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## Dietary Administration of Berberine or *Phellodendron amurense* Extract Inhibits Cell Cycle Progression and Lung Tumorigenesis

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### Abstract

*Phellodendron amurense* extract is a Chinese herbal remedy that has recently been studied for its antitumor, antimicrobial and other biological activities. It is previously unknown if these agents are bioavailable and effective against tumors when delivered as a dietary component. It is also unknown if the anti-tumorigenic properties of berberine, an isoquinoline alkaloid component of *P. amurense*, is equally effective when administered alone. There are contrasting reports on the cellular processes involved in anti-tumorigenesis by *P. amurense* and berberine. Here we find that berberine, when administered orally through the diet, inhibits *in-vivo* tumorigenesis of both p53 expressing and p53 null lung tumor xenografts equally whether administered in its pure form or as a part of *P. amurense* extract. We also show that berberine induces G1 cell cycle arrest, inhibits proliferative kinase signaling and arrests the growth of lung tumor cells in culture. Berberine administered in the diet was detectable by HPLC in the lungs of mice fed *P. amurense* or equivalent doses of berberine at concentrations of 455 and 518 ng/ml respectively and inhibited the growth of xenografted A549 cell tumors, which grew to 9.4 and 6.4 mm<sup>3</sup> respectively, compared to 58.9 mm<sup>3</sup> in control mice (p<0.001). Phosphorylation of Akt, CREB and MAPK was inhibited in A549 cells by *P. amurense*. Demonstration of oral bioavailability and anti-tumorigenic efficacy of dietary berberine, as well as further demonstration of signaling pathway modulation and cell-cycle arrest, implicate this relatively safe, natural compound as a potentially important therapeutic and chemopreventive agent for lung cancer.

### Keywords

lung cancer; herbal therapy; cell-cycle; berberine

### INTRODUCTION

Lung cancer is the leading cause of cancer death in both men and women in the United States [1]. Despite major advances in recent years, most lung cancers are disseminated at the

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time of presentation and have a mortality rate of about 90%, with a 5 year survival rate of only 16% [1]. This demonstrates an urgent need for better chemoprevention, treatment and adjuvant therapy for lung cancer.

Recently studies demonstrated that *Phellodendron amurense* (*P. amurense*) bark extract, prevented the development of prostate cancer in a murine model of prostate cancer chemoprevention [2] and inhibited prostate cancer cell proliferation [3] through inhibition of Akt/CREB mediated cyclin D1 activation. *P. amurense* extract inhibited CREB mediated Cox-2 activation [4], a signaling pathway involved in both prostate and lung cancer [5,6]. Berberine, an isoquinoline alkaloid present in many medicinal herbs including *P. amurense* extract, has pharmacological and biological activities including Cox-2 inhibition and other anti-inflammatory effects [7]. Recent reports have shown that berberine is one of the bioactive components of *P. amurense* extract [8]. Berberine has been studied for its inhibitory effect on proliferation and pro-apoptotic effect in several tumor cell lines, including non-small cell lung tumor cell lines [9–12], and has been shown to have tumor killing effects on lung cancer *in-vivo* when administered intraperitoneally [13,14]. Berberine has also been shown to inhibit prostate cancer and neuroblastoma cell growth through p53-dependent induction of apoptosis [15,16], and through p53-dependent cell cycle arrest and apoptosis in osteosarcoma cells induced by DNA damage [17].

Potential chemopreventive agents must be safe, effective, easily accessible and easily administered, preferably orally. We have previously demonstrated that the efficacy of chemopreventive/therapeutic agents can be dramatically affected by their inclusion in a complex mixture [18]. To our knowledge, no study has evaluated the efficacy of dietary berberine on tumorigenesis in an *in-vivo* model or compared the efficacy of berberine *in-vivo* to that of whole *P. amurense* extract. The goals of the current study were to determine the bioavailability and efficacy of berberine administered in the diet, to compare berberine alone to *P. amurense* extract and to determine the effect of inhibitory doses of dietary berberine on tumorigenesis related cellular phenotypes such as cell cycle arrest.

In demonstration of the efficacy of dietary berberine induced tumor growth arrest, we show that berberine, administered alone or in *P. amurense* extract are equally able to induce tumor cell growth arrest in association with G1 cell-cycle arrest regardless of p53 status. *P. amurense* extract and berberine induced G1 cell-cycle arrest in both p53 positive and null cells and dramatically inhibited the proliferation of these lung tumor cells *in vitro*. *P. amurense* extract concurrently reduced constitutive activation of Akt, CREB and MAPK indicating inhibition of an important proliferative signaling axis. Berberine was equally detectable in lung tissue following dietary administration of pure berberine or that as a part of *P. amurense* extract, indicating that relevant concentrations of berberine are achievable through oral administration whether given alone or in whole extract. No toxicity was observed even at the highest doses of berberine. Together, these findings show that dietary berberine and *P. Amurense* extract have significant potential as easily administered and safe lung cancer therapeutic agents and can inhibit proliferative kinase signaling pathways, arrest cell-cycle progression and prevent tumor growth.

## MATERIALS AND METHODS

### Proliferation Assays

The human lung tumor cell lines H1299 and A549 were cultured in RPMI-1640 plus 10% FBS (Life Technologies). Cells were counted using a hemacytometer and seeded onto 12-well tissue culture dishes at 500 cells per well. After attachment, cells were treated with DMSO solvent or the indicated concentrations of the *P. amurense* extract Nexrutine® (Solanova, Novato CA), or berberine (Sigma, St. Louis, MO) dissolved in DMSO. Final DMSO concentration was 0.025%. Mixtures were homogenized and were from a single batch. Cells were assayed for viable cell numbers in triplicate using the MTS based CellTiter 96 AQueous One Solution Cell Proliferation Assay kits (Promega). A490 was measured on a plate reader after attachment of cells (8 hours after seeding) for a baseline reading, and again throughout 5 days of growth in culture. Media and drugs were replaced every 2 days. p-values for biological triplicates were determined by a one-tailed Student's T-test.

### Tumorigenesis Models

A549 lung tumor cells were cultured, counted and resuspended in serum free media at a concentration of  $5 \times 10^6$  cells/mL. A volume of 200  $\mu$ L ( $1 \times 10^6$  cells) were injected subcutaneously into the right flank of athymic nude mice at an age of 4-6 weeks. Mice were monitored 3 times weekly and tumor sizes measured periodically until sacrifice at 4 weeks post-injection. Tumor volume was determined at 2 weeks post-injection by the formula for an ellipsoid  $\pi/6(l \times w \times h)$ . P-values were determined by one-tailed Student's t-test. Photographs were taken of tumors in live mice at 18 days post injection. At 4 weeks post-injection, mice were anesthetized by intra-peritoneal injection of a cocktail of 87mg/kg ketamine and 15mg/kg xylazine. Anesthesia was determined by absence of rear foot reflex. Blood was collected from the retro-orbital plexus into tubes containing 10 $\mu$ l of 0.5M EDTA. Mice were euthanized by CO<sub>2</sub> asphyxiation. Lung tissues were collected, pooled within experimental groups of 4 mice and homogenized in DMSO using an electric tissue homogenizer. Lung and blood samples were centrifuged at  $16,000 \times g$  for 15 minutes at 4°C. Serum and lung supernatants were collected and used for HPLC analysis as described below.

### HPLC Analysis of Berberine Concentrations

The concentrations of Berberine in lung and serum samples were determined by HPLC. The HP 1100 series HPLC system consisted of an autosampler, a binary pump, a thermostat equipped column compartment, and a diode array detector (Agilent Tech, Santa Clara, CA). The HPLC column was 4.6  $\times$  75 mm Zorbax SB-C18 3.5  $\mu$ m column, and the detection wavelength was 343 nm. Stock solutions were prepared by dissolving Berberine in acetonitrile, and Nexrutine in DMSO, respectively. The mobile phase composed of A (20 mM phosphate buffer) - B(acetonitrile) was used under gradient elution of 0 min, 80:20, v/v; 6.5 min, 60:40 v/v; 10 min, 80:20 v/v. The column temperature was controlled as constant at 25 °C. The flow rate of the mobile phase was 1 ml/min during the whole gradient cycle.

## Cell Cycle Analysis

After 48 hours in culture treated with the indicated concentrations of DMSO solvent, *P. amurense* extract, or berberine,  $10^6$  cells were washed and pelleted with PBS. Supernatant was removed and the pellet was dispersed by tapping. 500ul of cold 70% ethanol was added and incubated on ice for 20 minutes. Cell pellets were washed with PBS with 1% BSA, pelleted and resuspended in 100ul staining solution consisting of 10ug/ml propidium iodide and RNase A in PBS with 1% BSA. Cells in staining solution were diluted with 500ul PBS and analyzed on a Coulter flow cytometer for fluorescence associated with propidium iodide staining.

## Western Blotting

Cells on 6-well tissue culture dishes were treated with the indicated concentrations of drugs for 48 hours at which time the cells were approximately 80% confluent. Cells were lysed with 250  $\mu$ l of 1X sample buffer containing phosphatase and proteinase inhibitors, sonicated, and then boiled for 10 min. Gel loading was normalized by quantification of protein by Bradford assay. 20  $\mu$ l was resolved on SDS-PAGE and subjected to immunoblot with the indicated antibodies. Band intensities on photographed film were measured using Image J software, a public domain application from NIH.

## RESULTS

### ***Phellodendron amurense* extract and berberine inhibit lung tumor cell proliferation**

To determine the effect of *P. amurense* extract and berberine on lung tumor cell proliferation, we treated p53 negative H1299 and p53 positive A549 lung tumor cell lines with 0, 2.5 or 5  $\mu$ g/ml of *P. amurense* extract or 0, 25, or 50  $\mu$ M berberine. Cells were counted, seeded at low density and monitored for proliferation using an MTS assay. Both *P. amurense* extract and berberine treatment caused dose dependent inhibition of proliferation of lung tumor cell lines (Fig. 1). Treatment with *P. amurense* extract at 2.5 and 5  $\mu$ g/ml inhibited proliferation by 58 and 68% respectively in A549 cells and by 18 and 44% respectively in H1299 cells. Treatment with berberine at 25 and 50  $\mu$ M concentrations inhibited proliferation by 90 and 100% respectively in A549 cells and by 76 and 100% respectively in H1299 cells. Apoptosis was undetectable at 24 or 48 hours at highest doses as measured by Caspase3/7 activity (data not shown).

### ***P. amurense* extract and berberine induce G1 cell cycle arrest in lung tumor cells**

To determine if the inhibition of proliferation observed was associated with cell cycle arrest, both H1299 and A549 cells were treated with either DMSO, 5  $\mu$ g/ml *P. amurense* extract or 50  $\mu$ M berberine for 48 hours. DNA content was determined by propidium iodide staining and flow cytometry to analyze the cell cycle distribution of treated and control cell populations. Signal within the 2N DNA content peak was quantified to identify those cells in the G0/G1 phase of the cell cycle. Approximately 45% of H1299 cells treated with DMSO alone had 2N DNA content, compared to 67% of those treated with *P. amurense* extract and 81% of those treated with berberine (Fig. 2A). Likewise, A549 cells with 2N DNA content were increased from 63% in control cells to 74% and 77% with *P. amurense* extract and

berberine treatment, respectively. Cyclin B1, a cell cycle regulating cyclin that is present only during late S phase and G2 phase of the cell cycle was quantified by western blot analysis (Fig. 2B). Both *P. amurense* extract and berberine treatment resulted in decreased cyclin B1 content in A549 cells. These results are consistent with the concept that treatment of lung tumor cells with *P. amurense* extract or berberine results in cell cycle arrest at G1 phase. These cell lines exhibited the expected p53 status, with A549 expressing p53 and H1299 not expressing p53 (Fig. 2C).

### ***P. amurense* extract inhibits sustained activation of Akt, CREB and MAPK**

In order to determine if the Akt/CREB signaling axis is affected by *P. amurense* extract in lung tumor cells, as has been observed in prostate tumor cells [3], A549 cells were treated with *P. amurense* extract or DMSO solvent for 48 hours and subjected to western blotting for total and phosphorylated Akt, CREB and MAPK. With *P. amurense* extract treatment, phospho-Akt, phospho-CREB and phospho-MAPK levels were decreased by 47%, 44% and 33%, respectively, when compared to DMSO control (Fig. 2D). No difference in acute activation of Akt, CREB, or MAPK was observed after starvation for 50 hours followed by serum stimulation and drug treatment for 1 hour (data not shown).

### **Dietary *Phellodendron amurense* extract and berberine are delivered equally to lung tissue and equally inhibit tumorigenesis *in-vivo***

To determine the berberine content of commercially available *P. amurense* extract, and to learn whether an amount of *P. amurense* extract that contains an equivalent amount of berberine has additional therapeutic effects, we determined the berberine content of *P. amurense* extract using HPLC analysis. Berberine was detected at 343nm. Standard curves were established using known concentrations of berberine and *P. amurense* extract, yielding linear regressions with slopes (concentration/signal) of 0.0757 for berberine and 0.2289 for *P. amurense* extract (Fig. 3). This indicates that the extract contains 33% berberine.

Based on our HPLC analysis and effective dosage in previous studies [11], we administered berberine to athymic nude mice in powdered diet at 1000 or 1800 parts per million (ppm), and *P. amurense* extract at 3000 or 5400 ppm to give equivalent amounts of berberine. One week after initiation of these diets or a control diet consisting of base powdered diet alone, all mice were injected sub-cutaneously in the right flank with  $1 \times 10^6$  A549 lung tumor cells. Mice were monitored regularly for health, weight and tumor volume. All mice appeared healthy and gained weight appropriately throughout the study (data not shown). Both *P. amurense* extract and berberine inhibited tumor growth in a dose dependent manner. At 14 days post injection, the high doses of both *P. amurense* extract and berberine resulted in dramatically and significantly ( $p < 0.001$ ) decreased tumor volume at 9.4 and 6.4 mm<sup>3</sup> respectively compared to 58.9 mm<sup>3</sup> in control mice (Fig. 4). 3000 ppm *P. amurense* extract and 1000 ppm berberine also significantly decreased tumor growth at 28.8 ( $p < 0.02$ ) and 20.4 ( $p < 0.05$ ) mm<sup>3</sup> respectively compared to control mice. Hence, treatment with *P. amurense* extract did not appear to offer a distinct advantage to administering pure berberine.

To determine the physiological concentration of berberine in the nude mice used in these experiments, we performed HPLC analysis on pooled lung homogenates. Lungs were pooled

in order to achieve the concentrations necessary to detect berberine by HPLC. Lung homogenates from mice fed control diet, 3000 ppm *P. amurensis* extract and 1000 ppm berberine contained 0, 455 and 518 ng/ml berberine respectively (data not shown). Thus mice fed *P. amurensis* extract in doses intended to replicate the amount of berberine administered in the pure berberine group, exhibited similar physiologic concentrations of berberine in the lung. This demonstrates that berberine administered as a pure compound or in the extract is equally bioavailable and detectable by HPLC.

## DISCUSSION

We demonstrate the novel finding that berberine is equally effective in inhibiting the growth of lung tumors whether administered as pure berberine or *P. amurensis* extract, and that berberine is effective when administered orally through the diet. Furthermore, we show that this effect is in association with G1 cell cycle arrest and is observed regardless of p53 status. Sub-G1 DNA content, suggesting apoptosis, was only observed at the highest concentrations of berberine. The concentration of berberine used in previous studies demonstrating apoptosis in lung tumor cells [11] was equal to the highest dose we used, up to twice that dose. Berberine has been shown to induce mitochondrial dysfunction and apoptosis at higher doses in tumor cells [19] and even in promonocytic cells [20], although this raises concerns about toxicity at such doses. Berberine and the class of compounds known as protoberberines have topoisomerase poisoning properties [21]. Although berberine has low activity on topoisomerase compared to other protoberberines [21], this activity may play a role in its p53 dependent induction of apoptosis at higher doses. Our results suggest that berberine may have safer effects through cell cycle arrest in a p53 independent manner at a sub-apoptotic dose. Consistent with published data, we observed a greater effect on proliferation in A549 cells than in H1299 cells. The difference in effect between these two cell lines may be due to the difference in p53 status; however, variation between cell lines is expected, and it cannot be concluded that these differences reflect p53 dependent effects. The growth and cell cycle arrest observed cannot be compared for quantitative differences between *P. amurensis* extract and berberine because, as we later determined by HPLC analysis, the extract used in the assays contains 33% berberine. This resulted in a much lower berberine concentration in the *P. amurensis* extract treated cells than in the berberine treated cells.

Our *in-vivo* tumorigenesis models suggest that an equivalent concentration of pure berberine is equally effective as *P. amurensis* extract in preventing tumor growth; supporting the notion that berberine is indeed the major bioactive and anti-tumorigenic constituent of *P. amurensis* extract. Although we were unable to accurately measure dietary consumption, we casually observed slightly less consumption of the high dose *P. amurensis* extract, which may have resulted in less efficacy in preventing tumor growth. However, we did show similar levels of berberine in the lungs of mice fed berberine and *P. amurensis* extract. The levels of berberine found in the experimental lungs were less than 1 ug/ml, which is well under the NCI maximum concentration guidelines for potential anti-cancer drugs. No toxicity was observed with *P. amurensis* treatment in any of the mice as monitored by body weight. The highest dose used in this study is 50 fold less than the maximum safe oral dose in mice [22]. We demonstrate that *P. amurensis* extract inhibits Akt, CREB and MAPK phosphorylation in



lung tumor cells, further implicating this signaling axis in cell cycle arrest by berberine. Further investigation of these signaling molecules as underlying effectors of cell cycle arrest by berberine is warranted.

Demonstration that berberine is effective against tumorigenesis when administered orally through the diet, either alone or as a part of a whole plant extract, implicate berberine as a bio-available and effective anti-tumor compound. These results warrant further clinical investigation of berberine and berberine containing herbal products as natural and safe chemopreventive and therapeutic agents against lung cancer. Further mechanistic studies of berberine mediated anti-tumorigenic effects may delineate important targets for lung cancer prevention or treatment.

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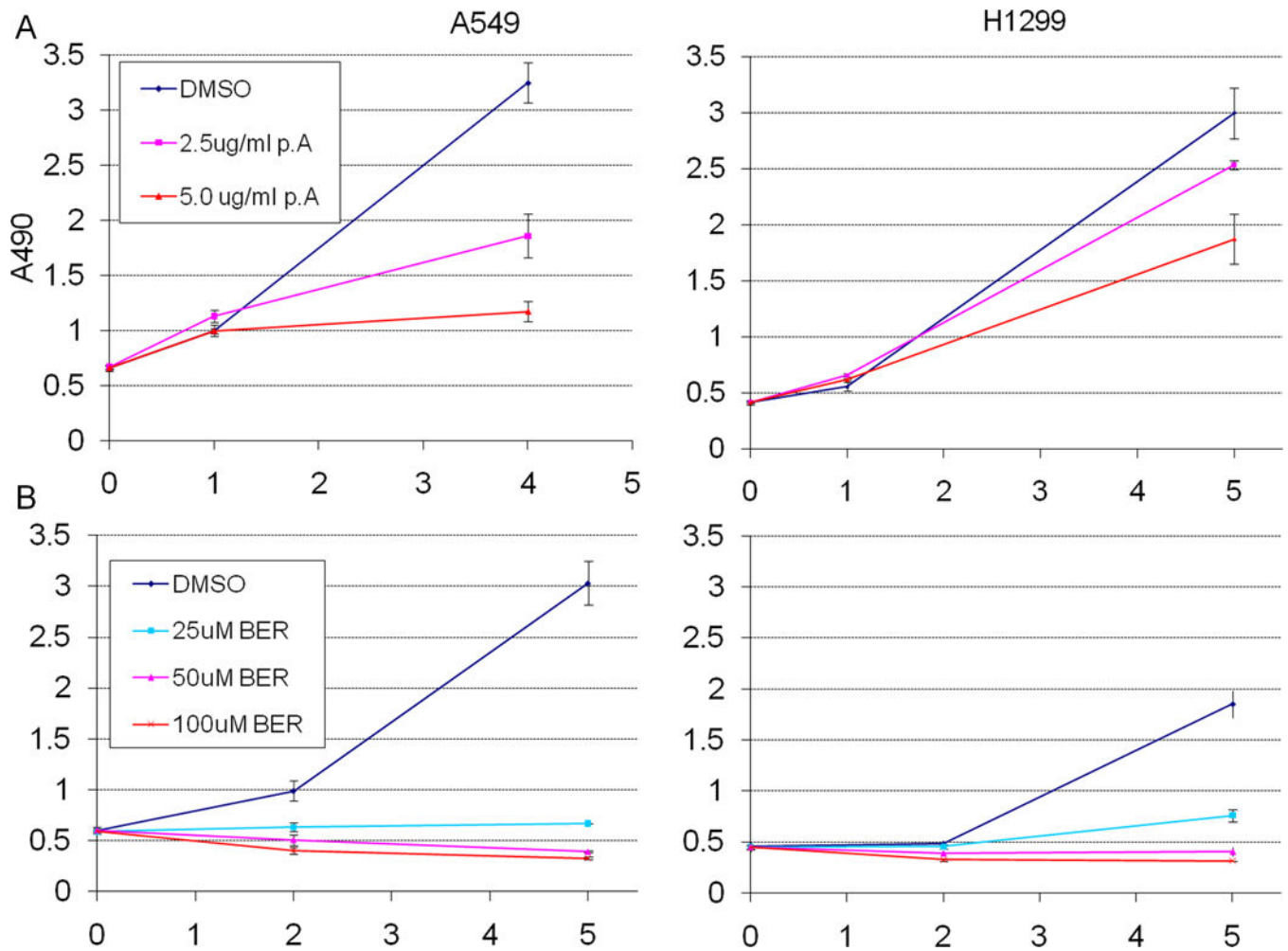
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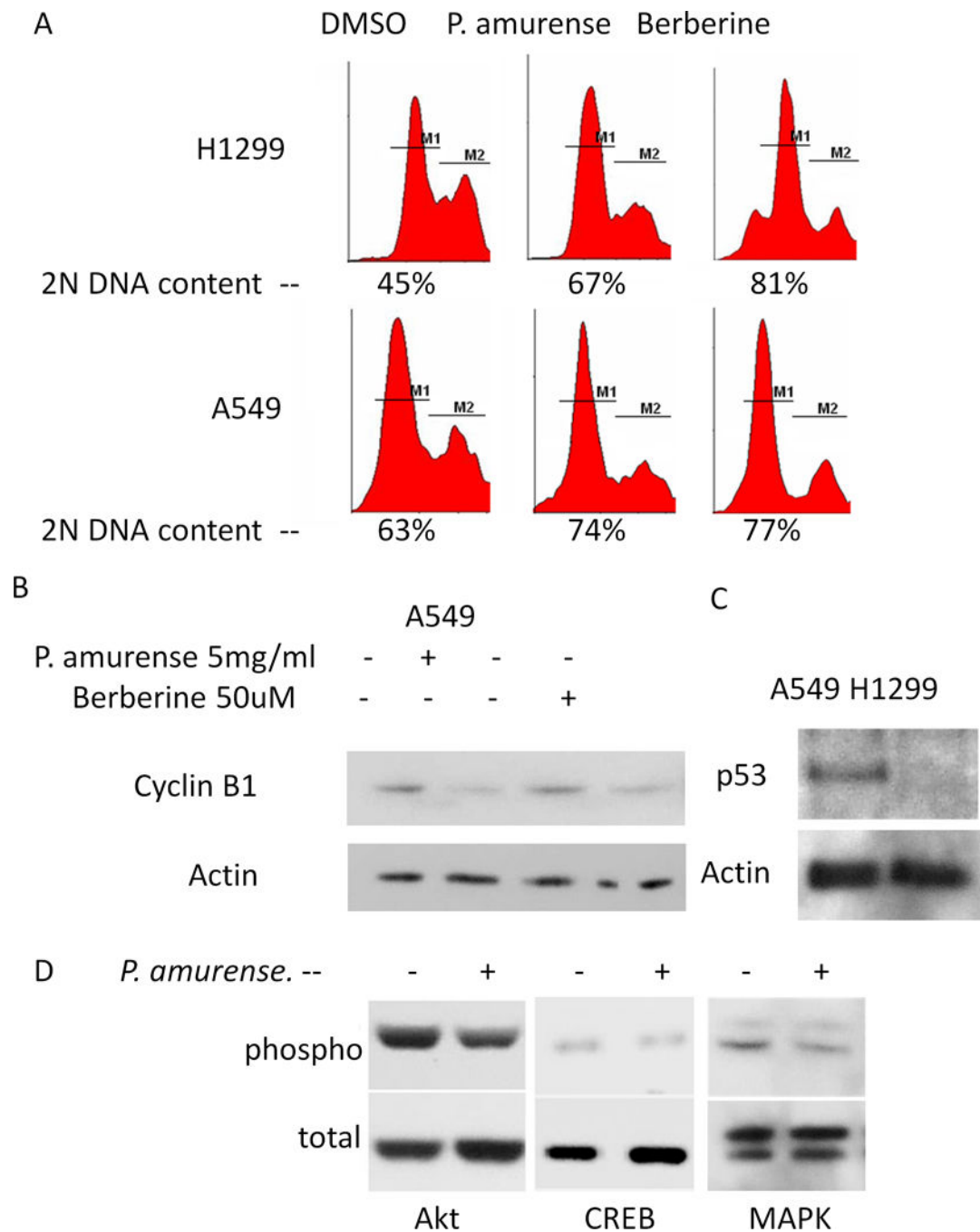
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**Figure 1. *P. amurensis* extract or Berberine treatment causes dose dependent inhibition of growth of lung tumor cell lines**

Cell proliferation over 5 days in culture as measured by MTS assay after (A) treatment with *P. amurensis* extract at 2.5 and 5 µg/ml in A549 cells and H1299 cells. (B) treatment with berberine at 25 and 50 µM in A549 cells H1299 cells. Error bars represent standard deviations of biological triplicates. All treatment groups were significantly less proliferative than controls ( $p < 0.005$  at day 5). The horizontal axes represent days post-seeding.



**Figure 2. *P. amurensis* extract and berberine induce G1 cell cycle arrest and decrease Akt, CREB and MAPK phosphorylation in lung tumor cells**

(A) DNA content analysis by PI staining and flow cytometry demonstrates accumulation of cells in G1 phase upon treatment of either *P. amurensis* extract or berberine. (B) A representative immunoblot for cyclin B1 in cells treated for 48 hours. *P. amurensis* extract and berberine treatment both resulted in decreased accumulation of Cyclin B1 indicating G1 arrest. (C) p53 status of A549 and H1299 cells represented by immunoblot. (D) Representative immunoblots for total and phosphorylated Akt, CREB and MAPK

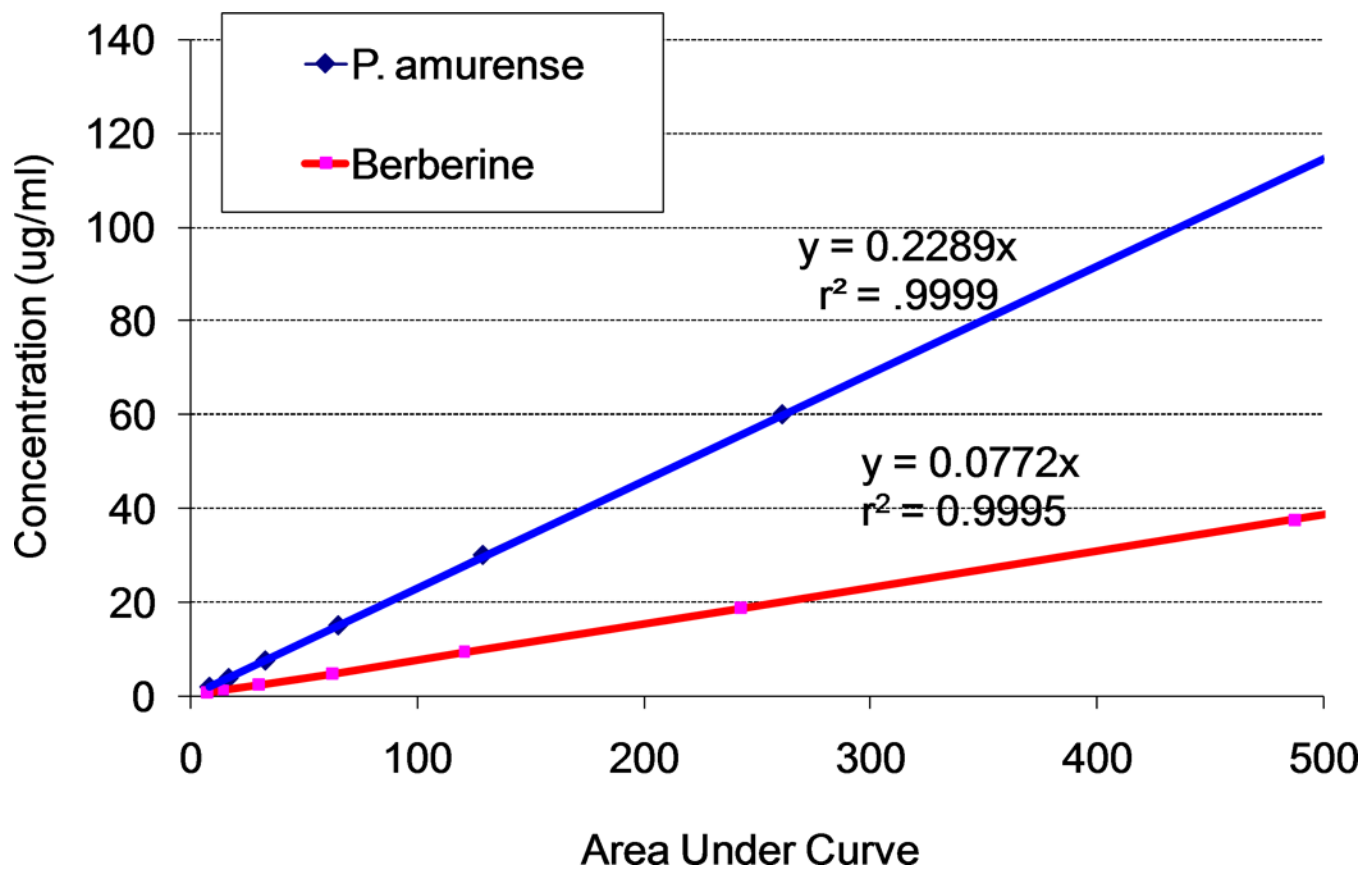
demonstrates decreased phosphorylation in A549 cells treated with 5µg/ml *P. amurensis* extract for 48 hours.

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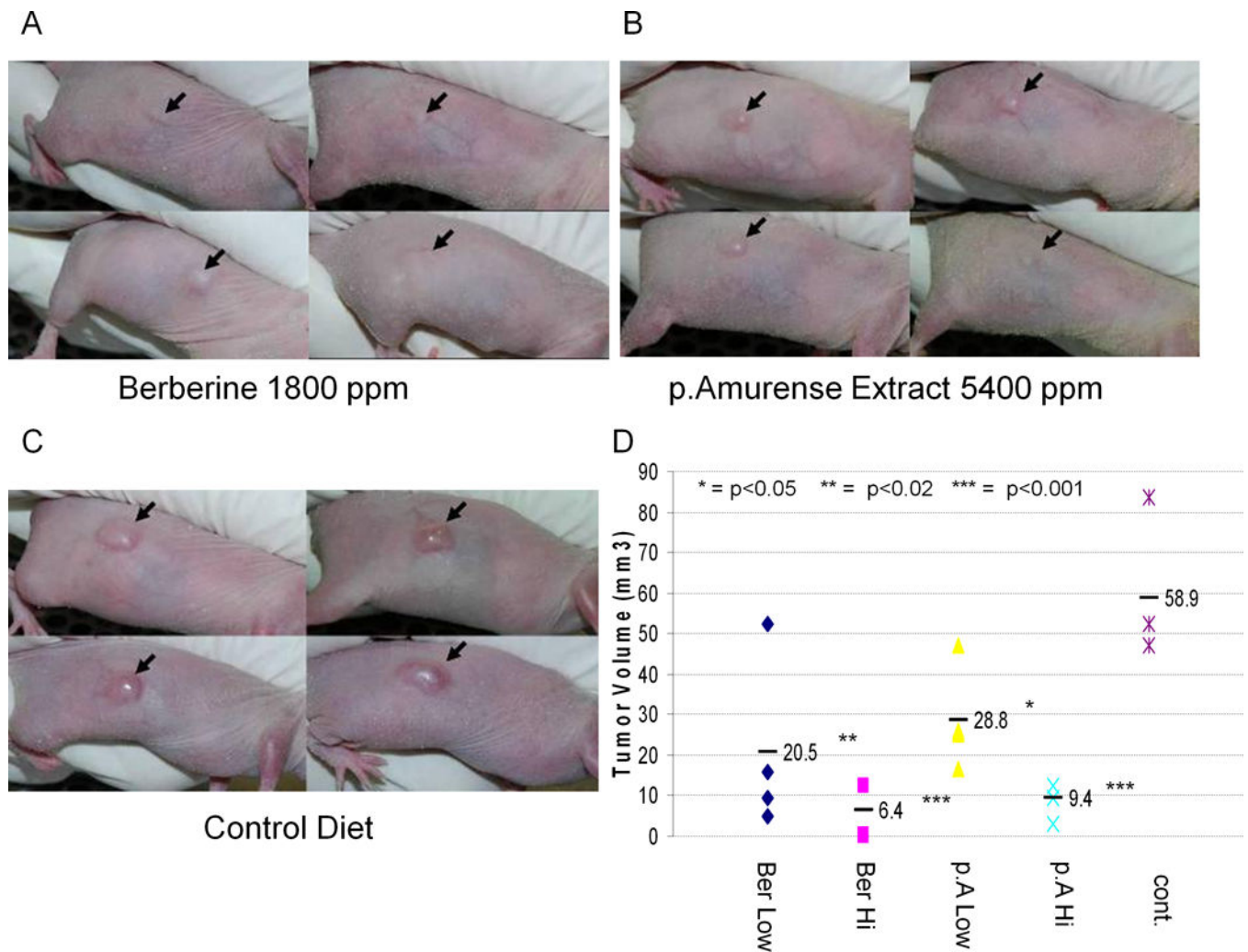
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**Figure 3. HPLC Detection of Berberine**

Standard curves for detection of the peak representing berberine in *P. amurensis* extract and pure berberine, yielding linear regressions with slopes (concentration/signal) of 0.0757 for berberine and 0.2289 for *P. amurensis* extract. The concentration of berberine in *P. amurensis* extract is 33% of that in purified berberine. Berberine was detected at 343nm.



**Figure 4. Dietary *P. amurensis* Extract and Berberine Equally Inhibit Tumorigenesis of Lung Tumor Cells *in-vivo***

(A) Xenografted A549 tumors in athymic nude mice fed 1800 ppm berberine in powdered diet *ad-libitum*. (B) Tumors in mice fed 5400 ppm *P. amurensis* Extract. (C) Tumors in mice fed control diet. (D) Tumor volumes at 14 days after injection of tumor cells, showing significant dose dependent inhibition of tumor growth by both treatments. n=4 mice per group.