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Curcumin enhances Vinorelbine mediated apoptosis in NSCLC cells by the mitochondrial pathway

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Abstract

Elderly lung cancer patients and those with poor performance status/co-morbid conditions are deprived of chemotherapy because of high toxicity of multidrug regimens. Human squamous cell lung carcinoma H520 cells treated with Curcumin were sensitized to the cytotoxicity caused by chemotherapeutic agent, Vinorelbine. Both caused apoptosis by increasing the protein expression of Bax and Bcl-xs while decreasing Bcl-2 and Bcl-X_L, releasing apoptogenic cytochrome *c*, and augmenting the activity of caspase-9 and caspase-3. Expression of Cox-2, NF-κB, and AP-1 was also affected. 23.7% apoptosis was induced in the H520 cells by treatment with Curcumin while Vinorelbine caused 38% apoptosis. Pre-treatment with Curcumin enhanced the Vinorelbine induced apoptosis to 61.3%. The findings suggest that Curcumin has the potential to act as an adjuvant chemotherapeutic agent and enhance chemotherapeutic efficacy of Vinorelbine in H520 cells in vitro. Thus, Curcumin offers the prospect of being beneficial in the above-mentioned patient groups.
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Keywords: Curcumin; Vinorelbine; NSCLC; Chemotherapy; Apoptosis; Mitochondrial pathway

Lung cancer is a leading cause of cancer deaths all over the world. It has the notorious distinction of being the most common cancer in males in developed as well as developing nations [1]. The role of chemotherapy in the treatment of patients with advanced non-small cell lung carcinoma (NSCLC) has been defined in a meta-analysis [2] wherein cisplatin based polychemotherapy is generally considered the most advisable frontline approach in these patients. However, some concerns still persist about the use of polychemotherapy especially with the inclusion of Cisplatin in particular subsets of patients with NSCLC such as older patients or those with poor performance status [3]. Physiological reduction of functional reserve and the presence of co-morbid conditions make elderly patients unsuitable for Cisplatin based multi-agent chemotherapy because of their high potential toxicity. [4–6]. Elderly Lung Cancer Vinorelbine Study Group (ELVIS), Multicenter Italian Lung Cancer in the Elderly Study (MILES), and some other studies concluded that a new single-agent like Vinorelbine which is a semi-synthetic vinca alkaloid is as effective and less toxic than the earlier Cisplatin based combinations and can be considered for elderly and unfit patients [7]. The benefit of chemotherapy to patients having poor performance status is still in doubt as some trials purposefully do not include such patients [8].

Curcumin (diferuloyl methane), a polyphenolic phytochemical, is a primary component of the dietary spice, turmeric. The pharmacological safety of Curcumin is well demonstrated by the fact that people in certain countries have consumed Curcumin as a dietary spice for centuries in amounts in excess of 100 mg/day without any side effects [9]. Curcumin is a well-known

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chemopreventive that blocks tumor initiation, promotion, and carcinogenesis [10,11]. The anticarcinogenic mechanisms of Curcumin action are not fully understood. It appears that Curcumin can suppress the cell growth pathway by inhibiting cellular protein kinases such as PKC, JNK, and the EGF receptor kinase, leading to growth inhibition [12–14]. It also has the ability to block the NF-κB cell survival pathway [15] and inhibit c-jun/AP-1 function [16]. Very few studies have looked into Curcumin's role as an adjuvant chemotherapeutic in cancer. This study is a step in this direction and the findings suggest that it has the potential to be used as a chemo-adjuvant in elderly patients or patients with poor performance status without increasing toxicity of the chemotherapeutic regimen.

Materials and methods

Cell culture and treatments. Human NSCLC (squamous cell carcinoma) cell line NCI-H520 was maintained in DMEM (Sigma) supplemented with 10% fetal calf serum and antibiotics in a humidified atmosphere of 5% CO_2 in air at 37 °C [17]. Logarithmically growing cells were treated with Curcumin (Sigma), Vinorelbine (a gift from Dabur Oncology Division, New Delhi, India), and a combination of the two for various time periods. Curcumin stock solutions were made up in dimethyl sulfoxide (DMSO) (Sigma); stored at −20 °C; and final working concentration used was 0.1% DMSO. Vinorelbine solutions were made in normal saline (0.9% w/v) just prior to each experiment. Combination treatment was given at optimized dose of Curcumin for 24 h followed by Vinorelbine for 24 h.

MTT assay. H520 cells were grown in 96-well microtiter plates and treated with Curcumin, Vinorelbine, and a combination of the two in replicate. Each experiment was repeated three times. One hundred microliters of MTT (3-[4,5-dimethyl thiazol-2yl]-2,5-diphenyltetrazolium bromide) (Sigma) solution (5 mg/ml) was added to each well followed by incubation for 4 h at 37 °C. The formazan crystals formed were dissolved in DMSO and the absorbance was measured at 570 nm using an ELISA reader [18].

In vitro drug dosage was standardized using the MTT assay, serum maximum levels (C_{max}), and area under curve (AUC) values. The concentration of Curcumin and Vinorelbine to be administered was chosen from a dose–response experiment showing moderate toxicity to the cells (data not shown). The lowest possible non-toxic dosage was used. A dose of 25 μM was fixed for Curcumin while for Vinorelbine 0.1 μg/ml was optimized. Duration of treatment for each drug was 24 h. Curcumin treatment alone (25 μM for 48 h) was used to assess the efficacy of the combination of Curcumin and Vinorelbine.

Fluorescence microscopy. H520 cells were grown on coverslips. After treatment with Curcumin, Vinorelbine, and a combination of the two for the designated time periods, cells were washed with PBS, fixed in 80% methanol, stained with 5 μg/ml propidium iodide (Sigma), and observed under a fluorescent microscope (Nikon Microphot FXA), and apoptotic cells were identified and counted [18].

Flowcytometric analysis. For flowcytometry, the cells were trypsinized, fixed in 70% ethanol, and incubated in the staining solution (20 μg/ml propidium iodide, 50 μg/ml RNase, 0.1% Triton X-100, and 0.1 mM EDTA) for 1 h at 4 °C in the dark. The cellular DNA content was analyzed using 488 nm for excitation and fluorescence measured at 600 nm by EPICS XL-MCL flowcytometer (Coulter Electronics, FL, USA). For each analysis 10,000 events were counted and the data were analyzed using Win MDI 2.8 software [18].

Immunocytochemical analysis. Protein expression and intracellular localization of transcription factors were assessed by this method. Cells were grown on coverslips and treated with Curcumin alone for 24 h, and for 48 h, Vinorelbine alone for 24 h and Curcumin (24 h) followed by Vinorelbine (24 h). The cells were fixed with 4% paraformaldehyde. Endogenous peroxidases were blocked with hydrogen peroxide in PBS containing 70% methanol and non-specific binding blocked using 5% bovine serum albumin (BSA). The cells were then incubated with antibodies against NF-κB and c-jun/AP-1 (Santa Cruz Biotechnology, CA) at a dilution of 1:100 for 24 h at 4 °C. Immunodetection was achieved by an avidin–biotin horseradish peroxidase-based colorimetric method (Vectastain Elite Kit from Vector laboratories, USA) with 3,3′-diaminobenzidine (DAB) as a chromogen and H_2O_2 as the substrate, followed by light counter staining with hematoxylin and examination under a microscope. The protein expression was determined semiquantitatively. Specimens were considered as immunopositive if at least 5% of the cells displayed distinct immunostaining. Scoring of immunopositivity was done on the basis of percentage of cells stained as well as the intensity of staining.

Western blot analysis. The cells were lysed in RIPA lysis buffer containing protease inhibitors. Equal amounts of protein extracts were electrophoresed on 10–15% SDS–polyacrylamide gels and electrotransferred to nitrocellulose membrane. The membrane was then incubated in 5% BSA for 3 h followed by overnight incubation with antibodies against rabbit Bcl-2, Bcl-X_L, Bcl-xs, p53, Bax, Cox-2, and poly ADP ribose polymerase (PARP) and β-actin (Santa Cruz Biotechnology, USA). β-Actin was used as a loading control. After washing, anti-rabbit alkaline phosphatase, conjugated antibody was added and incubated for 2 h. After washing, color development was done using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate from Promega Corporation, USA. The bands were analyzed and quantitated using scanning densitometer (Bio-Rad) [19].

Measurement of cytochrome c release. Cytosolic extracts of the cells were prepared as described [20]. The cytosolic fraction was collected by centrifugation (12,000 rpm for 30 min at 4 °C). One hundred and fifty micrograms of protein was electrophoresed on a 15% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. Cytochrome *c* was detected by Western blotting using mouse monoclonal anti-cytochrome *c* antibody (Santa Cruz Biotechnology, USA) (1:350 dilution) for 4 h. β-Actin was used as a loading control. After washing, anti-rabbit alkaline phosphatase-conjugated antibody was added and incubated for 2 h. After washing, color development was done using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate from Promega Corporation, USA. The bands were analyzed and quantitated using scanning densitometer (Bio-Rad) [19].

TUNEL assay. Apoptotic cells were visualized by the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique using the Dead End Colorimetric Cell Death Detection kit (Promega, USA) as described earlier [19]. The apoptotic index (AI) (number of apoptotic cells in 500 cells) was determined by microscopic examination of randomly selected fields containing at least 500 cells.

Caspase-3, -8, and -9 activity assay. Caspase-3, -8, and -9 activities were measured by using synthetic fluorogenic substrate (Ac-DEVD-AMC, substrate for caspase-3; Pharmingen, Germany; Ac-LETD-AFC, substrate for caspase-8, and Ac-LEHD-AFC, substrate for caspase-9; Genotech, USA) as described previously [17]. Amounts of fluorogenic AMC/AFC moiety released were measured using a spectrofluorimeter (ex. 380 nm, em. 420–460 nm for caspase-3; ex. 400 nm, em. 490–520 nm for caspase-8 and -9).

Statistical analysis. Results are expressed as means and standard error of mean (SEM). Statistical analyses were performed with Student's two-tailed paired *t* test using SPSS (Windows version 7.5). Values of $p < 0.05$ were considered statistically significant.

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