosis-inducing effect of gallic acid was almost the same in both parental and cisplatin-resistant subclone cells, although these cell lines showed a significant difference in their susceptibility to cisplatin cytotoxicity (Figure 4).

The effects of the caspase inhibitor BOC-Asp-FMK, and α-tochopherol, a vitamin with anti-oxidative properties, on the cytocidal effect of gallic acid were investigated (Figure 5). Although both compounds did

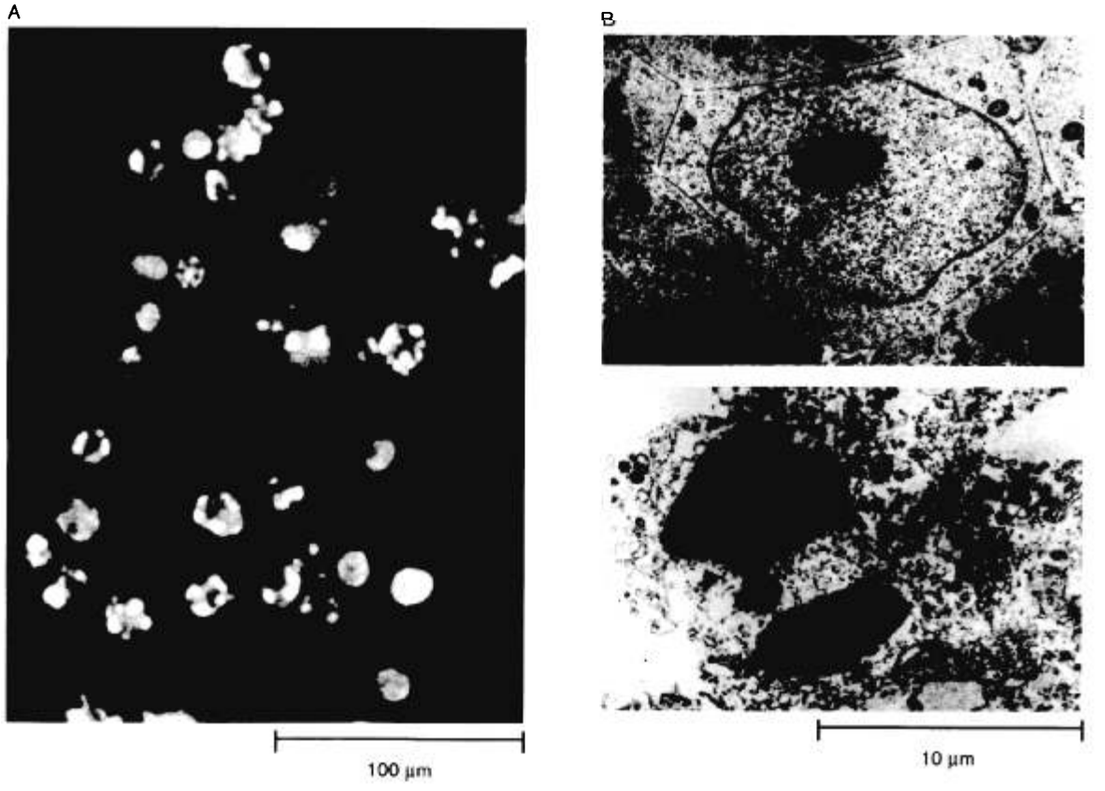


**Figure 2.** Morphlogical examination of gallic acid-treated SBC-3 cells by a fluoresent microscopy (A) and an electron microscopy (B). Cells were incubated for 12 h in the presence of 25 μg/ml gallic acid. (A) Nuclear fragmentation is apparent. (B) Different from untreated cells (upper panel), the gallic acid-treated cells (lower panel) showed typical apoptotic nuclei. Most of them were accompanied by secondary necrotic changes as represented by plasma membrane rupture.

not offer effective protection against the loss of cell viability caused by high doses of gallic acid (data not shown), significant protection from apoptotic cell death by these reagents was observed in the culture treated with low concentrations of gallic acid. The cell viability of SBC-3 cells decreased to around 50% of the control after 48 h of treatment with 6 μg/ml gallic acid. Treatment with BOC-Asp-FMK at 20 μM or α-tochopherol at 1 mg/ml significantly suppressed the gallic acid-induced apoptosis.

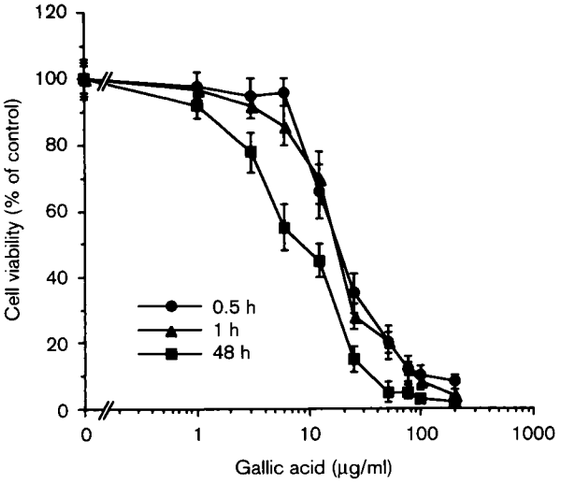


**Figure 3.** Effect of pulse treatment with gallic acid on cell viability of SBC-3 cells. Cells were incubated with varying concentrations of gallic acid for 0.5 (●), 1 (▲) or 48 (■) h. Culture cells which were pulse-treated with gallic acid for 0.5 or 1 h were washed once with PBS after the treatment and cultured further for a total of 48 h. Cell viability was measured with MTT assay after 48 h incubation and the values were plotted as a percentage of non-treated control culture. Results are presented as the mean ± SD from triplicate wells.

## Discussion

Apoptosis is a genetically encoded programmed cell death defined by characteristic morphological and biochemical changes.[17-19] Apoptosis plays a role opposite to that of mitosis in cell population kinetics in almost all tissues and apoptosis abnormalities are closely associated with multistep carcinogenesis.[20] Growth of tumor tissue is determined by the balance between cell proliferationa and apoptosis. In cancer cells, the inability of a cell to undergo apoptosis may provide a growth advantage and cells with defective apoptotic machinery may therefore have a better chance of surviving. Furthermore, genetic alterations reducing the susceptibility of cells to apoptosis are suggested to contribute to their resistance to radiation and chemotherapeutic agents. For these reasons, induction of apoptosis by gallic acid, irrespective of the resistance to cisplatin, is an attractive proposition. Investigations into the molecular mechanisms by which gallic acid induces apoptosis in lung cancer cells appear to be important for the development of effective therapeutic agents

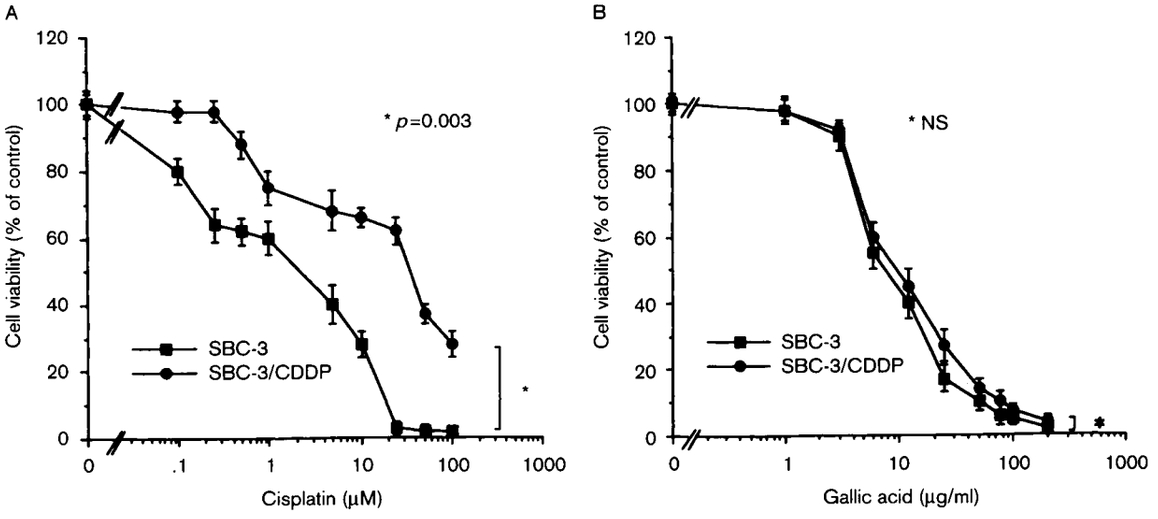


**Figure 4.** Different effect of cisplatin and gallic acid on SBC-3 and the cisplatin-resistant subclone of SBC-3 cells. Parental SBC-3 (■) and cisplatin-resistant SBC-3/CDDP (●) cells were treated either with cisplatin (left panel) or gallic acid (right panel) at various concentrations. Cell viability was measured with MTT assay after 48 h incubation and the values were plotted as a percentage of non-treated control culture. Results are presented as the mean ± SD from triplicate wells.

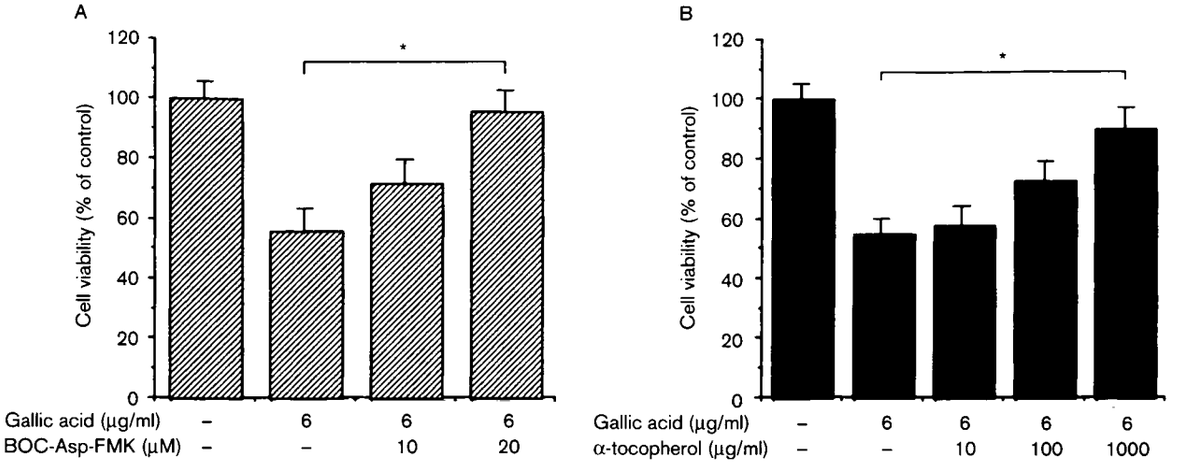


**Figure 5.** Effect of a caspase inhibitor BOC-Asp-FMK (A) and α-tocopherol (B) on gallic acid-induced apoptosis in SBC-3 cells. Cells were cultured in the presence or absence of gallic acid (6 μg/ml) with or without addition of the caspase inhibitor (A) or α-tocopherol (B) at concentrations as indicated. Cell viability was measured with the MTT assay after 48 h incubation and the values were plotted as a percentage of non-treated control culture. Results are presented as the mean ± SD from triplicate wells. *Significantly different at $p < 0.05$.

against lung cancer cells.

Plant polyphenols are secondary metabolites widely distributed in various sectors of the higher plant kingdom and well known as showing diverse biological and pharmacological action, including antiviral, anti-inflammatory and anti-oxidative activities.[9] Gallic acid is most frequently encountered in plants in ester forms such as hydrolyzable tannins and has been known to exibit similar activities.[21] Plant polyphenols have been shown to be effective not only against tumor initiation, but also against the promotion phase of tumorigenesis. Gallic acid and several of its derivatives also have anti-carcinogenic properties. Inoue *et al.* first reported the apoptosis-inducing activitiy of gallic acid with higher selectivity in cancer cells than in normal cells and they suggested that the apoptosis-inducing ability may be associated with its anti-cancer activity.[13,14]

The mechanisms for the apoptosis-inducing effect of gallic acid are still speculative. It has been reported that formation of reactive oxygen species such as hydrogen peroxide and superoxide anion may be involved in gallic acid-induced cell death.[22-27] It is well-recognized that oxidative stress is one of the causative factors of apoptosis and that various oxidizing agents such as hydrogen peroxide and UV irradiation induce apoptotic cell death in various tumor cell lines.[28] Conversely, anti-oxidants which act as radical scavengers, such as *N*-acetyl cysteine, α-tocophenol, reduced glutathione peroxidase, catalase and superoxide dismutase, protect cells from apoptosis. Some types of anti-oxidants are known to act as pro-oxidants and therefore it was suggested that some anti-oxidants may induce apoptotic cell death due to their pro-oxidant actions.[29] In the present study, we showed that gallic acid-induced apoptosis can be inhibited by an anti-oxidative vitamin, α-tocopherol, supporting the concept that gallic acid may induce apoptosis by its pro-oxidant action. Other possible mechanisms by which gallic acid induces apoptosis include inhibition of protein tyrosine kinases (PTKs).[23] Sarrano *et al.* suggested that gallic acid derivatives may induce apoptosis not only because of their contribution to the generation of reactive oxygen species, but also as a consequence of their inhibitory activity toward PTKs.[23]

According to the study of the relationship between the structure and cytotoxic activity of phenolic compounds, three adjacent phenolic hydroxyl groups are thought to be essential to the cytotoxicity of gallic acid, since the methylation or deletion of the phenolic hydroxyl groups in gallic acid abolishes the cytotoxic activity.[30-35] Because phenolic hydroxyl groups of gallic acid are rapidly metabolized in the liver, the *in vivo* cytotoxic effect of gallic acid may be limited. However, the present study demonstrated that short-term exposure of cells to gallic acid can effectively trigger apoptosis in lung cancer cells. Therefore, the *in vivo* anti-cancer effect of gallic acid is still practicable.

Intrinsic or acquired drug resistance is a major limiting factor in the effectiveness of chemotherapy. Cisplatin is an anticancer agent with a substantial

activity against a variety of human malignancies including lung cancer. In patients with small cell lung cancer, 65–95% overall response rates and 35–50% complete response rates were reported after treatment with cisplatin plus etoposide.[15] However, only a small fraction of patients remain in remission beyond 2 years. The emergence of drug resistance during induction chemotherapy is a major obstacle to improve the treatment outcome of patients with small cell lung cancer. The mechanism of cisplatin resistance is multifactorial. The decreased accumulation of intracellular cisplatin and activation of the detoxication system play important roles in the acquisition of cisplatin resistance. These cancer cell phenotypes are also responsible for resistance to other chemotherapeutic agents. The cisplatin-resistant cell line SBC-3/CDDP was reported to have cross-resistance to various anti-cancer drugs such as mitomycin C, vinca alkaloids, 5-fluorouracil and etoposide.[15] Therefore, applying gallic acid in the treatment of lung cancer to overcome resistance against chemotherapeutic agents is a promising treatment option.

## Conclusion

Gallic acid can effectively induce apoptosis in human lung cancer cells. The effect was suggested to be associated with its pro-oxidative property. The susceptibility to gallic acid-induced apoptosis was not altered by the acquisition of cisplatin resistance. Gallic acid was thus suggested to be a candidate drug for lung cancer treatment to circumvent the resitance to chemotherapeutic drugs.

## Acknowledgments

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