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# S29 ribosomal protein induces apoptosis in H520 cells and sensitizes them to chemotherapy

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**Abstract**

Non-small cell lung cancer (NSCLC) is the most prevalent type of lung cancer especially in India and displays resistance to anticancer treatment. In our earlier study we had isolated a cDNA clone from rat thymocytes induced to undergo apoptosis, which was found to encode S29 ribosomal protein [Biochem. Biophys. Res. Commun. 277 (2000) 476]. In the present study an attempt has been made to find out whether enhanced expression of S29 cDNA can kill NSCLC H520 cells. We found that S29 induced apoptosis and augmented the effect of anticancer drugs. Expressions of several molecular determinants of apoptosis were analyzed in order to understand the mechanism of apoptosis induced by S29. We observed downregulation of the expression of inhibitors of apoptosis proteins (IAPs) Bcl-2, Bcl-$X_L$, and survivin and upregulation of pro-apoptotic p53 and Bax as assessed by Western blotting. Mitochondrial release of cytochrome *c* and activation of initiator caspase-8 and -9 and effector caspase-3, followed by cleavage of nuclear substrate poly(ADP-ribose) polymerase, were also observed. Permeability transition as determined by changes in $\Delta\Psi_m$ was not a requirement for cytochrome *c* release. There was a marginal increase in the release of apoptosis inducing factor (AIF) and reduction of NF-κB dependent transcriptional activity. There was non-involvement of calcium and the telomerase activity, a proliferation marker.


*Keywords:* S29 ribosomal protein; H520 cells; Apoptosis; Mitochondria; Caspases; Cytochrome *c*

Apoptosis is a tightly controlled multistep mechanism of cell death. The molecular participants of apoptosis are located in mitochondria, plasma membrane, cytosol, and nucleus, with interplay between these compartments. The pathways converge at two points, the mitochondria and the caspases [2]. Recent studies demonstrate the ability of two main initiator caspase-8 and -9 to signal via distinct receptor or mitochondrial mediated pathway and activate the effector pro-caspase-3 within the cytosol [3]. During receptor mediated apoptosis activation of caspase-8 plays a key role. Active caspase-8 cleaves pro-apoptotic members of the Bcl-2 family followed by the release of mitochondrial constituents, the activation of the apoptosome complex, and the processing of executioner caspases [4]. In non-receptor mediated apoptosis the release of mitochondrial proteins and the activation of caspase-9 within the apoptosome complex seem to be the committed events [5]. The release of mitochondrial proteins is blocked by the anti-apoptotic Bcl-2 family members and promoted by pro-apoptotic members [6].

Apoptosis is critical in many physiological contexts including embryogenesis, immune cell maturation and response, tissue homeostasis, and in the cellular response to injury [7]. In pathologic states, while a failure to undergo apoptosis may cause abnormal cell overgrowth and malignancy, excessive apoptosis may contribute to organ injury [8–10]. Thus, a detailed understanding of apoptotic mechanisms and the factors that can compromise them is critical to the design of more potent, specific, and effective cancer therapies. Moreover, selective destruction or preservation of cells through modulation of the apoptotic program promises to impact the treatment of a variety of diseases.

In an attempt to isolate and characterize genes differentially expressed in apoptosis, we isolated and

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characterized a cDNA clone upregulated during apoptosis. This was found to represent the gene encoding the S29 ribosomal protein, a component of small ribosomal subunit [1]. Preliminary data revealed that enhanced expression of S29 resulted in increased apoptosis in HeLa cells. In order to further investigate the pro-apoptotic activity of S29, we transiently transfected the expression vector encoding full-length S29 ribosomal protein in different cancer cell lines of human organ origin (laryngeal cancer cell line, Hep2; glioma cell line, U87-MG; E6 transfected U87-MG; and non-small cell lung cancer cell line; H520). We observed that enhanced expression of S29 resulted in increased apoptosis in them but to different extents (unpublished data). Maximum amount (55%) of apoptosis was observed in H520 cells after S29 overexpression and the induction of apoptosis was evaluated on the basis of morphological changes, flow cytometry, and TUNEL assay.

Lung cancer is the major cause of cancer deaths worldwide and its incidence continues to rise. Based on histological features it is divided into two broad groups: SCLC (small cell lung cancer) and NSCLC (non-small cell lung cancer). There is difference in the sensitivity to treatment between these two types, which is perhaps due to their susceptibility to undergo apoptosis. NSCLC, the more prevalent type, shows lesser sensitivity to apoptosis, making it more resistant to various anticancer treatments whereas SCLC is more susceptible to apoptosis. In this paper we have attempted to identify the molecular targets of S29 induced apoptosis and have looked for the involvement of both pro-apoptotic and anti-apoptotic genes, the role of mitochondria, and the initiator and executioner caspases in NSCLC, H520 cells. Our results show that S29 induces apoptosis by increasing p53, Bax and decreasing Bcl-2, Bcl-$X_L$, survivin, and NF-κB. Mitochondrial involvement was seen by increased release of cytochrome *c* and increase of downstream caspase-3. Moreover, in a combination experiment with chemotherapeutic agents (cisplatin, etoposide), evidence is provided that S29 mediated downregulation of Bcl-2, Bcl-$X_L$, and survivin has the potential to augment the chemotherapeutic response of these chemotherapeutic agents. The results indicate that S29 over expression induces two cell death pathways in H520 cells, which appear to co-exist.

## Materials and methods

DeadEnd colorimetric detection Kit and Dual luciferase Assay Kit were obtained from Promega, Madison, USA. Caspase-3 assay kit and caspase-8 substrate Ac-IETD-AFC were from Pharmingen, San Diego, USA. PCR-ELISA kit for telomerase was from Roche Biochemicals, USA. Antibodies against Bcl-2, Bax, Bcl-$X_L$, p53, NF-κB [p65], β-actin, and cytochrome *c* for Western blotting were all purchased from Santa Cruz Biotechnology, CA, USA. Caspase-9 and PARP (in the form of serum) antibody were purchased from Neo Markers, Germany.

### *Cell culture and treatments*

The human non-small cell lung carcinoma (NSCLC) cell line, H520, was obtained from National Center for Cell Science, Pune, India. The cell line was maintained in DMEM supplemented with 10% fetal calf serum and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) in a humidified atmosphere of 5% $CO_2$ in air, at 37 °C. Logarithmically growing cells were used for all experiments.

Twenty four hours post-transfection, cells were treated with z-DEVD-cmk (200 μM; *N*-benzyloxycarbonyl–Asp–Glu–Val–Asp–chloromethylketone) or Ac-YVAD-CHO (50 μM; acetyl–Tyr–Val–Ala–Asp–aldehyde) for 24 h and the growth inhibition of H520 cells was assessed as described earlier [11]. In another set of experiments, 24 h post-transfection, cells were treated with either actinomycin D (2.5 μg/ml) or cycloheximide (2.5 μg/ml) or cisplatin (2.5 μg/ml) or etoposide (10 μg/ml) for 24 h and growth inhibition of NCI-H520 cells was assessed.

### *Expression vector*

pcD-S29, a eukaryotic expression vector containing the full-length S29 ribosomal sequence along with 5′ non-coding sequence, and empty vector; pcD-X (vector from which the S29 coding part had been removed by *Bam*HI digestion) as control were used for transfections [1]. pcD vector was used for the construction of pcD-S29 [12]. In this vector, a DNA fragment containing both the SV40 early region promoter and two introns normally used to splice the virus 16S and 19S late mRNAs is placed upstream of the cDNA cloning site to ensure transcription and splicing of the cDNA transcripts. An SV40 late region polyadenylation sequence occurs downstream of the cDNA cloning site, so that the cDNA transcript acquires a polyadenylated 3′ end. This vector also ensures that if the cDNA contains the entire protein coding sequence, then it can direct the production of the relevant protein.

### *Transfection of H520 cells*

The cells ($5 \times 10^4$) were seeded in 6-well plates, a day before transfection. The cells were transfected with 10 μg pcD-S29 vector (containing S29 cDNA fragment) using the calcium phosphate co-precipitation method as described previously [13]. Control cells were transfected with pcD-X. Transfection efficiency was similar in all experiments as seen by co-transfection with pEGFP plasmid. After 48 h of transfection all cells were scored for apoptosis.

### *Detection of apoptosis*

*Morphological characteristics.* Apoptotic cell death was evaluated by observing morphological changes typical of apoptosis, such as cell shrinkage, chromatin condensation, membrane blebbing, and formation of apoptotic bodies, by light microscopy [14].

*Flow cytometry.* Briefly $2 \times 10^6$ cells were washed once in phosphate-buffered saline (PBS) and fixed in 70% ethanol at −20 °C overnight. Fixed cells were washed and resuspended in a buffer containing 50 μg/ml propidium iodide (PI), 0.1% sodium citrate, and 1% Triton X-100. PI stained cells were analyzed using a FACScan cytometer (Coulter) equipped with an argon laser using Win MdI 2.8 software.

*TUNEL assay.* Apoptotic cells were further visualized using DeadEnd colorimetric detection system from Promega, Madison, USA, according to the manufacturer's instructions. This system end labels the fragmented DNA of apoptotic cells using a modified TUNEL (TdT-mediated dUTP Nick-End Labelling) assay.

### *Cell viability assay*

Growth inhibition of H520 cells was determined by use of the colorimetric MTT cell viability assay as described previously [11]. Briefly, $1 \times 10^4$ cells were seeded in 96-well microtiter plate. Cells were

transfected with S29 ribosomal protein. The transfection was performed for 24 and 48 h, after which they were either left untreated or stimulated with drugs/inhibitors for 24 h. One hundred μl of 5 mg/ml of MTT was added followed by incubation for 4 h at 37 °C. The Formazan crystals thus formed were dissolved in DMSO and the absorbance was measured at 570 nm using an ELISA reader.

*Measurement of caspase-3 and -8 activity*

Caspase-3 activity in control and S29 transfected H520 cells was analyzed using caspase-3 assay kit according to the manufacturer's instructions (Pharmingen, San Diego, USA). Caspase-3 activity in cell lysates was determined fluorometrically using a synthetic tetrapeptide fluorogenic substrate Ac-DEVD-AMC. Fluorescence of the released AMC was measured spectrofluorometrically using an excitation wavelength of 380 nm and an emission wavelength range of 420–460 nm.

Caspase-8 was measured using IETD-AFC as the substrate. The fluorescence of the released AFC was measured with an excitation wavelength of 400 nm and emission wavelength range of 480–520 nm.

*Immunoblot analysis*

Control and S29 transfected cells were lysed in RIPA lysis buffer containing protease and phosphatase inhibitors. Equal amounts of protein extracts were electrophoresed on 12–18% SDS–polyacrylamide gels and transferred to a nitrocellulose membrane. Western blotting was done using the kit from Promega, USA, as described by the manufacturer. The bands were visualized with anti-rabbit or anti-mouse or anti-goat IgG antibodies conjugated with alkaline phosphatase (AP) using BCIP and NBT (Promega Western blot kit) and comparative densitometric analysis was performed using NIH 1.52 image analysis software [15].

*Detection of cytochrome c and AIF release*

Control and S29 transfected cells were washed with PBS and resuspended in buffer containing 250 mM sucrose, 20 mM Hepes–KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride. The cells were then homogenized and centrifuged three times at 3000*g* for 10 min to separate cellular debris and nuclei. The mitochondria-enriched fraction was pelleted by centrifugation at 18,000*g* for 15 min. The supernatant was subjected to Western blot analysis.

*Measurement of mitochondrial membrane potential by flow cytometry*

Control and S29 transfected H520 cells were incubated with 40 nm of 3,3′ dihexyloxacarbocyanide iodide ($DiOC_6$, Molecular Probes, USA) for 30 min in culture medium at 37 °C, 5% $CO_2$. The cells were then washed with PBS and collected by centrifugation at 200*g*. Incorporation of $DiOC_6$ by H520 cells was determined by flow cytometry using excitation of a single 488 nm argon laser.

*Assay of NF-κB transcriptional activity*

To analyze the effect of S29 ribosomal protein on NF-κB transcriptional activity, pGL-3 luciferase vector with and without NF-κB binding sites was used. H520 cells were co-transfected with luciferase vectors and pcD-S29 using calcium phosphate method. Forty-eight hours post-transfection, cells were lysed and luciferase activity was analyzed using Dual luciferase reporter assay system and measured in luminometer (Turner 20, Promega, USA).

*Calcium estimation*

Cellular calcium levels in control and S29 transfected cells were estimated by *O*-cresolphthalein complexone method (CPC). Calcium ions form a violet complex with *O*-cresolphthalein in an acidic medium. Chromogen consisting of *O*-cresolphthalein 0.152 mmol/L, 8-hydroxyquinone 6.65 mmol/L, and HCl (57.07 mmol/L) was incubated with 20 μl of cellular extract/lysate, and the change in color from yellow to violet was read spectrophotometrically at 570 nm. The calcium concentration is expressed as μM/mg protein [16].

*Assay of telomerase activity*

This was measured using the PCR-ELISA kit (Roche Molecular Biochemicals, Germany). The samples were lysed and an aliquot containing 2 μg protein was used for the assay. Telomerase positive embryonic kidney cell line (HEK-293) and untreated HeLa cells were used as positive controls while heat inactivated HeLa extract was used as negative control. The telomerase activity detected was expressed in relation to the positive control and expressed as relative units (RU) [17].

## Results

### *Enhanced expression of S29 ribosomal protein induces apoptosis in H520 cells*

The morphological characteristics of apoptosis were evident 48 h post-transfection with pcD-S29. Fifty-five percent of H520 cells underwent apoptosis, as compared to the control cells, which showed 4% apoptosis. Besides the morphological changes, apoptosis was also quantified by measuring the fraction of hypodiploid cells using flow cytometry. In situ DNA fragmentation was measured by the TUNEL technique and similar findings were obtained further supporting the above data (Table 1, Figs. 1A and B).

### *Effect of macromolecular synthesis inhibitors on S29 ribosomal protein induced apoptosis*

To test the requirement for macromolecular synthesis in S29 induced apoptosis, H520 cells were treated with

Table 1
Percentage apoptosis as observed by morphological characteristics, TUNEL and FACS in control H520 and S29 transfected H520 cells

| | Control H520 cells | S29 H520 cells |
|---|---|---|
| % Apoptosis | 4 | 55 |
| Caspase-8 activity | 1662 | 8877 |
| Caspase-3 activity | 366 | 820 |
| *Integrated density values* | | |
| Procaspase 9 | 9488 | 4255 |
| Bax | 8710 | 22,374 |
| Bcl-2 | 59,884 | 21,072 |
| Bcl-$X_L$ | 90,472 | 46,842 |
| Survivin | 68,320 | 37,089 |
| AIF | 13,241 | 88,075 |
| Cytochrome *c* | 17,678 | 108,076 |
| P53 | 372,172 | 579,937 |
| NF-κB | 83,895 | 58,125 |
| *PARP cleavage* | | |
| 116 kDa | 233,496 | 50,065 |
| 86 kDa | 74,290 | 103,455 |

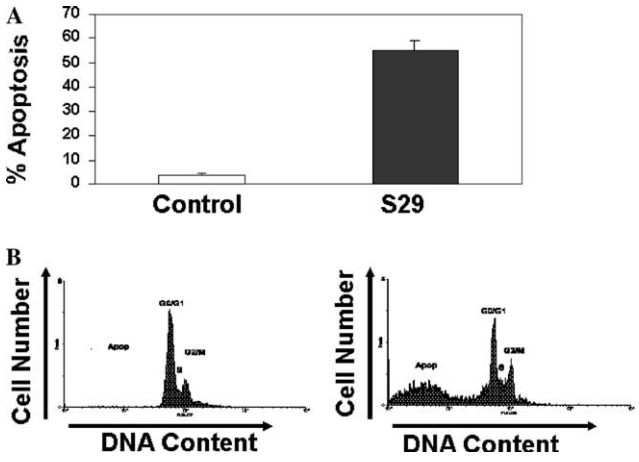


Fig. 1. (A) Percentage apoptosis. Percentage apoptosis as observed in control and S29 transfected H520 cells as assessed by morphological characteristics and TUNEL. (B) Flow cytometric analysis. (a) H520 cells transfected with control vector and (b) H520 cells 48 h after transfection with pcD-S29.

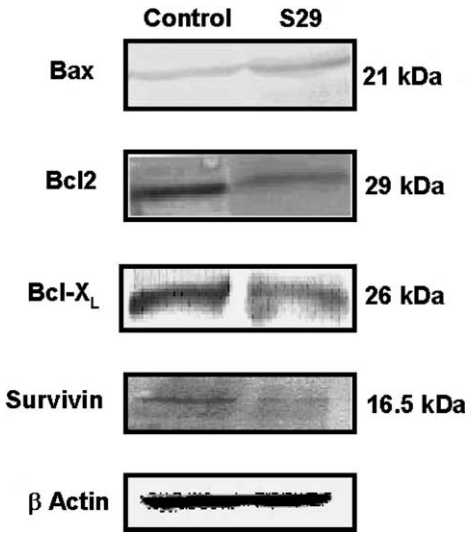


Fig. 2. Western blot densitometric analysis of protein expression of Bax, Bcl-2, Bcl-$X_L$, and survivin in control and S29 transfected H520 cells.

actinomycin D or cycloheximide, either in the presence or absence of the S29 ribosomal protein. In spite of using a very low dose of cycloheximide (2.5 μg/ml), cell viability as determined by MTT assay showed that cycloheximide itself induced 35% apoptosis but this was augmented to 86% in the S29 ribosomal protein transfected H520 cells. On the other hand actinomycin D by itself induced 9.5% apoptosis and this was further augmented to 85% apoptosis in the S29 transfected cells. Our results show that the cell death machinery may exist in a quiescent state but may be activated by the S29 ribosomal protein in H520 cells without transcription or translation as apoptosis occurred in the presence of actinomycin D or cycloheximide (Fig. 8).

*S29 overexpression causes downregulation in the protein expression of anti-apoptotic Bcl-2, Bcl-$X_L$, and survivin but upregulation of pro-apoptotic Bax*

In order to understand the role of Bcl-2 family, which is an important regulator of apoptosis, we investigated whether S29 overexpression would alter the expression of the pro- and antiapoptotic members. We observed that the basal level of Bcl-$X_L$ was high in H520 cells and S29 over expression resulted in a 1.6-fold decrease in its level. Low basal levels of Bcl-2 were found in H520 cells and these were further decreased by 2.5-fold on S29 overexpression as compared to the control. Survivin was moderately expressed in H520 cells and S29 overexpression decreased this by 2-fold (Fig. 2). Moderate level of Bax was seen in control and S29 overexpression increased it to 1.5-fold (Table 1 and Fig. 2). Loading was normalized using β-actin. Taken together these data suggest that S29 causes a reduction in the cellular levels of the anti-apoptotic proteins and a moderate increase in those of the pro-apoptotic protein, irrespective of their endogenous levels.

*S29 ribosomal protein activates initiator and effector caspases*

Since the key players in apoptosis cascade are thought to be caspases and since the expression of pro-caspase-8 is also observed in all NSCLC cell lines [18], we tested the effect of S29 on it and we focused our attention on both receptor and non-receptor mediated caspases. As shown in Table 1 and Fig. 3A, S29 over expression resulted in a 2.2-fold increase in caspase-3 activity as compared to the controls. This was further confirmed using the caspase-3 peptide inhibitor, Ac-DEVD-CHO, which significantly reduced the S29 induced apoptosis (Fig. 8). The possibility of the involvement of other caspases was investigated by using the caspase inhibitor Ac-YVAD-CHO. This inhibitor also inhibited the S29 ribosomal protein induced apoptosis in H520 cells (Fig. 8), thereby suggesting the possibility of involvement of other caspases besides caspase-3. Hence, we also examined the activity of caspase-8, a receptor mediated caspase, and found it to be increased by 5.3-fold (Table 1 and Fig. 3B). In non-receptor mediated apoptosis the release of mitochondrial protein and the activation of pro-caspase-9 seem to be the committed events. Immunoblot analysis for capase-9 was also performed and a 2-fold decrease in pro-caspase 9 (46 kDa band) was observed in S29 transfected cells. Loading was normalized using β-actin antibody. The above findings suggest that S29 particularly activates caspase-8, accompanied by moderate activation of caspase-9 and -3.

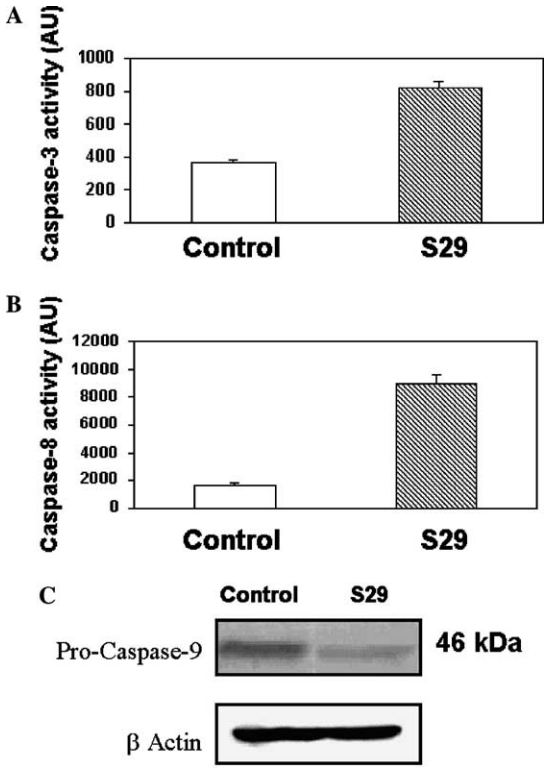


Fig. 3. (A) Caspase-3 activity in control and S29 transfected H520 cells. (B) Caspase-8 activity in control and S29 transfected H520 cells. (C) Western blot analysis of protein expression of pro-caspase-9 in control and S29 transfected H520 cells.

*S29 induces cytochrome c release from the mitochondria*

Recently, it has been reported that cytochrome *c* released from the mitochondria during apoptosis activates caspase-9 and -3 [4,19,20] and as we had observed their activation we hence looked for mitochondrial involvement, if any. As shown in Table 1 and Fig. 4A, S29 transfected H520 cells showed a significant increase of 3.3-fold in cytochrome *c* in the cytosolic fraction, thereby suggesting the involvement of mitochondria. The released cytochrome *c* in turn may activate downstream events of the execution phase of apoptosis. The slight but non-significant increase (1.3-fold) in AIF protein in the cytosol from mitochondria as compared to control ruled out the involvement of this protein (Table 1 and Fig. 4A). Loading was also normalized using β-actin antibody.

*S29 causes no change in the mitochondrial membrane potential*

The release of AIF from the mitochondria has been shown to be dependent on the opening of mitochondrial permeability transition pores [21]. Since we observed a slight increase in AIF protein in the cytosol from mitochondria as compared to control, we examined the

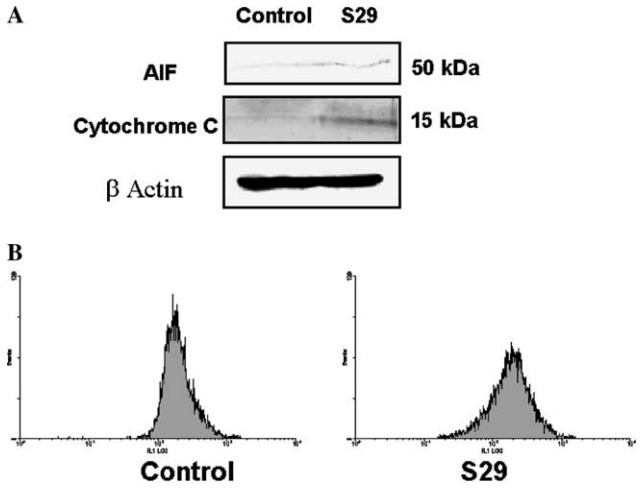


Fig. 4. (A) Western blot analysis of protein expression of cytochrome *c* and AIF in cytosolic fractions of control and S29 transfected H520 cells. (B) Mitochondrial membrane potential of control and S29 transfected cells by flow cytometry with excitation at 488 nm and the resulting emission at 530 nm.

change of mitochondrial transmembrane potential upon induction of apoptosis with S29. Mitochondrial inner membrane potential was monitored by the incorporated fluorescence of the cationic lipophilic dye $DiOC_6$, a potential-sensitive dye. The pattern of the fluorescence of $DiOC_6$ taken up by control H520 cells showed a peak with higher fluorescence representing cells with intact high mitochondrial membrane potential (Fig. 4B). The amount of $DiOC_6$ dye taken up in the S29 transfected cells remained unaltered, thereby suggesting that mitochondrial permeability transition is not involved in S29 induced apoptosis.

*S29 induces PARP cleavage*

Since PARP is a substrate for caspase-3 and -7 [22,23] and cleavage of this nuclear protein is one of the biochemical hallmarks of apoptosis, we investigated its protein expression and its cleavage. As shown in Table 1 and Fig. 5, in control H520 cells, the native 116 kDa band along with an additional 89 kDa band corresponding to the cleavage product of PARP was detected by immunoblotting. This spontaneous cleavage corre-

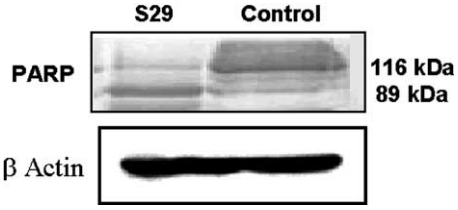


Fig. 5. Western blot analysis of protein expression of PARP in control and S29 transfected H520 cells.

lated with the observed high basal levels of caspase-3 activity in H520 cells. However, in S29 transfected cells there was disappearance of the 116 kDa band, accompanied by a 2-fold increase in the 89 kDa cleavage product, thereby confirming the role of caspase-3 and -7 in S29 induced apoptosis in H520 cells. Loading was normalized using β-actin.

*S29 ribosomal protein moderately increases pro-apoptotic transcription factor p53 but decreases NF-κB*

P53 acts as a transcription factor that upregulates or represses the activity of a range of genes that modulate apoptosis. As shown in Table 1 and Fig. 6A, p53 protein expression was increased by 1.5-fold in S29 transfected H520 cells.

To examine the role of nuclear factor-κB (NF-κB) in S29 induced apoptosis, we determined the levels of NF-κB, p65 by Western blotting. A moderate decrease of 1.6-fold in the protein expression of NF-κB, p65 was seen (Table 1 and Fig. 6A). To investigate whether NF-κB was transcriptionally active, we further examined NF-κB-dependent reporter activity using luciferase assay system. The results show that S29 resulted in a downregulation of NF-κB transcription activity similarly by 1.3-fold (Fig. 6B). These results demonstrate that S29 ribosomal protein induces apoptosis by moderately increasing p53, by reducing NF-κB protein, and by diminishing its transcriptional activity.

*Non-involvement of calcium in S29 induced apoptosis*

Since intracellular signaling mechanism implicated in most instances of the process of apoptosis includes influx of calcium, we measured the intracellular calcium

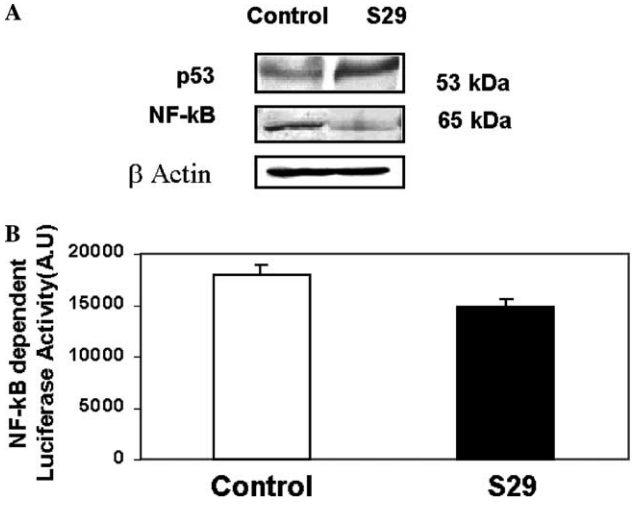


Fig. 6. (A) Western blot densitometric analysis of p53 and NF-κB in control and in S29 transfected H520 cells. (B) NF-κB dependent luciferase activity in control and in S29 transfected H520 cells.

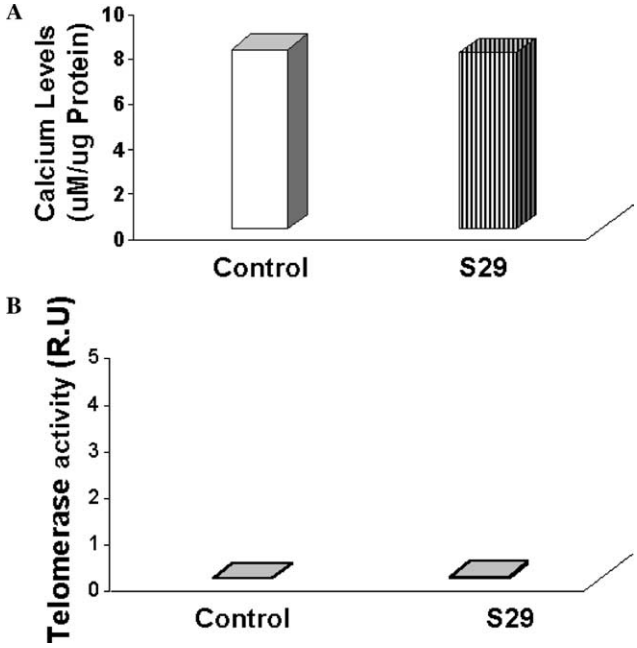


Fig. 7. (A) Calcium levels. (a) Control vector transfected cells, (b) S29 transfected H520 cells after 48 h. The results represent means ± SD of three separate experiments. (B) Telomerase activity. (a) Control vector transfected cells, (b) S29 transfected H520 cells after 48 h. The results represent means ± SD of three independent experiments.

levels. Even though moderate basal level of calcium was seen in H520 cells, no significant changes were observed on S29 overexpression (Fig. 7A).

*S29 does not affect telomerase activity*

Telomerase activity was assessed in both control and S29 transfected H520 cells. We observed that there was no difference in telomerase activity in control and S29 transfected cells (Fig. 7B), thereby suggesting that S29 induces apoptosis but does not affect proliferation.

*Overexpression of S29 ribosomal protein augments the chemotherapeutic response in H520 cells*

Since we observed downregulation of antiapoptotic proteins and upregulation of pro-apoptotic proteins in S29 transfected cells, we investigated whether overexpression of S29 ribosomal protein would augment the apoptotic response of chemotherapeutic agents. Twenty-four hours post-S29 transfection, H520 cells were treated with cisplatin or etoposide for 24 h and the percentage apoptosis was calculated using MTT assay and flow cytometry. As shown in Fig. 8, we observed that cisplatin and etoposide by themselves induced 50 and 13% apoptosis, respectively. In the presence of S29 ribosomal protein, there was moderate augmentation of cisplatin induced apoptosis to 74% whereas marked augmentation of 90% apoptosis was seen in etoposide treated cells.

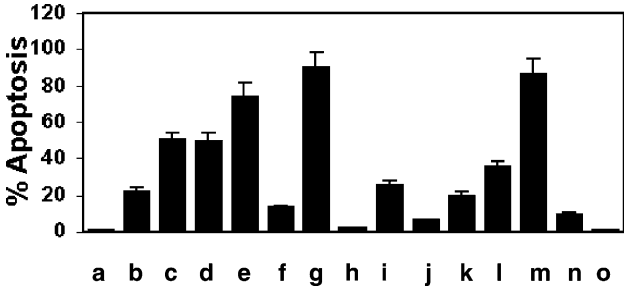


Fig. 8. Percentage apoptosis as observed in control and S29 transfected H520 cells in the presence or absence of caspase inhibitors as assessed by MTT assay and flow cytometry. (a) Control H520 cells, (b) S29 transfected H520 cells after 24 h, (c) S29 transfected H520 cells after 48 h, (d) cisplatin treatment for 24 h, (e) S29 transfection for 28 h followed by cisplatin treatment for 24 h, (f) etoposide treatment for 24 h, (g) S29 transfection for 28 h followed by etoposide treatment for 24 h, (h) Ac-DEVD-CHO caspase inhibitor treatment for 48 h, (i) S29 transfection for 24 h followed by Ac-DEVD-CHO caspase inhibitor treatment for 24 h, (j) Ac-YVAD-CHO treatment for 48 h, (k) S29 transfection for 24 h followed by Ac-YVAD-CHO caspase inhibitor treatment for 24 h, (l) CHX treatment for 24 h, (m) S29 transfection for 24 h followed by CHX treatment for 24 h, (n) Act D treatment for 24 h, (o) S29 transfection for 24 h followed by Act D treatment for 24 h. These data are means ± SD of three independent experiments.

Our results show that S29 ribosomal protein sensitizes/augments the chemotherapeutic response of both cisplatin and etoposide but to different extents.

## Discussion

In our earlier study we isolated differentially expressed genes associated with apoptosis using subtractive hybridization. Out of several clones obtained one clone on sequencing revealed homology to the ribosomal protein S29, a 56 amino acid protein with a zinc finger motif. Preliminary data revealed that enhanced expression of S29 resulted in increased apoptosis in HeLa cells. These findings provided the first hint of an apoptotic role for S29 ribosomal protein. Similar to our findings, it has been reported that human ribosomal protein L13a induces apoptosis, presumably by arresting cell growth in the G2/M phase of the cell cycle. In addition, constitutive expression of a closely related ribosomal protein L7 arrests Jurkat cells in G1 and also induces apoptosis [24–26]. However, there are also reports where elevated expression of ribosomal protein has been observed in human tumors and their suppression induced apoptosis [27,28]. These contradictory findings give rise to the speculation that components of the translation apparatus can act as multifunctional proteins, thereby illustrating "one gene dual function" phenomenon.

In order to further investigate the pro-apoptotic activity of S29 in other tumor cell lines, we transiently transfected the expression vector encoding full-length S29 ribosomal protein in different cancer cell lines of human organ origin (laryngeal cancer cell line, Hep2; glioma cell line, U87-MG; E6 transfected U87-MG; and non-small cell lung cancer cell line; H520). We observed that enhanced expression of S29 resulted in increased apoptosis in them but to different extents (unpublished data, Table 2). Maximum amount (55%) of apoptosis was observed in H520 cells after S29 overexpression and the induction of apoptosis was evaluated on the basis of morphological changes, flow cytometry, and TUNEL assay.

Caspases are a family of intracellular cysteine proteases that lie in a latent (zymogen) state in cells but become activated in response to a wide variety of apoptotic stimuli [29]. Two major pathways of caspase activation have been reported: (1) the receptor mediated apoptosis pathway where the TNF family of death receptors activates upstream caspase-8 [30,31] and (2) the mitochondrial-mediated apoptosis pathway where cytochrome *c* is released from the mitochondria and activates upstream caspase-9 [32,33]. Both pathways culminate in the activation of a major downstream effector caspase-3 [34,35]. Once activated, the downstream caspase cleaves specific protein substrates (PARP, DFF45), leading to execution of apoptosis [36]. From our findings it appears that though in S29 transfected H520 cells there is activation of both caspase-8 and -9, caspase-8 appears to be more significantly increased. Our data also demonstrate that S29 triggers release of cytochrome *c* from mitochondria to cytosol, which in turn activates downstream caspase-3. This is followed by cleavage of its substrate, PARP. Thus S29 induces caspase dependent apoptosis in H520 cells. Similar to our findings there are reports in the literature that suggest that caspases play an important role in apoptosis [29,37] and that cytochrome *c* release from mitochondria activates caspase-9, which is thought to mediate apoptosis triggered by signals such as chemotherapeutic drugs [38–41]. The analysis of our results suggests that it is likely that in non-small cell lung carcinoma H520 cells at least two apoptotic pathways coexist and there is a cross talk between them. This is substantiated by an another study [42] which has also shown that in lung carcinomas, two cell death pathways co-exist.

At the surface of the mitochondria the pro- and anti-apoptotic Bcl-2 family members compete to regulate the

Table 2
Effect of S29 on various cancer cell lines — Percentage apoptosis as observed by FACS of various cancer lines after overexpression of S29

| | Control (empty vector transfected cells) (%) | S29 transfected cells (%) |
|---|---|---|
| U87-MG | 1 | 3 |
| E6 transfected U87-MG | 2 | 9 |
| Hep2 | 5 | 35 |
| H520 | 4 | 55 |

exit of cytochrome *c* and for this release three models have been proposed in the literature: physical rupture of the outer mitochondrial membrane resulting from mitochondrial swelling, channel formed by pro-apoptotic Bax or a novel channel formed by voltage dependent anion channel (VDAC) and Bax [30,43,44,49,50]. Bcl-2 and Bcl-$X_L$ are well-established inhibitors of apoptosis and both block mitochondrial cytochrome *c* release [51]. These molecules guard against cytochrome *c* release by heterodimerizing with Bcl-2 family members and thus neutralizing, death agonists such as Bax that might have the capacity to form pores in the mitochondrial membranes [21]. The observed downregulation of Bcl-2 and Bcl-$X_L$ and upregulation of Bax by S29 in our study thus facilitate cytochrome *c* release. The released cytochrome *c* might then be promoting assembly of the apoptosome, perhaps by activating a caspase (caspase-9) which can promote further caspase activation events and subsequent cellular demolition. This was further confirmed by the use of caspase inhibitors which prevented S29 induced apoptosis. Inhibitors of apoptosis proteins (IAPs) block a common step in cell death, downstream of mitochondrial cytochrome *c* release by inhibiting caspase-3, -7, and -9. Because caspases are central for most apoptotic pathways, it is not surprising that IAPs protect cells from several anticancer drugs and other inducers of apoptosis [52]. Survivin is an inhibitor of apoptosis overexpressed in almost all human cancers. In the present study the observed decrease in survivin levels may be contributing to an increase in caspase-3 activity.

Apoptotic signaling via mitochondria appears to occur via several distinct pathways. It may be dependent on the induction of permeability transition (PT) for the mitochondrial release of cytochrome *c*, and thus can be inhibited with inhibitors of the PTP, such as cyclosporine (CyA) or signaling may be independent of PT, and cytochrome *c* release is dependent on caspases or Bid. The results observed and presented here for S29 induced apoptosis in H520 cells are consistent with apoptotic signaling via a PT independent, caspase dependent pathway, as S29 induced apoptosis is not inhibitable by the PT-inhibitors.

Besides accumulating evidence on the role of mitochondria in many forms of apoptosis by releasing cytochrome *c*, there is also evidence of the release of another protein, apoptosis-inducing factor (AIF), from the mitochondria. The release of AIF is dependent upon the occurrence of membrane permeability transition (PT) [45] characterized by membrane potential loss, while cytochrome *c* release can be either dependent on [46,47] or independent of PT [48]. Our results also indirectly suggest that S29 induced apoptosis is not characterized by membrane potential loss since we observed increase in the release of cytochrome *c*, unaltered mitochondrial membrane potential, and non-significant increase in the release of AIF from mitochondria.

MTT assay using macromolecular synthesis inhibitors showed that S29 ribosomal protein induced apoptosis can exist in a quiescent state and can be activated by mechanisms that do not require synthesis of RNA or protein.

Thus in an attempt to identify transcriptionally regulated molecules of the "activation" or "signaling" stage of the cell death process we looked for the protein expression of the transcription factors NF-κB and p53. NF-κB has been identified as a crucial protective factor against apoptosis in several experimental systems [53]. Since we observed a decrease in NF-κB which is upstream to p53, our results suggest that NF-κB was not able to effect the observed moderate increase in p53 levels. NF-κB induces the expression of IAPs such as Bcl-2, and Bcl-$X_L$ [33,54,55] but since it was decreased itself, this might perhaps explain the observed decreased levels of Bcl-2, Bcl-$X_L$, and survivin.

High expression of anti-apoptotic members such as Bcl-2 and Bcl-$X_L$ is commonly found in several human cancers including prostrate, colorectal, lung, gastric, renal, and neuroblastoma, thereby contributing to neoplastic cell expansion and interfering with the therapeutic action of many anticancer drugs [56]. Downregulation of these proteins can either restore the apoptotic process in tumor cells or sensitize tumor cells for chemotherapy and radiotherapy. In the present study it was observed that S29 induced apoptosis occurs not only by decreasing Bcl-2, Bcl-$X_L$, survivin, and NF-κB protein expression, but can also sensitize these lung cancer cells to chemotherapeutic drugs such as etoposide and cisplatin.

In conclusion, we have demonstrated that S29 is a potent apoptosis-inducing agent in human NSCLC, H520 cells which acts through both receptor mediated and mitochondria mediated caspase-dependent cell death pathways. Based on these findings we suggest that S29 ribosomal protein may be a good candidate for additional evaluation as a cancer therapeutic agent for human lung cancer as well as other types of cancers. Work is in progress in our laboratory and we are in the process of trying RNAi based knockdown experiments to determine if S29 depletion renders cells less susceptible to apoptotic inducing stimuli.

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