CANCER BIOLOGY & THERAPY
2016, VOL. 17, NO. 10, 1022–1029
http://dx.doi.org/10.1080/15384047.2016.1219810

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RESEARCH PAPER

Synergistic effect of fenretinide and curcumin for treatment of non-small cell lung cancer

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ABSTRACT

Curcumin and fenretinide are 2 well-known and promising chemotherapeutic compounds via various molecular mechanisms. However, the anticancer capacity of either curcumin or fenretinide is limited. This prompted us to examine the combined anticancer effects of curcumin and fenretinide. Our results demonstrate for the first time that there is synergistic anticancer effect of combined treatment with these 2 agents, leading to enhanced cytotoxicity and enhanced expression level of pro-apoptotic protein cleaved PARP in non-small cell lung cancer (NSCLC) cells while showed little toxicity to rat cardiomyoblast normal cells. The combination treatment was also demonstrated to inhibit lung carcinoma growth *in vivo*. Furthermore, we show that fenretinide or the ER stress inhibitor 4-PBA decreased curcumin-induced Glucose-regulated protein 78 (GRP78) upregulation, and produced a similar enhanced cytotoxic effect. In addition, GRP78 knockdown by siRNA also enhanced the cytotoxic effect of curcumin in A549 and H1299 cells. Our findings suggest that the 2 small molecules, when used in combination, can potentially be effective therapeutic agents for treating NSCLC, at least in part, by regulating endoplasmic reticulum (ER) chaperone protein GRP78.

Abbreviations: NSCLC, non-small cell lung cancer; GRP78, Glucose-regulated protein 78; ER, endoplasmic reticulum; 4-PBA, 4-phenylbutyrate; LLC, Lewis lung carcinoma

ARTICLE HISTORY
Received 13 January 2016
Revised 10 July 2016
Accepted 29 July 2016

KEYWORDS
Curcumin; fenretinide; GRP78; non-small cell lung cancer

Introduction

Lung cancer is the leading cause of cancer-related death among males in both developed and developing countries, and has surpassed breast cancer as the leading cause of cancer death among females in more developed countries.[1,2] Improving survival in lung cancer is still a challenge for modern oncology considering that less than 15% of patients are alive 5 y after diagnosis.[3] Based on differences in biological characteristics, therapy and prognosis, lung cancer falls into 2 major classes: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC).[4] NSCLC accounts for approximately 85% of all lung cancer cases.[5] A review of the recent clinical evidence indicates that drug combination chemotherapy could significantly improve therapeutic efficacy in the treatment for NSCLC.[6] Hence, developing new therapeutic approaches based on combination use of anticancer agents, and improving therapeutic efficacy, are urgently needed.

Curcumin, an active component of Curcuma longa, is a yellow polyphenol and first purified in 1815.[7,8] Curcumin is commonly used as a flavoring agent in food and ingested daily in many parts of the world. It has been reported to possess several promising biological properties, including antioxidant,[9] anti-inflammatory,[8] anticancer,[10] antimicrobial properties[11] and chemopreventive activities.[12] Some studies also show that curcumin exhibits ROS-inducing or pro-oxidant activity.[13,14] It was reported that, in phase I/II human clinical trials, curcumin showed good tolerance, low toxicity, and therapeutic potential against a wide variety of cancers at different sites.[15] However, the anticancer utility of curcumin is limited due to its low bioavailability and poor aqueous solubility. In recent years, an increasing number of preclinical studies have clearly demonstrated that therapy of using curcumin combined with various anticancer agents represents one way of overcoming its limitations in order to establish more efficient and less toxic therapeutic approaches to cancer.[15,16]

Fenretinide [N-(4-hydroxyphenyl) retinamide] is a synthetic retinoid acid (RA), which was first synthesized by RW Johnson Pharmaceutical Research Institute in the 1960s.[17] Nowadays, fenretinide is capturing the attention of cancer researchers and diabetes investigators from all over the world.[18] The mechanisms of fenretinide-induced cell apoptosis in cancer cells are complex, mainly involving Rac **(regulatory subunit of NADPH oxidase complex) activation** in neck squamous cell carcinoma cells[19] and mTOR signaling pathway in NSCLC

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cells.[20] In a phase III breast cancer prevention trial, the analysis of a 15-year follow-up showed that fenretinide significant reduces the risk of second breast malignancies in premenopausal women.[21] However, the poor bioavailability of fenretinide limits its further therapeutic development for clinical use.[22]

Glucose-regulated protein 78 (GRP78), also referred to as Bip, is one of the best-characterized endoplasmic reticulum (ER)-chaperone proteins, acting as a central regulator of ER. Although the development of anticancer drug resistance is likely to be multifactorial, the induction of GRP78 may be a major contributing cause in a wide range of cancers, including lung cancer, breast cancer, prostate cancer and glioma.[23] Therefore, downregulation of GRP78 may represent a new approach to increasing the chemosensitivity of anticancer agent-induced cell death.

Accumulating evidence from preclinical studies has demonstrated that chemotherapeutic agents combined with curcumin[24-26] or fenretinide[27,28] for cancer therapy often provide promising results. Here, we tested the combined effect of curcumin and fenretinide on the growth of NSCLC both *in vitro* and *in vivo*. For the first time, we show that fenretinide could increase curcumin sensitivity to chemotherapy both *in vitro* and *in vivo*, and describe the underlying mechanism by which this action occurred.

Materials and methods

Chemicals and reagents

Curcumin, fenretinide and 4-phenylbutyrate (4-PBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The purity of all the chemicals used in this study was > 98%. RPMI Medium 1640, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine Serum (FBS), 0.25% trypsin-EDTA (1X) and penicillin-streptomycin were obtained from Gibco/Invitrogen (Carlsbad, CA, USA). PARP, GAPDH and β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). GRP78 antibody, GRP78 siRNA and scrambled siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

The NSCLC A549 was grown in RPMI 1640 medium with 10% FBS, 100 U/ml penicillin-streptomycin. H9c2 cardiomyoblast cells and mouse Lewis lung carcinoma (LLC) cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin-streptomycin. Cells were cultured at 37°C with 5% CO^2 and appropriate humidity. The cells were used until they reached 7080%– confluence.

MTT assay

Cells were seeded at 1×10^4 cells per well in 96-well plate and incubated overnight. The cells were exposed to curcumin or fenretinide alone, or in combination. As the control, cells were incubated with fresh medium. Cell viability was determined after 24 h of incubation by replacing the medium with 100 μl fresh medium prior to the addition of 20 μl of MTT (5 mg/ml). After 4 h of incubation at 37°C, the medium was removed and 100 μl of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan followed by gently shaking the plate. The absorbance at 570 nm was measured with a microplate reader.

Hoechst 33342 staining

To analyze the apoptotic cells, Hoechst 33342 staining was applied. Briefly, cells were cultured in 96-well plates for 24 h as described for the MTT assay. After 24 h of treatment with curcumin or 4-PBA, alone, or in combination, the cells were incubated with Hoechst 33342 (10 μg/ml) for another 15 min at 37°C, and then visualized by the In Cell Analyzer 2000 system. Apoptosis was assessed according to the method of a previous study,[29] based on stereotypic morphological changes, including chromatin condensation, nuclear fragmentation cytoplasmic shrinkage, and the formation of apoptotic bodies (with nuclear fragments). At least 12 fields in each group were observed and at least 400 cells per field were counted. The apoptotic cells were analyzed using Developer Toolbox software and apoptosis (%) was calculated as: apoptotic cells/total cells counted$\times$100%. The quantification process was blinded to avoid experiment bias.

siRNA transfection

According to the manufacturer's instructions, using lipofectamine 2000 liposome, the GRP78 siRNA and scrambled siRNA were transfected into the A549 cells. The expression levels of GRP78 were detected with Western blotting to determine the effect of RNA interference. At 24 h thereafter, cells were seeded into 96-well plates at a concentration of 1×10^5 cells in 0.1 ml DMEM/well and incubated overnight, and then treated with curcumin or DMSO (vehicle) for 24 h and analyzed by MTT assay.

Protein extraction and western blot analysis

After treatment, A549 cells were collected and washed with phosphate-buffered saline (PBS). Pre-cooled cell lysis buffer with PMSF and cocktail were added for 30 min. After centrifugation at 12,500 g and 4°C for 20 min, the supernatant was separated. Protein concentrations were determined with the BCA Protein Assay Reagent Kit (Pierce-Thermo Scientific, Rockford, IL, USA). The extracted proteins were boiled in loading buffer at 95°C for 5 min. Protein samples were electrophoresed on 8% SDS–polyacrylamide gel, and then transferred to polyvinylidene difluoride (PVDF) blotting membrane. After blocking with 5% skim milk PBST at room temperature for 1 h, the membranes were incubated with different primary antibodies overnight and then washed with PBST (0.1% Tween-20 in PBS) thrice. After that, the blots were incubated with secondary antibodies labeled with horseradish peroxidase at room temperature for 1 h. The membranes were washed again thrice in PBST buffer. The transferred proteins were detected by an enhanced-chemiluminescence (ECL) system (GE Healthcare, Little Chalfont,

Buckinghamshire, UK). Semi-quantifications were performed with densitometric analysis by Quantity One software (Bio-Rad Laboratories, Inc., Hercules, California, USA).

LLC mice model

All protocols were performed in accordance with guidelines established by the Guide for the Care and Use of Laboratory Animals of University of Macau. LLC cells in logarithmic phase were used in this study. Adherent cells were digested, harvested, counted and re-suspended in PBS. The density of cells was then adjusted to 1×10^7 cells/ml. Tumor cell suspension (200 μl/mouse) was implanted carefully subcutaneously into the right side of the back of C57BL/6 mice weighing 20–22 g. Five days after LLC inoculation, mice were randomly divided into 4 groups (6–8 mice/group) and given an intraperitoneal injection of vehicle (PEG 400), curcumin (Cur, 40 mg/kg), fenretinide (Fen, 1 mg/kg) or combination every 2 d. The tumor volumes were measured every 2 d with a caliper and calculated according to the formula [(length$\times$width^2)/2], where length stands for the larger tumor diameter and width represents the smaller tumor diameter. After 25 d of treatment, mice were sacrificed under deep anesthesia. Tumors were immediately harvested at necropsy, and their weights were determined on the balance. All mice were kept at a constant temperature of 25°C, under a 12-h light/12-h dark cycle with 60% relative humidity.

Statistical analysis

Data are presented as the means ± standard deviation (SD) from at least 3 independent experiments. Differences between groups were compared by one-way ANOVA followed by Turkey's multiple comparison tests. A p-value of less than 0.05 was considered statistically significant.

Results

Fenretinide enhances the cytotoxic effect of curcumin in NSCLC cells

A549 and LLC NSCLC cells were exposed to various concentrations of curcumin (10–30 μM), fenretinide (2–6 μM) or their combination for 24 h, and cell cytotoxic activity was determined. As shown in Fig. 1A, the combination of both agents produced a restraining effect on cell proliferation. This observation is consistent with the results of the MTT assay (Fig. 1BC). In particular, at the combinatorial concentrations of 20 μM curcumin and 6 μM fenretinide yielded a strikingly cytotoxic effect, going down to about 10% viability (Fig. 1B) in A549 cells. However, at the concentration of curcumin and fenretinide, curcumin resulted in 44% viability while fenretinide yielded about 53% viability. A similar result was observed when another NSCLC cell line H1299 was analyzed (Fig. S1). These combined data indicated that fenretinide enhanced the cytotoxic effect of curcumin in NSCLC cells.

Curcumin and fenretinide combination treatment show low toxicity in H9c2 cells

To investigate the cytotoxic effects of curcumin (10–30 μM) in combination with fenretinide (2–6 μM) on normal cells, these agents were applied to H9c2 cells, and the cell viability was analyzed by MTT assay. As shown in Fig. 2, curcumin up to 30 μM, or low-concentration curcumin (10–20 μM) combined with fenretinide (2–6 μM) showed no significantly cytotoxic effect in H9c2 cells ($p > 0.05$). In contrast, high-concentration curcumin (30 μM) combined with fenretinide (4–6 μM) showed cytotoxicity. It is worth noting that the combination of 20 μM curcumin and 4 μM fenretinide, which produced the greatest improvement on apoptosis in both cancer cell lines, showed no toxicity to H9c2 cells, suggesting that it may be highly active in chemotherapy and has low toxicity to normal cells.

Fenretinide decreases curcumin-induced GRP78 upregulation and increases curcumin-induced PARP cleavage

In order to clarify the key molecular-mediated mechanism of the effect conferred by fenretinide on curcumin treatment in A549 and H1299 cells, the expression level of GRP78 and Cleaved PARP was determined by Western blotting. As shown in Fig. 3 and Fig. S2, it was found that 20 μM curcumin alone significantly increased the expression of GRP78 and cleaved PARP ($p < 0.05$), while 4 μM fenretinide had no obvious influence on the expression of these 2 proteins ($p > 0.05$). In the combination treatment group, GRP78 expression level was remarkably reduced compared to the curcumin treatment group (Fig. 3AB and Fig. S2AB); and cleaved PARP expression level was dramatically elevated (Fig. 3CD and Fig. S2CD), which was consistent with toxicity data observed using MTT assay (Fig. 1BC and Fig. S1). Therefore, the regulation of GRP78 and cleaved PARP may be necessary to induce toxicity in NSCLC after combination treatment with curcumin and fenretinide.

Four-PBA plus curcumin exhibits a similar improvement in anticancer effects to that of fenretinide

We replaced fenretinide with 4-PBA, an ER stress inhibitor that can suppress GRP78 expression. The combination of curcumin and 4-PBA produced a similarly enhanced cytotoxic effect in A549 (Fig. 4AB) and H1299 cells (Fig. S4). In addition, 4-PBA significantly reduced GRP78 expression in a highly similar manner to fenretinide (Fig. 4C and Fig. S3) in both NSCLC cell lines. Taken together, these results indicated that GRP78 may act as a key modulator of curcumin-induced apoptosis in A549 and H1299 cells and inhibition of curcumin-induced GRP78 upregulation play an important role in improvement of cytotoxicity.

GRP78 knockdown enhances the cytotoxic effect of curcumin in NSCLC cells

We studied the cell viability in scrambled-siRNA and GRP78 knockdown by siRNA after the addition of different

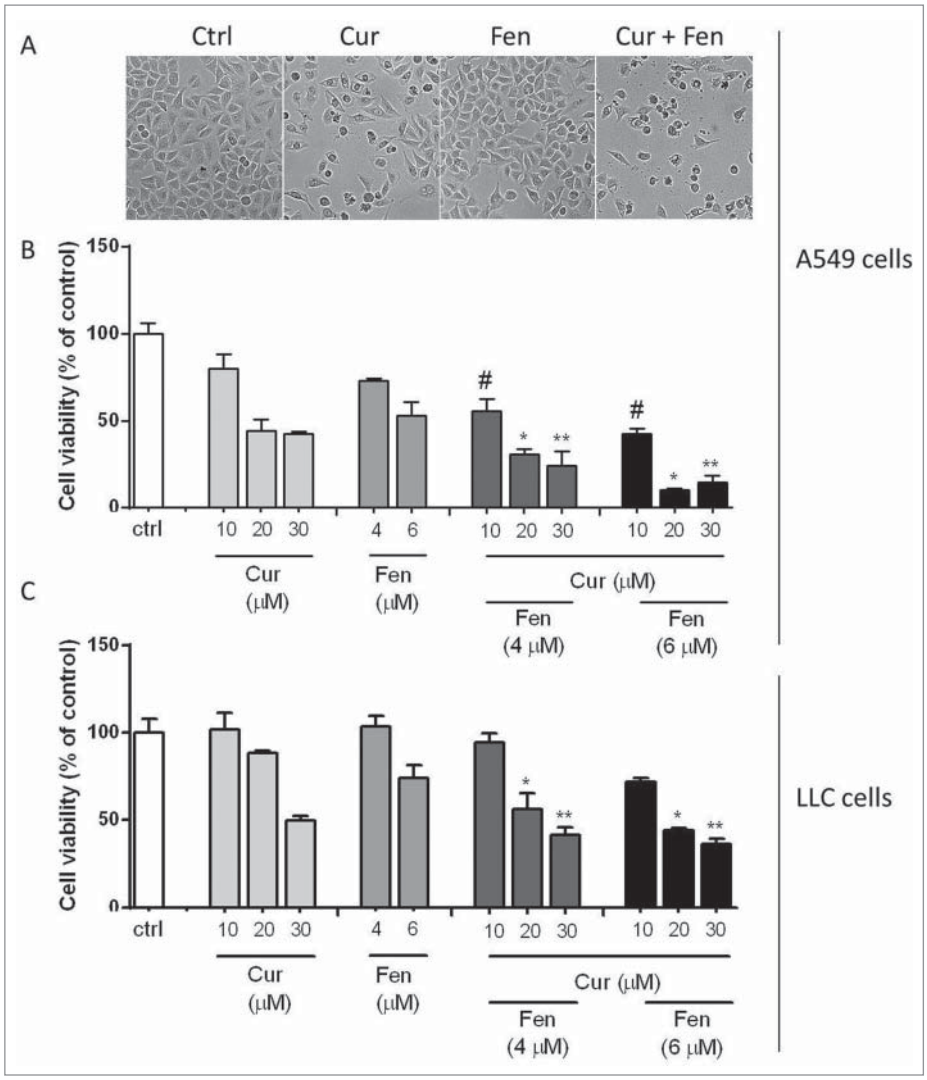

Figure 1. Effect of the combination of curcumin and fenretinide on the growth of A549 and LLC cells *in vitro*. A549 cells and LLC cells were treated with different concentrations of curcumin (Cur), fenretinide (Fen) or both in combination (Cur+Fen) for 24 h. Representative images of A549 cells after various treatments (A). Cell viability of A549 cells (B) and LLC cells (C) was determined by MTT assay. $^{\#}p < 0.05$ versus 10 μM curcumin treatment group, $^{*}p < 0.05$ vs. 20 μM curcumin treatment group, $^{**}p < 0.05$ versus 30 μM curcumin treatment group.

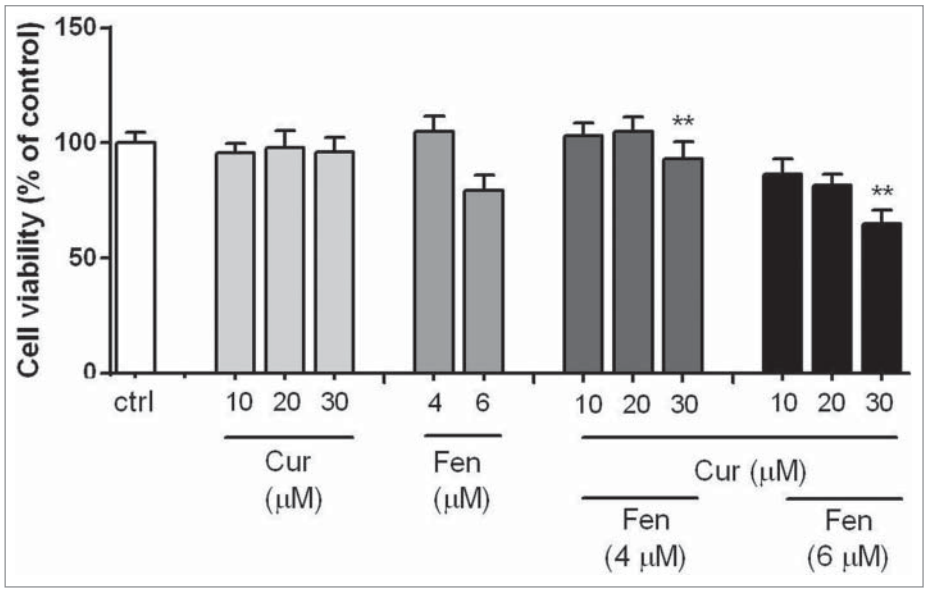

Figure 2. Effect of the combination of curcumin and fenretinide on the growth of H9c2 cells *in vitro*. H9c2 cells were co-treated with different concentrations of curcumin and fenretinide for 24 h. The effects on cell growth were evaluated by MTT assay. $^{**}p < 0.05$ vs. 30 μM curcumin treatment group.

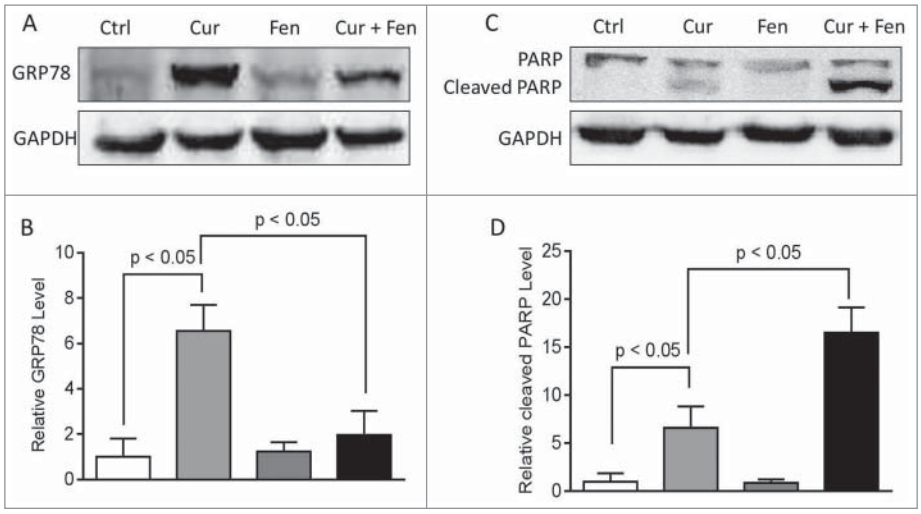

Figure 3. Effect of curcumin or fenretinide alone or in combination on the expression level of GRP78 and cleaved PARP in A549 cells. After exposure to each compound alone (20 μM curcumin, 4 μM fenretinide) or in combination (20 μM curcumin plus 4 μM fenretinide) for 24 h. Western blotting was carried out with antibodies against GRP78 (A) and PARP (C). Densitometry analysis of GRP78 (B) and PARP (D) was performed.

concentrations (20 μM or 30 μM) of curcumin for 24 h in A549 and H1299 cells. Our results show that cell viability was significantly decreased in the GRP78 knockdown cells compared with the scrambled control cells after 24 h of curcumin treatment (Fig. 5 and Fig. S5). This may imply that reduction of GRP78 expression could sensitize NSCLC cells to curcumin.

Curcumin and fenretinide in combination inhibit tumor growth in mouse xenograft tumor model

Based on the synergism of curcumin and fenretinide *in vitro*, we evaluated the anticancer activity of curcumin and fenretinide in mice with subcutaneously implanted LLC cells. As indicated in Fig. 6A, curcumin (40 mg/kg) or fenretinide (1 mg/kg) as single agent inhibited the tumor volume slightly. In contrast, curcumin combined with fenretinide exhibited distinct tumor growth inhibition to a greater extent than seen in fenretinide-treated mice ($p < 0.05$). No significant difference was observed between the curcumin treatment and combined treatment groups ($p > 0.05$). Moreover, Fig. 6C demonstrates that tumor weight significantly decreased in the combination group compared with the vehicle or fenretinide group ($p < 0.05$). The curcumin and fenretinide combination could therefore significantly decrease tumor growth *in vivo*, demonstrating the synergistic effect of these 2 drugs used in combination. Body weights of the mice in the curcumin and combination groups decreased slightly, while those in the fenretinide group did not show obvious changes (Fig. 6B).

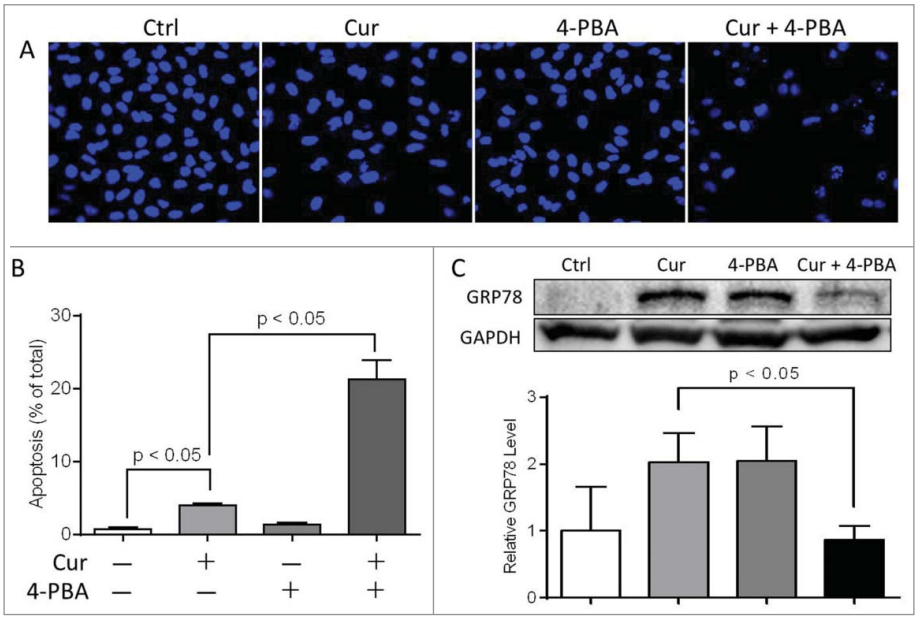

Figure 4. Effects of 4-PBA on curcumin treatment in A549 cells. A549 cells were treated with curcumin and 4-PBA (an ER stress inhibitor), alone or in combination for 24 h. The cells were observed by fluorescence microscope and effects on cell apoptosis were measured after Hoechst staining. (A) Representative images of A549 cells after various treatments. (B) Quantification of histograms indicates the percentage of apoptotic cells. (C) Western blotting was performed with antibody against GRP78.

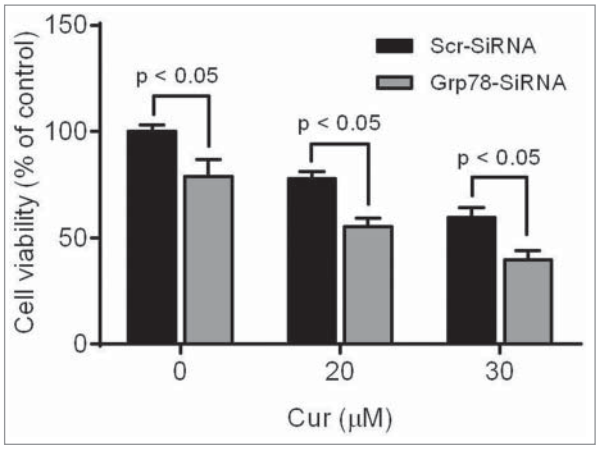

Figure 5. Effect of the GRP78 silencing with siRNA on the cytotoxic effect of curcumin in A549 cells. The GRP78 silenced cells were treated with different concentrations of curcumin for 24 h. The effects on cell growth were evaluated by MTT assay.

Discussion

This study highlights the intriguing pharmacological interaction of 2 well-known anticancer agents, curcumin and fenretinide, for treating NSCLC both *in vitro* and *in vivo*. An interesting new finding of our study is that curcumin combined with fenretinide has a synergistic effect for treatment of non-small cell lung cancer, leading to inhibited cell viability and enhanced expression level of pro-apoptotic protein cleaved PARP in NSCLC cells, as well as suppressing tumor volume in an LLC mouse model. In contrast to the lung cancer cell lines, simultaneous administration of curcumin and fenretinide showed little toxicity to rat cardiomyoblast normal cells at the same concentrations and exposure time in NSCLC cells. Therefore, the combination of the 2 agents may be an effective and alternative therapeutic approach for treatment of NSCLC.

Despite the current advances in chemotherapy options, highly effective, low-toxicity approaches for treating NSCLC are still needed. For these reasons, chemotherapeutic regimens for NSCLC use multiple drugs, including platinum agent and docetaxel, in combination. This approach is characterized by appreciable efficacy and acceptable toxicity, and has been suggested as the reference standard therapeutic approach.[30] In the present study, we showed that the viability of 3 NSCLC cell lines was significantly decreased by curcumin and fenretinide combination treatment compared to single agent treatment in a concentration-dependent manner (Fig. 1 and Fig. S1). Western blotting analysis showed that cleaved PARP (cPARP), a marker of apoptosis, was increased by combination treatment, but not by curcumin or fenretinide (Fig. 3CD and Fig. S2CD), which suggests that combination treatment initiates lung cancer cells apoptosis.

We show for the first time that fenretinide decreased curcumin-induced GRP78 upregulation was involved in NSCLC therapy. Previously, the role of GRP78 in cancer treatment with curcumin has raised considerable concerns for researchers. After curcumin exposure, the expression level of GRP78 was significantly up-regulated in in human NSCLC cell line NCI-H460 [31] and in MDA-MB-231 breast cancer cells.[32] Similarly, we found that curcumin treatment increased GRP78 expression in A549 and H1299 cells (Fig. 3AB and Fig. S2AB). Due to its anti-apoptotic property, elevated GRP78 is one of the critical pro-survival mechanisms of tumor cells, to survive and thrive

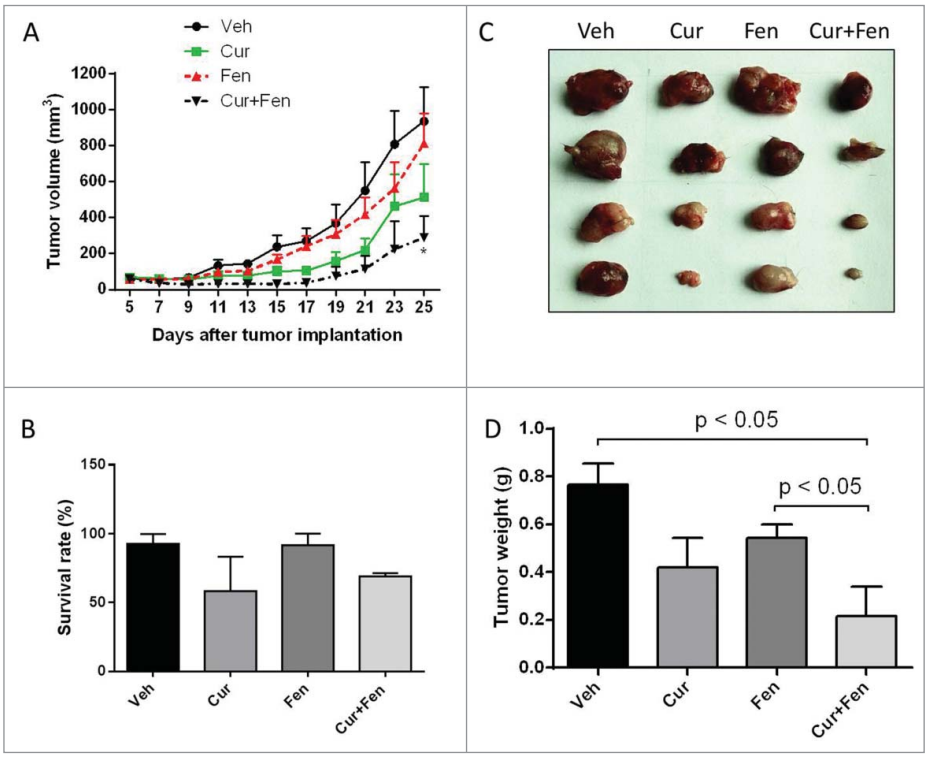

Figure 6. Effect of combination of curcumin and fenretinide on the growth of LLC xenograft in mice. The mice transplanted with LLC xenograft were randomly divided into 4 groups and given injection of vehicle (Veh), curcumin, fenretinide or combination every 2 d. After 20 d of treatment, tumors were harvested at necropsy. (A) Volumetric growth of LLC xenograft. (B) Mice survival rate and tumor weight (D) were determined. $^{*}p < 0.05$ versus fenretinide treatment group.

under detrimental microenvironmental conditions.[33] Recent progress of GRP78 research, utilizing overexpression and siRNA approaches, demonstrates that GRP78 contributes to tumor growth and endows cancer cells with drug resistance. [23]

In the current study, we found that fenretinide or the ER stress inhibitor 4-PBA decreased curcumin-induced GRP78 upregulation, and produced similar enhanced cytotoxic effects, reflecting by a pro-apoptotic action in A549 and H1299 cells (Fig. 4AB and Fig. S4). Furthermore, GRP78 knockdown by siRNA also enhanced the cytotoxic effect of curcumin in the both cells (Fig. 5 and Fig. S5). Thus, knock-down of this pro-survival ER stress GRP78 may contribute to the remarkably increased chemosensitization effect of combined curcumin plus fenretinide. Contrary to our findings, other studies have concluded that silencing GRP78 generated resistance to curcumin treatment of hepatocellular carcinoma[34] and colon cancer.[35] Collectively, it seems that GRP78 plays varying roles in different cancers, and perhaps in cancer progression.

In conclusion, this study shows that curcumin combined with fenretinide has a synergistic cytotoxic effect in NSCLC cells. Their combination also confers an enhanced anticancer effect in LLC mice. In addition, suppression of curcumin-induced ER protein, such as GRP78, by fenretinide may play equally important roles in its ability to confer the synergistic function, although the precise mechanism underlying the reciprocal signaling pathways modulated by these 2 agents has yet to be determined. Therefore, our study establishes a combinational therapy involving curcumin and fenretinide for the treatment of NSCLC.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

This work is supported by the research fundings from the National Natural Science Foundation of China (Grant No. 81660602), Overseas and Hong Kong, Macau Young Scholars Collaborative Research Fund by the National Science Foundation of China (Grant No. 81328025), Premier-Discipline Enhancement Scheme Supported by Zhuhai Government Fund, Macau Science and Technology Development Fund (Grant No. 078/2011/A3 and No. 134/2014/A3) and Research Committee, University of Macau (MYRG2015-00182-ICMS-QRCM and MYRG139(Y1-L4)-ICMS12-LMY).

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