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## Effects of four Chinese herbal extracts on drug-sensitive and multidrug-resistant small-cell lung carcinoma cells

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**Abstract** *Purpose:* We examined the pharmacology, cell biology and molecular biology of small-cell lung carcinoma cells treated with four extracts of Chinese herbal medicines. Many cancer patients take these medicines, but their effects at the cellular level are largely unknown. We were especially interested in the effects on drug-resistant cells, as resistance is a significant clinical problem in lung cancer. *Methods:* Drug-sensitive (H69), multidrug-resistant (H69VP) and normal lung epithelial cells (BEAS-2) were exposed to extracts from two plants used in Chinese herbal medicine for lung cancer: *Glycyrrhiza glabra* (GLYC) and *Olenandria diffusa* (OLEN), and to extracts of two commercially available combinations of Chinese herbal medicines, SPES (15 herbs) and PC-SPES (8 herbs). Cytotoxicity was measured in terms of cell growth inhibition ( $IC_{50}$ ). The kinetics of DNA fragmentation after exposure to the herbal extracts was measured by BudR labeling followed by ELISA. Apoptosis was measured by the TUNEL assay followed by flow cytometry. Expression of apoptosis- and cell cycle-related genes was measured by reverse transcription of mRNA followed by filter hybridization to arrays of probes and detection by chemiluminescence. *Results:* In each case, the four herbal extracts were equally cytotoxic to H69 and H69VP and less cytotoxic to BEAS-2. All four extracts induced DNA fragmentation in the lung carcinoma

cells. The kinetics showed DNA fragments released to the medium (an indication of necrosis) in GLYC-exposed cultures, but inside the cells (an indication of apoptosis) in OLEN-, SPES- and PC-SPES-exposed cultures. TUNEL analysis confirmed that exposure to the latter three extracts, but not to GLYC, led to apoptosis. Compared to untreated and GLYC-treated cells, H69 and H69VP cells treated with OLEN, SPES and PC-SPES showed elevated expression of a number of genes involved in the apoptotic cascade, similar to cells treated with etoposide and vincristine. *Conclusion:* The Chinese herbal medicine extracts OLEN, SPES and PC-SPES are cytotoxic to both drug-resistant and drug-sensitive lung cancer cells, show some tumor cell specificity compared to their effect on normal cells, and are proapoptotic as measured by DNA breaks and gene expression. The reaction of the tumor cells to these extracts was similar to their reaction to conventional chemotherapeutic drugs.

**Keywords** Lung cancer · Drug resistance · Chinese herbal medicines

### Introduction

Small-cell lung cancer (SCLC) accounts for about 20% of all lung cancers and is aggressive, with a 5-year survival rate at diagnosis of less than 10% [19]. Patients commonly present with disseminated disease, and so treatment is by chemotherapy, with combinations commonly involving cisplatin, etoposide, doxorubicin, 5-fluorouracil and taxol [12]. Unfortunately, this treatment eventually becomes ineffective because most SCLC tumors develop multidrug resistance [18]. Since there are many resistance mechanisms [15], overcoming resistance is a major clinical challenge.

Faced with palliative care, many cancer patients use alternative medicines, including herbal therapies [6, 23]. Among these therapies, traditional Chinese medicine is probably the best established and codified,

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dating back several thousand years. The theoretical basis of this medical system concerns the opposing forces of *yin* and *yang*, generative and destructive cycles, and a life force, or *qi* [2]. Specific herbal extracts, and combinations, have been devised to treat specific diseases including cancers [11, 27]. Although there is some empirical evidence that the herbs are effective in treating cancer, their mechanisms of action at the cellular level are largely unknown. This takes on added importance if patients are combining their use with conventional chemotherapy. Also, the effects of herbal extracts on drug resistance have not been reported.

We investigated the effects of four Chinese herbal medicines on three cell lines: SCLC, multidrug-resistant SCLC and normal lung epithelium. Two of the four extracts were from single plants often prescribed for lung cancer, *Glycorrhiza glabra* (GLYC) and *Olenandria diffusa* (OLEN). The other two were from commercially available combinations of herbs called SPES and PC-SPES. OLEN (Chinese name *Bai hua she-she cao*) has antitumor, antimutagenic and immunostimulating activities [1, 25, 29]. GLYC (Chinese name *gan cao*) is antiinflammatory, antitumorigenic and antimutagenic [7, 11]. SPES, developed by BotanicLabs (Brea, Calif.), is in the form of capsules containing lyophilized extracts of 15 Chinese herbs and has been shown to decrease pain in patients with advanced cancers [14]. Among the herbs in SPES are the immune stimulant *Cistanche desitcola*, the antitumor agent *Rabdosia rubescens* and the antiinflammatory agent *Zanthoxylum nitidum* [1, 11]. PC-SPES, also prepared by BotanicLabs, contains eight herbs, including the antiandrogenic *Serenoa repens* that inhibits prostatic hyperplasia [21], the protein kinase C inhibitor *Scutellaria baicalensis* [13], the antitumor agent *Panax ginseng* [30], and *Glycorrhiza glabra* (see above).

While rigorous evidence for clinical antitumor efficacy of GLYC, OLEN and SPES is lacking, there is some evidence for the efficacy PC-SPES. In both prostate cancer cells and animal models, PC-SPES is proapoptotic and cytotoxic [8, 9, 10, 28]. Clinical trials have indicated that it is effective in lowering levels of prostate-specific antigen and in stopping tumor growth in both androgen-dependent and androgen-independent prostate cancer [4, 5, 17, 20, 26].

Although cancer patients doubtless use herbal extracts, there is scant preclinical or clinical data on the effects of these extracts on SCLC. Our goal was to begin this analysis with an examination of the pharmacology (cytotoxicity), cell biology (method of cell death) and molecular biology (gene expression) in drug-sensitive and drug-resistant SCLC cells.

## Materials and methods

### Extracts and chemicals

GLYC and OLEN were obtained as dried plants from Jen-On Medical Group (Monterey Park, Calif.). For preparation of an

extract, 0.5 g was ground to a fine powder with a mortar and pestle and suspended in 30 ml distilled water. In Chinese medicine, the plant extracts are consumed as teas after heating. Therefore, the suspension was incubated with shaking at 70°C for 18 h. Following centrifugation at 1500 g for 10 min, the supernatant was sterilized by filtering through a 0.45- $\mu$ m filter using a syringe. The resulting extract was adjusted with distilled water to 17 mg/ml based on the original plant material. The extracts were stored at 4°C for up to 1 week until use.

SPES and PC-SPES were obtained from BotanicLabs (Brea, Calif.) as capsules. The extraction method used has been employed in previous studies of these preparations [8, 9, 10]. The contents of one capsule (320 mg) were dissolved in 1 ml 95% ethanol and the suspension incubated with shaking at 37°C for 1 h. After centrifugation at 3000 g for 10 min, the supernatant was filter-sterilized as above. The final concentration was 300 mg/ml based on the original plant material. The extracts were stored at -20°C for up to 1 week until use.

### Cell cultures and cytotoxicity measurement

NCI-H69 SCLC cells were grown at 37°C in an atmosphere containing 5% CO<sub>2</sub> as a suspension in AIM-V serum-free medium (Life Technologies, Grand Island, N.Y.). A multidrug-resistant cell line (H69VP) selected in etoposide [16] was also grown in AIM-V and showed cross-resistance to etoposide (9-fold), doxorubicin (20-fold) and vincristine (10-fold) (data not shown). These cells have multiple mechanisms of drug resistance, including alterations in topoisomerase II and expression of the membrane pumps, MDR1 and MRP. BEAS-2 normal lung epithelial cells [22] were grown in DMEM-F12 supplemented with 10% fetal bovine serum.

For cytotoxicity testing, extracts were added as indicated to logarithmically growing cells in 1-ml cultures containing 6000 cells/ml. After 4 days of continuous exposure, cells were counted in a Coulter Z-1 counter. Counts were validated microscopically by hemocytometer after staining with trypan blue. All experiments were done in triplicate and repeated at least three times. The IC<sub>50</sub> values, defined as the concentration of extract that had reduced cell counts on day 4 by 50%, were calculated as compared to solvent controls. Mean values were calculated and compared by ANOVA for the three cell lines for that extract.

### Cell death assays

The mechanism of cytotoxicity was investigated by the kinetics of cellular DNA fragmentation (Boehringer-Mannheim kit, Indianapolis, Ind.). Briefly, 5 $\times$ 10<sup>5</sup> cells/ml were incubated for 16 h at 37°C in an atmosphere containing 5% CO<sub>2</sub> in AIM-V medium containing 10  $\mu$ M BudR. The cells were washed and resuspended at 10<sup>5</sup>/ml in BudR-free medium, and 200  $\mu$ l of this culture was incubated with herbal extract at 2 $\times$ IC<sub>50</sub> for the indicated time, after which 100  $\mu$ l of the culture medium was removed for quantitation of DNA fragments by ELISA. This is a measure of cell necrosis. DNA fragments within the cells, generated by apoptosis, were measured following cell lysis in bovine serum albumin (BSA)/Tween 20 at 21°C for 30 min. Following centrifugation, the lysate was used for ELISA.

ELISA was performed in a round-bottomed microtiter plate coated with anti-DNA antibody (mouse anti-human DNA monoclonal, clone MCA-33) overnight at 4°C. Following blocking with BSA, 100  $\mu$ l labeled DNA fragment solution was added to the coated wells followed by incubation for 90 min at 21°C. The DNA was fixed and denatured by microwave irradiation at 500 W for 5 min. Mouse anti-BudR (mouse monoclonal BMG 6HB) conjugated with peroxidase was added and, following incubation in the dark for 120 min at 21°C, the reaction was stopped by the addition of 500  $\mu$ l concentrated H<sub>2</sub>SO<sub>4</sub>. The resulting color was quantitated by absorbance at 450 nm. Extract-treated samples were compared to untreated controls as background.

Apoptosis was confirmed by TUNEL analysis. Logarithmically growing cells were exposed to herbal extract at 1 $\times$ IC<sub>50</sub> for

3 h. They were then washed twice in phosphate-buffered saline (PBS) containing 1% BSA and resuspended in PBS/BSA at  $5 \times 10^5$  cells/ml. To 500  $\mu$ l cells, 100  $\mu$ l 4% paraformaldehyde was added and the cells were fixed at 21°C for 1 h. Following washing in PBS, the cells were resuspended in 100  $\mu$ l cold permeabilization buffer (0.1% Triton X-100, 0.1% sodium citrate) for 2 min at 4°C. The cells were washed twice in PBS and then resuspended in 50  $\mu$ l TUNEL mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP (Boehringer-Mannheim kit). After incubation in the dark at 37°C for 1 h, the cells were counterstained with propidium iodide and analyzed by flow cytometry.

#### Gene expression analysis

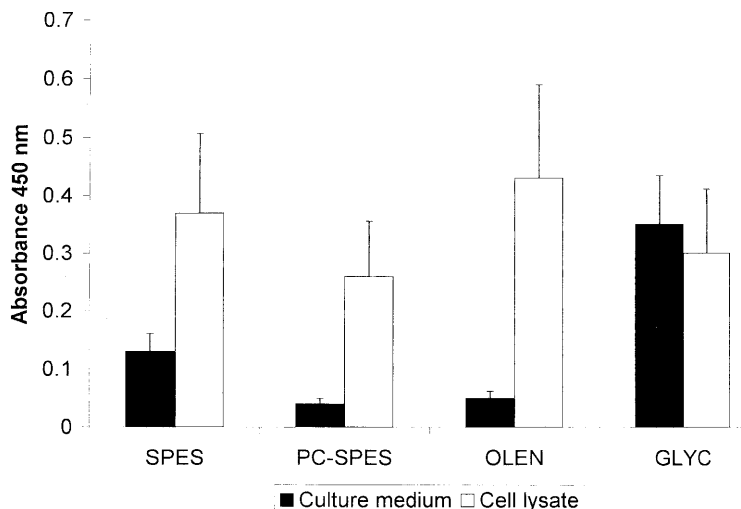
Cultures (6 ml) of logarithmically growing cells ( $5 \times 10^5$  cells/ml) were incubated in medium containing herbal extract at  $1 \times \text{IC}_{50}$  at 37°C in an atmosphere containing 5%  $\text{CO}_2$  for 3 h. Following washing twice in PBS, the cell pellets were frozen at -70°C overnight. RNA was isolated by the RNeasy Mini protocol (Qiagen, Valencia, Calif.). Generally this method yielded 0.5  $\mu$ g RNA. This amount of RNA was reverse-transcribed to cDNA and labeled with biotin-dUTP, and hybridized to denatured probes to apoptosis- and cell cycle-related genes on filters. Hybridization was detected by binding of alkaline phosphatase-streptavidin to the cDNA using a chemiluminescent substrate, CDP-Star (SuperArray, Bethesda, Md.), followed by exposure to X-ray film, generally for 1–2 min.

**Table 1** Cytotoxicities of herbal extracts in drug-sensitive and drug-resistant SCLC cells, and normal lung epithelial cells. Values are mean  $\pm$  SD  $\text{IC}_{50}$  (mg/ml). Control cultures (no extract) had  $2 \times 10^5$  cells/ml (H69, H69VP) and  $4 \times 10^4$  cells/ml (BEAS-2) after 4 days

Extract	Cell line		
	H69, SCLC	H69VP, resistant	BEAS-2, normal
<i>Olenandria diffusa</i>	$0.80 \pm 0.24$	$0.99 \pm 0.41$	$6.53 \pm 1.66^*$
<i>Glycorrhiza glabra</i>	$0.90 \pm 0.39$	$0.79 \pm 0.32$	$8.30 \pm 2.85^*$
SPES	$1.05 \pm 0.57$	$1.71 \pm 0.74$	$6.75 \pm 2.25^*$
PC-SPES	$0.90 \pm 0.34$	$1.23 \pm 0.55$	$7.83 \pm 3.90^*$

\*Significantly different from the other two lines for that extract

**Fig. 1** DNA Fragmentation in H69 cells treated with herbal extracts. Triplicate 5-ml cultures ( $5 \times 10^5$  cells/ml) were pre-labeled with BUdR and then exposed to herbal extract at the  $\text{IC}_{50}$  for 90 min. DNA fragments released to the medium or within the cells (lysate) were determined by ELISA. Error bars indicate SD. Controls treated with solvent only gave absorbances of 0.02 (medium) and 0.03 (lysate)



Control gene targets on each filter included  $\beta$ -actin and GAPDH as positive controls, and pUC18 as a negative control. The following cell cycle- and apoptosis-related genes were hybridization targets: bad, bax, bcl-2, bcl-w, bcl-x, caspases 1–10, c-myc, E2F, fas, fas ligand, gadd45, iNOS, mdm2, NFkB, p21<sup>Waf1</sup>, p53, pig7, pig8, Rb, DR5, trail, casper, CRADD, DAXX, IAP-1, IAP-2, NIK, TNFR2, TRAF2, TRAF3, TRAF5, and DR5. The intensity of hybridization was scored visually in tandem duplicate spots on the filter in relation to the positive controls.

#### Results

Our initial experiments tested the cytotoxicities of extracts of Chinese herbs on drug-sensitive and drug-resistant SCLC cells, as well as normal lung epithelial cells. As shown in Table 1, the four herbal extracts were cytotoxic at low concentrations. These findings are similar to those found for SPES and PC-SPES in other tumor cell lines [8]. In each case, the  $\text{IC}_{50}$  values for multidrug-resistant cells (H69VP) were not different from those for the drug-sensitive cells (H69). Also, the  $\text{IC}_{50}$  values for normal lung epithelial cells were significantly higher than those for the tumor cell lines.

We then investigated the mechanism of cytotoxicity using the kinetics of DNA fragmentation as an indication of apoptosis (fragments slowly formed within cells) or necrosis (fragments rapidly formed and released from the damaged cells). Cells were incubated in herbal extract for between 30 min and 8 h, and then the DNA fragments were estimated by ELISA in both the culture medium and cell lysates. Figure 1 shows representative data for H69 cells exposed to the four extracts for 90 min. With three of the extracts (SPES, PC-SPES, OLEN), most of the DNA fragments came from within the cells (lysates), indicating apoptosis. However, with GLYC, the fragments were released to the medium from damaged cells, indicating necrosis. Similar results were obtained with H69VP cells (data not shown).

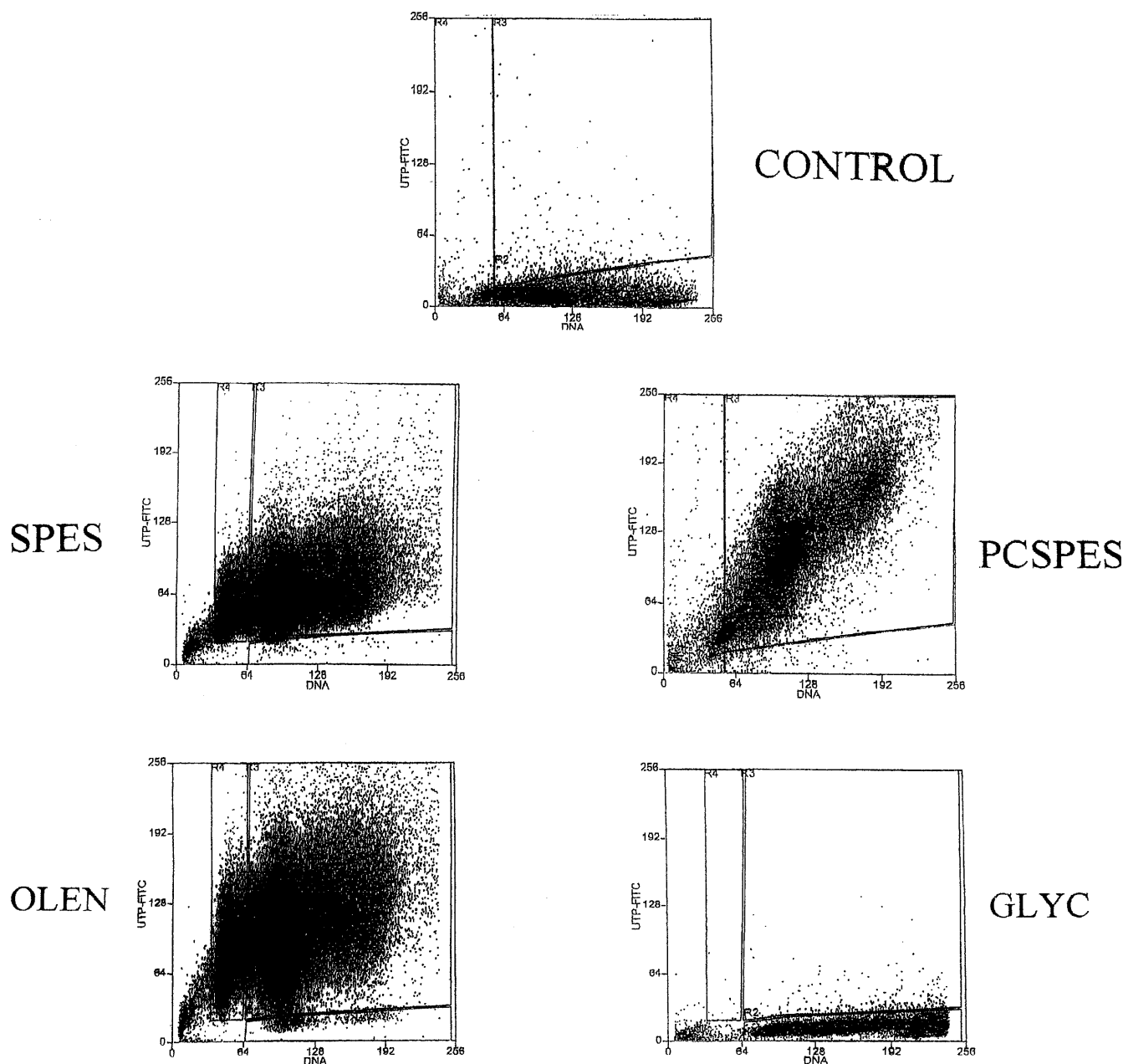
These cytotoxicity experiments were followed by TUNEL assays of DNA breaks in situ. Compared to

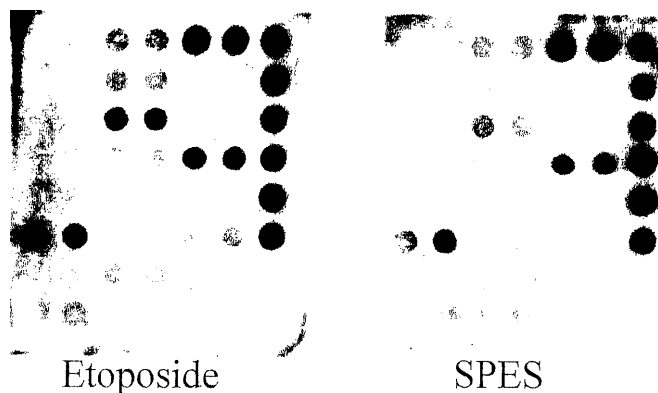
untreated controls (1–3% apoptotic cells in four experiments), SCLC cells treated with SPES (58–85%), PC-SPES (63–77%) and OLEN (49–81%) showed TUNEL positivity, while cells treated with GLYC did not (1–2%) (Fig. 2). Similar results were obtained with H69VP cells (data not shown).

We analyzed gene transcription in response to herbal extracts using GeneArrays. These arrays have tandemly arranged genes related to the cell cycle and

apoptosis. We performed these experiments twice with similar results. Figure 3 shows representative array autoradiograms of H69 cells exposed to etoposide or SPES. Our results for H69 cells are summarized in Table 2. While untreated controls had detectable expression of only a few of the genes studied, cells treated with conventional chemotherapeutic drugs (etoposide and vincristine) showed strong signals for a number of genes involved in cell cycle regulation and apoptosis. Cells treated with OLEN, SPES and PC-SPES showed a pattern similar to those obtained with the two drugs. In general, cells treated with GLYC, did not appear to show this transcription pattern, and instead showed strong expression of the antiapoptosis gene, *bcl-2*.

**Fig. 2** TUNEL analysis of H69 cells treated with herbal extracts. Cells were exposed to extract at  $2 \times IC_{50}$  for 3 h and then fixed, permeabilized and stained with a fluorescent TUNEL reaction. After counterstaining with propidium iodide, the cells were analyzed by flow cytometry. Controls were untreated H69 cells from the same culture





**Fig. 3** Autoradiograms of gene arrays of H69 cells treated with etoposide (left) and SPES (right). Cells were exposed for 90 min to 1  $\mu$ M etoposide or 1 mg/ml SPES. RNA was isolated and reverse-transcribed to biotin-labeled cDNA, which was hybridized to DNA targets on filters. Hybridization was detected by AP-streptavidin and chemiluminescence. The spots on the right row of each filter are positive controls (GAPDH and  $\beta$ -actin). The other genes are in tandem duplicate spots. The positive spots on both filters are: *top row* mdm2 and caspase 10, *third row* bax, *fourth row* p21, *sixth row* trail. The one positive spot pair (*second row*) seen only in the etoposide-treated cells is the gene for etoposide-specific mRNA

## Discussion

We undertook these studies for two reasons. First, there is an ancient tradition of Chinese herbal medicine which is based on its own theories and philosophy [2], and we wanted to begin to understand in Western medical terms the effects of the herbal remedies. These herbs are used as mixtures, and while there has been some progress in breaking them down to individual chemical constituents [11], our studies focused on them as they are used in traditional practice. Our second motivation was that many cancer patients use these herbs along with conventional treatments [6, 23], and understanding the cellular effects of the herbs could provide important information for clinical decision-making.

The cytotoxicity data (Table 1) showed that the four herbal extracts were indeed cytotoxic to tumor cells, more so on a dosage basis than to normal lung epithelial cells, indicating some tumor specificity and possibly a favorable therapeutic index. The similarities in  $IC_{50}$  between drug-sensitive and multidrug-resistant cells indicates that these extracts are not affected by the drug resistance mechanisms displayed in H69VP cells, including overexpression of two drug transporters, MDR1 and MRP.

The kinetics of DNA fragmentation showed that extracts SPES, PC-SPES and OLEN were proapoptotic, while GLYC was pronecrotic (Fig. 1). This was confirmed by TUNEL staining in situ (Fig. 2). Most conventional chemotherapeutic drugs are proapoptotic [3], and a group of apoptotic and cell cycle regulatory genes are upregulated in the cells in response to the drugs [24]. We found that the pattern of expression of some of the genes expressed in response to the three proapoptotic herbal extracts was similar to that observed in these cells after exposure to conventional chemotherapeutic drugs (Fig. 3, Table 2). The response to GLYC alone appeared to be different, at least at the time-point analyzed in these experiments. Interestingly, GLYC is part of PC-SPES, which was proapoptotic, while GLYC alone was not. Perhaps other components of PC-SPES are responsible for its proapoptotic activity. While these results do not show how the herbal extracts damage cells, they suggest that H69 cells respond to the damage at the molecular (transcription) and cellular (apoptosis) levels in a manner similar to their response to chemotherapy.

Our studies indicate the potential usefulness of these Chinese herbal extracts in treatment of SCLC, particularly drug-resistant disease.

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**Table 2** Expression of apoptosis-related genes in H69 cells treated with etoposide, vincristine or herbal extracts, or untreated (CON) (0 undetectable expression, 1 low expression, 2 high expression)

	Control	Etoposide	Vincristine	OLEN	GLYC	SPES	PC-SPES
Mdm2	2	2	2	2	2	2	2
Bcl-2	1	0	1	0	2	0	0
Bcl-x	0	0	0	1	0	0	0
Bax	0	1	2	2	2	1	1
Bad	0	1	1	1	0	1	1
P21	0	2	1	2	1	2	2
Caspase6	0	2	2	2	0	2	2
Caspase9	0	1	1	2	0	2	2
Caspase10	0	1	1	2	0	2	2
Trail	0	0	1	1	1	1	1
Pig8	0	2	1	0	0	0	0
c-myc	0	2	1	0	1	0	0
CRADD	0	1	2	0	1	1	0
TRAF5	1	0	0	1	1	1	0
TNFR2	2	1	1	0	2	2	0
NIK	0	1	1	1	0	1	1

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