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A herbal medicine for the treatment of lung cancer

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Abstract

Lung cancer is the leading cause of cancer-related deaths throughout the world. Extracts of medicinal plants are believed to contain different chemopreventive or chemotherapeutic compounds. In this study, we determined the anti-cancer property of one of the traditional Indian medicine *Rasagenthi Lehyam* (RL) for the treatment of lung cancer. Two lung cancer cell lines (A-549 and H-460) and one normal bronchial epithelial (BEAS-2B) cell line were used to test the chemotherapeutic effect of RL. Out of five fractions of RL, chloroform fraction of RL (cRL) demonstrated a significant inhibition of cell proliferation and induction of apoptosis in A-549 and H-460 cells but not in normal BEAS-2B cells. The cRL fraction up-regulated the pro-apoptotic genes *p53* and *Bax* and induced caspase-3 activation, and down-regulated the pro-survival gene *Bcl-2* in both the lung cancer cell lines. Also, nuclear export of *p53* was seen in cRL-treated lung cancer cells. In addition, cRL induced G_2/M arrest of cell cycle and enhanced the radio-sensitivity of both the lung cancer cell lines. This study suggests that cRL may prove to be a potent anti-cancer agent that may be used for the treatment of lung cancer. However, further studies are required to bring cRL into the mainstream of medicine in the treatment of lung cancer. (Mol Cell Biochem **280:** 125–133, 2005)

Key words: anti-cancer activity, apoptosis, herbal medicine, lung cancer, radio-sensitisation

Introduction

Lung cancer is the most common cancer throughout the world, particularly in the United States [1] and it accounts for 14% of all cancers and 28% of all cancer-related deaths worldwide [2, 3]. Chemotherapy is the standard treatment for lung cancer patients, but in spite of its ability to improve the symptoms and the quality of life of the patients with lung cancer, only a minimal increase in survival rate can be achieved [4, 5]. Along with palliative care, many cancer patients tend to use alternative medicines, among which herbal therapies are more common [6, 7]. Natural products are lead molecules for many of the drugs that are currently in use [8]. Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical conditions; however, the scientific basis regarding their modes of action is limited. Extracts of medicinal plants are believed to contain different chemopreventive or chemotherapeutic compounds, which possess more than one mechanism of action.

The induction of apoptosis is known to be an efficient strategy for cancer therapy. Several studies have demonstrated that extracts from herbal medicines or mixtures have anti-cancer potential [9, 10] *in vitro* and *in vivo* [11, 12]. Recently, several dietary phytochemicals that play a significant role in the anti-carcinogenic process have been identified. Apoptosis is regulated by various genes such as *p53*, Bcl_2 and *Bax* [13, 14]

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and in this, *p53* gene has come to the forefront of cancer treatment, because it is commonly mutated and its functions are inhibited in many cancer types [15, 16]. The p53 tumour suppressor protein is a transcription factor that regulates several genes, especially those involved in the cell cycle, DNA repair and apoptosis [17]. The p53 protein can also activate the expression of Bax and mediate Bcl-2 suppression, leading to cellular apoptosis [18]. Regulation of apoptosis is a complex process and involves a number of cellular genes, including *Bcl-2* [19] and *Bcl-2*-related family members such as $Bcl\text{-}x_L$, $Bcl\text{-}x_s$, *Bad* and *Bax* [20]. *Bcl-2* and $Bcl\text{-}x_L$ exert their anti-apoptotic effect, at least in part by binding to Bax and related pro-apoptotic proteins. The members of *Bcl-2* family regulate the initiation of mitochondrial apoptotic pathway. The major function of *Bcl-2* is to inhibit apoptosis and to prolong cell survival. Over-expression of Bcl-2 protein is associated with enhanced oncogenic potential and poor response in lung cancer treatment [21]. Apoptosis proceeds through caspase activation cascades, known as the extrinsic and intrinsic pathways. The extrinsic pathway-induced apoptosis is mediated by receptors (FADD), which activate initiator caspase-8 or -10 signaling that leads to activation of executioner caspases such as caspase-3, -6, -7 and -9. Steps in the intrinsic pathway, which is induced by stress, radiation and chemotherapeutic drugs, include cytochrome-*c* release from mitochondria, caspase-9 activation and then activation of effector caspases, particularly caspase-3 [22].

Rasagenthi Lehyam (RL) is a *Siddha* medicine, which is a poly-herbal formulation for the treatment of cancer in India. Recently, we reported that chloroform extract of RL (cRL) inhibited the growth of prostate cancer cells and induced apoptosis in prostate cancer cell line PC-3 [23]. In this study, we demonstrate that cRL possess anti-tumor activity in A-549 and H-460 human lung cancer cell lines, which inhibits the pro-survival genes and up-regulates the pro-apoptotic genes. These results may advance our understanding of the molecular mechanisms of action of this herbal medicine in the treatment of lung cancer.

Materials and methods

Extraction of RL

A total of five extracts (*n*-hexane, chloroform, ethyl acetate, *n*-butanol and water) of methanolic fraction of RL were obtained, lyophilised and analysed adopting high performance liquid chromatography (HPLC) as described earlier [23].

Cell cultures

Two lung cancer cell lines, A-549 and H-460, obtained from American-type tissue culture (ATCC), were used to test the anti-tumour activity of RL fractions. Both the cell lines were maintained and propagated in RPMI 1640 medium containing 2 mM L-glutamine, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate and 10% fetal bovine serum (FBS). Normal lung epithelial cell line (BEAS-2b) was cultured in bronchial epithelial medium (Cambrex, MD) in monolayer. All the cell lines were maintained at 37 °C in 5% CO_2/95% air-humidified atmosphere.

Irradiation

A 100 kV industrial X-ray machine (Phillips, Netherlands) was used to irradiate the cultures at room temperature. The dose rate with a 2 mm Al plus 1 mm Be filter was $\sim$2.64 Gy/min at a focus-surface distance of 10 cm [23, 24].

MTT assay

MTT assays were performed as described earlier [24].

Clonogenic inhibition assay

The colony-forming assay was performed as described previously [23]. Two different cell concentrations in quadruplet sets were used for one dose point. After incubation for 10 or more days, each flask was stained with crystal violet, and the colonies containing more than 50 cells were counted. The surviving fraction (SF) was calculated as discussed earlier [23, 25].

Cell cycle analysis

The cell cycle analysis was carried out using propidium iodide (PI) staining and subsequent flow cytometry. Flow cytometry analysis was performed as described earlier [25].

Western Blot analysis

Total protein extracts from untreated cells or cells treated with cRL at various time intervals were subjected to Western Blot analysis as described earlier [25] using Bcl-2 monoclonal antibody (sc-509) (Santa Cruz, CA), Bax monoclonal antibody (sc-7480) (Santa Cruz, CA), or p53 monoclonal antibody (sc-126) (Santa Cruz, CA), with β-actin antibody (Sigma Chemical Co, St Louis, MO) as the loading control.

Quantitation of apoptosis

To quantitate apoptosis, we used AnnexinV-FITC and PI staining by flow cytometry as described earlier [25].

Flourimetric assays for caspase-3/7 activation

Caspase activation was analysed using the Apo-ONE Homogeneous caspase 3/7 Assay kit (Promega MD, USA). Cells (5×10^3/well) were plated in 96-well plates and treated either with different concentrations of cRL alone, or radiation alone or in combination. After 3, 6, 12 and 24 h, caspase activation was measured using a fluorimeter at excitation and emission wavelengths of 485 and 530 nm respectively.

Indirect immunofluorescence

Cells were grown in the 8-well chamber slide and treated with different concentrations of cRL or left untreated. Then the cells were fixed with 10% buffered formalin and permeabilised with chilled acetone (Histological grade, EMD Biosciences). The fixed cells were then blocked with goat serum and incubated with the primary antibody p53 (sc-6243, Santa Cruz, CA) in a humidified chamber at 37 °C. After incubation with the primary antibody, the cells were washed with PBS and conjugated with secondary antibody with the fluorescent dye Alexa Fluor 594 (red) (Molecular Probes Inc., Oregon). The chambers were removed and the slide was air dried, the nuclei were then stained with $4',\beta'$-diamidino-2-phenylindole (DAPI) hydrochloride by mounting the slide with Vectashield mounting medium containing DAPI (Vector Laboratories Inc., CA). The slide was then observed under a confocal microscope using UV lasers and thereby the localisation of the p53 protein studied.

Statistical analysis

All experiments were performed three times to ascertain reproducibility. Statistical analysis was performed to calculate the radio-sensitisation properties of cRL on the lung cancer cell line as described previously [23–25].

Results

cRL-induced cytotoxicity in A-549 and H-460 lung cancer cells

All the five fractions were lyophilised into powder and dissolved separately in DMSO for *in vitro* testing. Control groups were treated with DMSO alone. *N*-hexane, ethyl acetate, *n*-butanol and water fractions of RL (50 μg/ml) either failed to inhibit the growth of lung cancer cells or the inhibition was very minimal (data not shown). In A-549 and H-460 cells, cRL (5–30 μg/ml) reduced the cell viability in a dose-dependent manner with the lowest values being 22 and 27.87% respectively (Fig. 1A). A similar pattern of cytotoxic effect was seen when the lung cancer cell lines were exposed to cRL by clonogenic inhibition assays (Fig. 1B). Both assays demonstrated that cRL exhibits cytotoxic effect on the lung cancer cell lines.

cRL-induced apoptosis in lung cancer cells

In this study, cRL at 30 μg/ml induced apoptosis in A-549 cells (26.51, 31.02, 75.5 and 91% at 6, 12, 24 and 48 h respectively) and at 40 μg/ml in H-460 cells (34.08, 43.94, 59.2 and 88% at 6, 12, 24 and 48 h respectively) by annexinV-FITC staining (Fig. 1B). Further, PI staining revealed similar results in both lung cancer cell lines (data not shown). To ascertain the role of cRL on normal cells, we used normal bronchial epithelial cells, BEAS-2B, within six passages. Interestingly, up to 40 μg/ml concentration cRL did not alter either the cell proliferation (data not shown) or induce apoptosis in BEAS-2B cells (Fig. 1C). These results demonstrated that cRL specifically targets cancer cells and not normal lung epithelial cells.

cRL induces p53 and Bax and down-regulates Bcl-2 proteins in lung cancer cells

The p53 is a tumour suppressor protein, which can function as a transcription factor. It controls cell proliferation and apoptosis in response to various types of cellular stress or damage [17, 26]. It is known that in most human cancer cells, loss of functional p53 impairs the response of the cells to apoptotic stimuli [27] and leads to poor prognosis in patients. In our study, cRL induced p53 expression in both A-549 (Fig. 2A) and H-460 cells (data not shown). The up-regulation of p53 began to increase 3 h after treatment with cRL and reached the maximum expression at 24 h. The comparison of the results between apoptotic response and induction of p53 indicated that the up-regulation of p53 would have played a role in the induction of apoptosis. On the other hand, cRL significantly down-regulated Bcl-2 protein at 24 h and elevated the levels of Bax protein (5-fold) in A-549 cells.

Nuclear export of p53 in lung cancer cell lines by cRL

Having established that cRL induces p53 protein in both lung cancer cell lines, we examined sub-cellular localisation of p53 before and after treatment with cRL by indirect immunofluorescence method. The p53 was localised in the cytoplasm in untreated lung cancer cells whereas it was exported into nucleus in both A-549 and H-460 cells after the treatment with cRL (Fig. 2B). Morphological changes in the cells treated with cRL indicated that they were undergoing apoptosis. The results were in agreement with the apoptosis and Western Blot analysis.

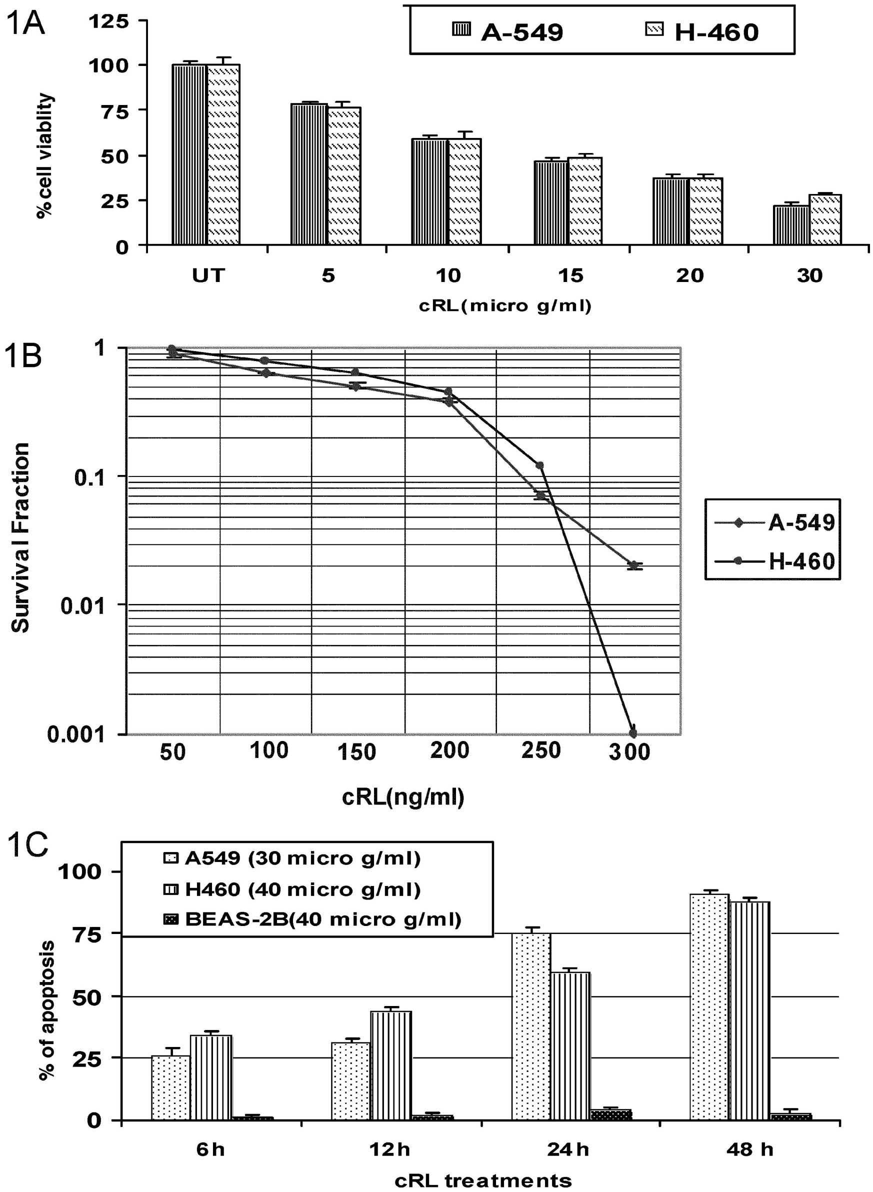

Fig. 1. (A) Effect of cRL on lung cancer cell lines. The lung cancer cell lines A-549 and H-460 were treated with different concentrations of cRL for 24 h, and the cell viability was measured by MTT assay. Each data point represents the mean for four wells from three independent experiments (mean ± S.E.). (B) cRL-induced clonogenic inhibition in lung cancer cells. Lung cancer cells treated with different concentrations of cRL (0–300 ng/ml) and clonogenic inhibition assays were performed in these cells. Cell survival curve of lung cancer cells with treatments as assayed by colony forming ability. The data shown are representative of the combined mean of three independent experiments. (C) cRL-induced apoptosis in lung cancer cell lines. Cells were treated with either 30 μg/ml (A-549) or 40 μg/ml (H-460 or BEAS-2B) of cRL dissolved in DMSO and apoptotic assays were performed at 6, 12, 24 and 48 h. Bar graph shows the percentage of apoptotic cells. The base-line apoptosis in the untreated group was normalised with data on the treated group. Each data point represents the mean of three independent experiments (mean ± S.E.).

The cRL enhances radio-sensitisation effect on lung cancer cell lines

For testing the radio-sensitisation effect of cRL, we exposed the cells to either radiation alone (2 Gy) or in combination with cRL. For the combination experiment, the cells were treated with cRL, an hour before irradiation. The results indicated that A-549 cells are more resistant (2 Gy = 2.8%) to radiation compared to H-460 cells (2 Gy = 8%). Interestingly, when radiation (2 Gy) was combined with cRL,

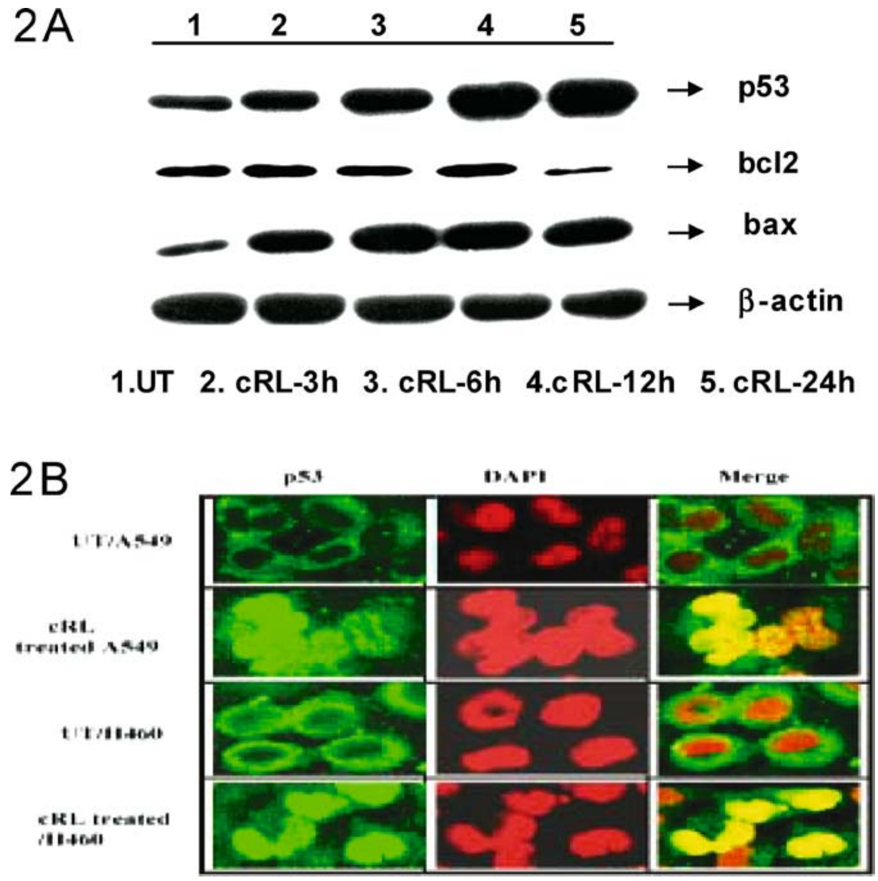

Fig. 2. (A) Regulation of apoptotic gene expression by cRL. Whole cell protein extracts were prepared from A-549 cells that were either left untreated or treated with cRL for the time intervals indicated. The blot was probed with antibodies for p53, Bcl-2, Bax and β-actin. (B) Effect of cRL on the localization of p53 in lung cancer cell lines. Indirect immunofluorescence analysis for p53 in A-549 and H-460 lung cancer cell lines following 24 h treatment with cRL. The superimposed images show the cells undergoing apoptosis.

radiation-induced inhibition of cell proliferation elevated from 2.8 to 91.5% in A-549 cells and from 8 to 87% in H-460 cells (Fig. 3A).

Furthermore, the combination of cRL with radiation caused a significant induction of apoptosis after 24 h (54%) and 48 h (70%) when compared to radiation (5 Gy) alone (3.23% at 24 h and 7.7% at 48 h) in A-549 cells (Fig. 3B). In H-460 cells radiation (5 Gy) alone induced 4.86% apoptosis at 24 h and 13.05% at 48 h but combination of cRL enhanced apoptosis to 52% at 24 h and 69.5% at 48 h (Fig. 3C). Similar results were observed from PI staining, scoring the cells by flow cytometry (data not shown).

Caspase-3 activation in lung cancer cell lines by cRL

To ascertain whether the induction of apoptosis is due to the up-regulation of pro-apoptotic genes, we measured the levels of caspase activation in A-549 and H-460 cells after treating them with cRL. In this study, cRL enhanced caspase-3 activation in A-549 (6.2-fold) (Fig. 4A) and in H-460 cells (5-fold), (Fig. 4B) which was observed till 24 h. In combination experiments the peak activation of caspase-3 was found at 24 h in A-549 and 3 h in H-460 cells. However, no significant caspase-3 activation was seen in radiation (5 Gy)-alone treated lung cancer cells.

cRL-released radiation-induced G_2/M phase block of cell cycle in lung cancer cell lines

Our results presented *vide supra*, that cRL in combination with irradiation increased the percentage of apoptosis, prompted us to find whether cRL mediated abrogation of G_2/M phase of cell cycle. cRL at 20 μg/ml (H-460) concentration accumulated the cells in G_2/M phase of cell cycle

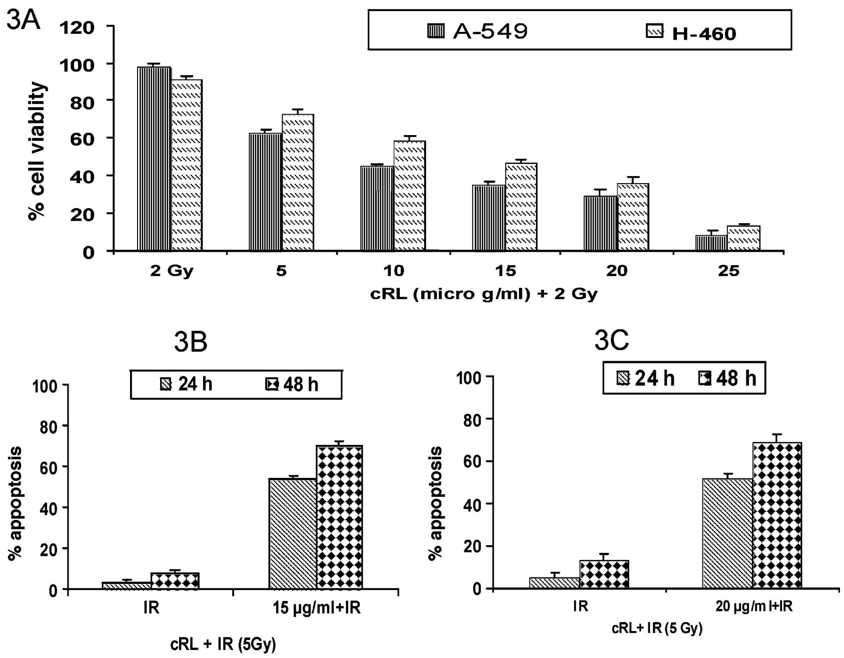

Fig. 3. (A) Radio-sensitisation effect of cRL on lung cancer cell lines. The cells (A-549 and H-460) were treated either with radiation alone or radiation in combination with various concentrations of cRL for 24 h, and the cell viability was measured by MTT assay. Each data point represents the mean for four wells from three independent experiments (mean ± S.E.). Enhancement of radiation-induced apoptosis in lung cancer cells by cRL. (B) A-549; (C) H-460. Cells were treated with radiation alone (5 Gy) or radiation combined with cRL (A-549; 15 μg/ml; H-460; 20 μg/ml) and apoptotic assay was performed after 24 h of exposure. Each data point represents the mean of three independent experiments (mean ± S.E.).

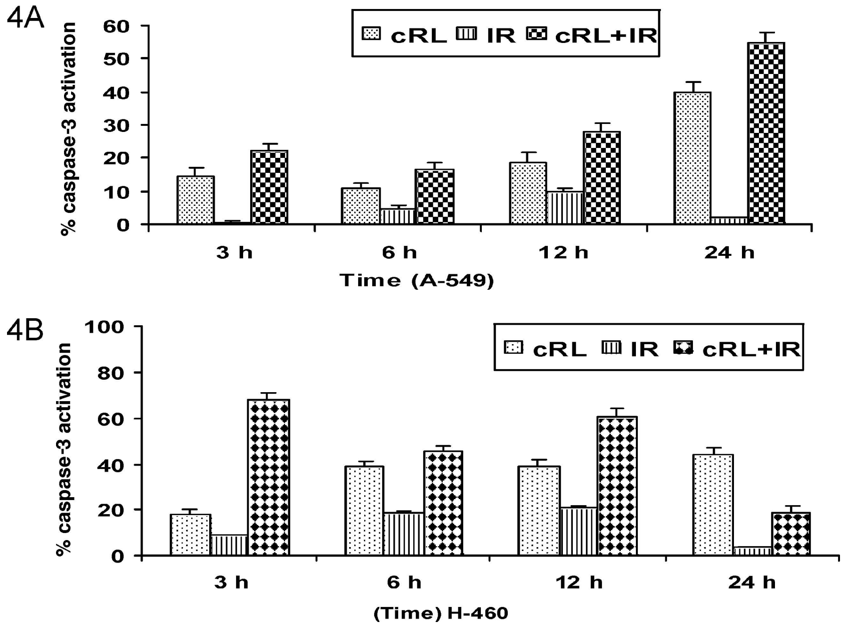

Fig. 4. Induction of caspase-3 activation in lung cancer cell lines. (A) A-549; (B) H-460. Cells were left untreated or treated with cRL, radiation or the two in combination and caspase activity was determined after 3, 6, 12 and 24 h. Caspase activities are expressed as the percentage of caspase activity as compared to control and presented as mean ± S.E. of two samples in triplicates.

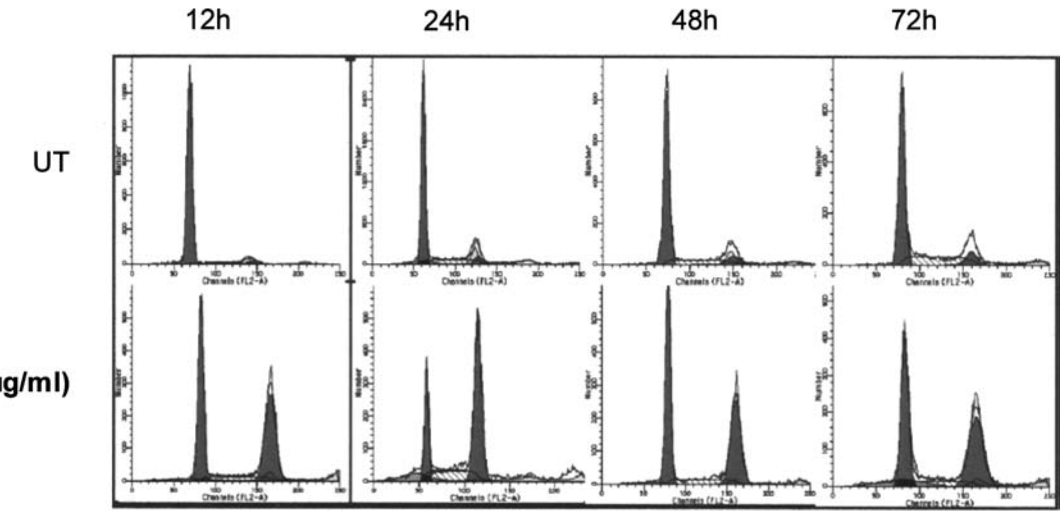

Fig. 5. The effect of cRL in cell cycle distribution of H-460 cells. Asynchronous cultures of H-460 cells were treated with cRL for 12, 24, 48 and 72 h and the cells were resuspended. At each time point the treated as well as untreated cells were stained with PI, and analysed by flow cytometry. The results were analysed by MODFIT LT statistics program.

(Fig. 5 and Table 1) compared to untreated cells. Observation of the time course analysis of the cell cycle confirmed that radiation caused an increase of the G_2/M fraction but no significant induction of radiation-induced apoptosis was seen either in H-460 cells or A-549 cells (data not shown). However, the combination of cRL with irradiation caused complete abrogation of G_2/M arrest within 12 h and abrogation of G_2/M arrest would have led to radiation-induced apoptosis on lung cancer cells.

Table 1. Effects of cRL, ionizing radiation or their combination, on cell cycle distribution of H-460 cells

Treatments	12 h	24 h	48 h	72 h
G_0/G_1				
UT	44.3 ± 2.0	46.8 ± 2.0	61.8 ± 1.4	70.2 ± 3.0
20 μg/ml	23.9 ± 3.2	17.0 ± 1.7	13.5 ± 1.2	10.7 ± 0.7
IR	22.9 ± 2.7	33.9 ± 2.6	35.9 ± 3.7	41.5 ± 1.8
20 μg/ml + IR	14.9 ± 2.8	12.6 ± 1.0	25.5 ± 2.1	27.2 ± 1.1
S				
UT	38.4 ± 3.7	37.3 ± 4.2	30.6 ± 1.4	24.1 ± 5.0
20 μg/ml	18.3 ± 1.6	13.4 ± 2.5	36.9 ± 2.0	51.2 ± 2.9
IR	3.3 ± 0.2	5.0 ± 0.6	10.4 ± 1.5	9.2 ± 2.2
20 μg/ml + IR	28.9 ± 2.9	44.4 ± 3.0	28.6 ± 1.0	33.6 ± 1.5
G_2/M				
UT	17.3 ± 2.7	15.9 ± 1.8	7.6 ± 0.36	5.7 ± 2.4
20 μg/ml	57.8 ± 1.4	69.6 ± 1.5	49.9 ± 3.1	38.1 ± 1.3
IR	73.8 ± 2.1	61.1 ± 3.2	58.7 ± 1.4	54.3 ± 3.8
20 μg/ml + IR	56.2 ± 1.6	43.0 ± 2.7	38.9 ± 2.7	24.2 ± 3.6

Discussion

The Indian systems of medicine, *Ayurveda* and *Siddha*, which originated several centuries ago, are holistic approaches to health. Interest in herbal products for the treatment and prevention of cancer has gained momentum in recent years. Many natural compounds, especially plant products and dietary constituents, have been found to possess chemopreventive and chemotherapeutic potential both *in vitro* and *in vivo* [28]. Our aim of this study is to determine the anti-cancer effect of cRL on lung cancer cells and delineate the possible mechanism of action on lung cancer cells. The results demonstrate that cRL significantly inhibits the cell proliferation and induces apoptosis in A-549 and H-460 cells. Inhibition of cancer cell growth and induction of apoptosis are the two major goals in cancer treatment.

The up-regulation of p53 and Bax and induction of caspase-3 by cRL together would have played a key role in the induction of apoptosis in lung cancer cells. The p53 protein plays an important role in cell cycle control and induction of apoptosis during the treatment period in cancer patients [29]. Moreover, the sensitivity of cancer cells to chemotherapeutic agents is greatly influenced when the function of p53 is abrogated [30]. Bax is one of the transcriptional targets of p53, which plays an important role in the induction of apoptosis. The pro-apoptotic Bcl-2 family protein Bax and the anti-apoptotic protein Bcl-2 play important roles in the regulation of apoptosis [31]. When Bcl-2 is up-regulated or produced in excess, cells are protected from Bax-induced apoptosis. On the other hand, if Bax levels are high, the cells proceed into apoptosis. Therefore, the ratio between Bcl-2 and Bax will determine whether or not cells will undergo apoptosis. In this study, cRL down-regulated Bcl-2 protein expression while at the same time up-regulation of Bax protein was seen in both cell lines. In addition, our results demonstrated that cRL not only induces p53 expression, but facilitates its translocation into the nucleus in A-549 and H-460 cells.

Mitochondria play a major role in apoptosis triggered by many stimuli. Disruption and permeation of the mitochondrial membrane are general phenomena associated in initiating the apoptotic cascade and necrotic cell death [32, 33]. An excessive mitochondria Ca^{2+} influx has been suggested to be a potent cell death stimulus leading to mitochondrial membrane depolarisation and cytochrome-*c* release [32, 33]. Activation of caspases by translocation of cytchrome-*c* from mitochondria to the cytosol is a downstream event through which the mitochondrion's role as a regulator of cell life and death has become inevitable [34]. We demonstrated that in this study, cRL up-regulated Bax and induction of capase-3 activation indicates that cRL may cause mitochondrial membrane disruption, which would lead to apoptosis in both cell lines.

Usually, ionising radiation is known to cause G_2/M arrest of cell cycle and induce apoptosis in irradiated cells. However, in our study, radiation caused G_2/M block of cell cycle, but failed to induce apoptosis to significant levels in lung cancer cells. The G_2/M checkpoint is an important cellular response to DNA damage, and plays a key role in the sensitivity of tumour cells to many therapies [35, 36]. Abrogation of the G_2/M checkpoint often leads to a marked increase in the sensitivity of cells to ionising radiation and some types of chemotherapy, for example agents like methylxanthines, phosphatase inhibitors or UCN-01 [37]. We found that cRL is one such agent that enhances radiation-induced cytotoxicity and induction of apoptosis in lung cancer cells. Our findings in this study demonstrate that arrest of G_2/M phase of cell cycle by cRL may be an additional mechanism of its action in lung cancer cells. Hence, the G_2/M arrest by cRL might provide for growth inhibition and induction of apoptosis in lung cancer cells.

In summary, we have demonstrated that exposure of lung cancer cell lines to cRL results in cytotoxic effect, cell cycle arrest at G_2/M phase and induction of apoptosis sparing the normal bronchial epithelial cells. Our data have also demonstrated that cRL enhances radio-sensitising effect in lung cancer cells. The up-regulation of the pro-apoptotic genes and down-regulation of the pro-survival genes suggests that cRL may prove to be an effective therapeutic agent, which may either be used alone or in combination with conventional therapies (chemotherapy or radiation therapy) for lung cancer.

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