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Polo-like kinase1 (Plk1) knockdown enhances cisplatin chemosensitivity via up-regulation of p73α in p53 mutant human epidermoid squamous carcinoma cells

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ABSTRACT

Polo-like kinase 1 (Plk1), a critical regulator of mitotic entry, progression and exit, has been shown to be involved in a variety of cancers and thus is becoming an attractive target for cancer management. In case of DNA damage, Plk1 not only inhibits p53 independent apoptosis by dysfunctioning p73α but also allows cells to recover from growth arrest. Here, we showed the effects of knocking down *plk1* gene through small interference RNA (siRNA) on cell cycle progression, proliferation and chemosensitivity of p53 mutant A431 cells to cisplatin (CDDP). The expression of Plk1 was measured by RT-PCR and Western blotting. Anti-proliferative response accompanied with cell cycle arrest in G(2)/M phase and induction of cell death was recorded following Plk1 knockdown. Furthermore, cells following knockdown of Plk1, which induced increase of Cyclin B1, p-Cdc2 and p73α with a decrease in p-Cdc25C, were more sensitive to CDDP. CDDP treatment induced nuclear translocation and co-localization of Plk1 with p73α whereas combination of CDDP and Plk1siRNA upregulated the expression of p73α protein in a synergistic manner thereby leading to an increase up to ~5 folds in CDDP-induced cell death. The increase in caspase-3 activity indicated apoptosis as a contributor in the total cell death. Conclusively, *plk1* gene silencing can enhance the sensitivity of A431 cells to low doses of CDDP by upregulating p73α expression and thus can be a revolutionary approach in cancer chemotherapy.

1. Introduction

Developing novel strategies to prevent skin cancer represents an urgent goal due to increase in incidence of skin cancer epidemic. Worldwide, one in three cancers is skin-related. By WHO estimates, 132,000 cases of malignant melanoma (66,000 deaths) and more than 2 million cases of other skin cancers occur annually with squamous cell carcinoma (SCC) being the second most common form of skin cancer after basal cell carcinoma (BCC) [1]. Cisplatin (cis-diammine dichloroplatinum (II); CDDP) is the most constant ingredient of chemotherapy regimens used for non-melanoma SCC. In the treatment of head and neck cancer, the biology and histogenesis of which resemble that of SCC of skin, CDDP has been most widely used as a base of single and combination chemotherapy as well as a radiosensitizer [2,3]. However, resistance to this drug often develops, and additional administration decreases its chemotherapeutic efficacy. Besides, there are significant dose-limiting toxicities (e.g., neuropathy, nephrotoxicity, and ototoxicity) [4,5]. Thus, it would be useful to identify specific mitotic molecular targets whose disruption would result in tumor cell apoptosis without the side-effects of CDDP.

In the process of neoplastic transformation, it is worth noting that polo-like kinase 1 (Plk1), a mitotic cyclin-independent serine-threonine kinase is significantly over-expressed in BCC and SCC clinical samples as well as in SCC cell lines, viz. A253 and A431 [6]. Plk1 has been shown to play key roles in the regulation of mitotic progression, including mitotic entry, spindle formation, chromosome segregation, and cytokinesis. It is often over-expressed in many different tumor types, and overexpression often correlates with poor prognosis [7]. In addition, mitotic arrest and inhibition of

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proliferation, apoptosis, and tumor growth inhibition have been observed in preclinical studies using small interfering RNAs (siRNA) or small molecules that inhibit Plk1 [8–13]. Currently, several Plk inhibitors are in phase I or II clinical studies [14–16]. Plk1 is a target whose depletion can specifically kill tumor cells without affecting the normal cell survival [17]. Moreover, in case of dysfunctional p53 typical G1 arrest gets lost in response to DNA damage and cells display a stronger dependency on the G2 DNA damage checkpoint for protection against genotoxic insults. Inhibition of Plk1 is an efficient tool to establish an irreversible DNA damage-induced G2 arrest [18]. Finally, because Plk1 expression and activity are tightly coupled to mitosis, nondividing cells should not be affected by Plk1 inhibition, perhaps mitigating the side-effects seen with CDDP [19]. The status of p53 is thought to be an important mediator in the cellular response to chemotherapy [20] and about 50% of all skin cancers show mutations in p53, particularly C → T and CC → TT transitions [6]. Hence, we hypothesize that in cancers with non-functional p53 Plk1 inhibition would serve as a potent adjuvant therapy when combined with a DNA-damaging regimen.

In cancers lacking p53, p73α protein gets activated by DNA-damaging-agents such as γ-irradiation or treatment with chemotherapeutic drugs like CDDP, and plays an important pro-apoptotic role to produce substantial cytotoxic effect [21]. p73 is a newly characterized member of tumor suppressor p53 family, with structural similarity to p53 that mimics many of the p53's biological activities [22] including transactivation of an overlapping set of target genes such as *p21/WAF1*, *bax* and *PUMA*; induction of apoptosis, cell cycle arrest and cellular senescence [23,24].

In order to determine the importance of Plk1 in SCC, we investigated the effects of Plk1 knockdown on the biological characteristics of A431 cells by taking advantage of siRNA against Plk1. Our results elucidate the role of Plk1 in pathogenesis of SCC and may help to develop a novel strategy targeting p73α via Plk1 to improve the efficiency of chemotherapy delivered to patients with SCC having mutated p53.

2. Materials and methods

2.1. Reagents

Propidium iodide (PI), dimethyl sulfoxide (DMSO), 4′,6-diamidino-2-phenylindole (DAPI), 3[4-dimethylthiazol-2-71]-2-5-diphenyl tetrazolium bromide (MTT), CDDP and β-actin (clone AC-74) were purchased from Sigma–Aldrich (St. Louis, Columbia, USA). The p-Cdc2 (Tyr15), p-Cdc25C (Ser198), Plk1 and Cyclin B1 antibodies were procured from Cell Signaling Technology, Inc. (Danvers, MA, USA) while p73α antibody was from Abcam (Cambridge, UK). Ribonuclease A (RNase A) was from Bangalore Genei (Bangalore, India) while polyvinylidene fluoride (PVDF) membrane was obtained from Millipore (Bedford, MA, USA). Caspase-3/CPP32 Fluorometric Assay kit was procured from Biovision Research Products (CA, USA). Fluorescein isothiocyanate (FITC), Alexa Fluor-568 and horseradish peroxidase conjugated anti-mouse/anti-rabbit secondary antibodies were procured from Bangalore Genei (Bangalore, India), Molecular Probes (Invitrogen, Carlsbad, CA) and Cell Signaling Technology, Inc. (Danvers, MA, USA), respectively. Rest of the chemicals were of analytical grade of purity and procured locally.

2.2. Cell culture and Plk1 RNAi experiment

A431 cells were obtained from National Centre for Cell Science (Pune, India) and cultured in Dulbecco's Modified Eagle Media (DMEM) F12 supplemented with 10% heat inactivated fetal bovine serum, 100 μg/ml penicillin streptomycin (Gibco Lifetech, Karlsruche, Germany) and maintained in a humidified atmosphere of 95% O_2 and 5% CO_2 at 37 °C. Transfection of cells with Plk1 (GenBank accession no. NM_005030) siRNA sense sequence 5′-GGAGGUGUUCGCGGGCAAGtt-3′; antisense sequence 5′-CUUGCCCGCGAACACCUCCtt-3′ (Ambion, Austin, TX) (siPlk1), targeting regions of the Plk1 transcript at exon 1, was performed by using siPORT Neo Fx transfection reagent (Ambion, Austin, TX) according to the manufacturer's instructions with slight modifications. Briefly, 3×10^4 cells were seeded on 24-well plates in normal medium without antibiotics and transfected at 30–40% confluency. Transfection complex was prepared, with 1.875 μl (0.8 μg) from 20 μM stock siRNA and 2.5 μl of siPORT NeoFX in 100 μl Opti-MEM I medium (Invitrogen, Carlsbad, CA) per well. 100 μl of newly formed transfection complexes were incubated for 25 min at RT and then added to wells. Next, 150 μl Opti-MEM1 was poured in the wells, making the total volume to 250 μl Opti-MEM1 medium per well. 150 μl of DMEM F12 medium with 20% FBS was added to wells after 18 h. Transfected cells were cultured for up to 48 h and then harvested for further analysis. A scrambled siRNA was purchased from Ambion (Negative control siRNA #1) (Ambion, Austin, TX) and used as the control. For CDDP treatment, the time point chosen for the addition of CDDP to the transfected cells was 24 h after transfection, and was based on preliminary experiments (data not shown).

2.3. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from the treated and control samples using the Invitrogen Purelink Micro to Midi Total RNA purification Kit (Carlsbad, CA), in accordance with the manufacturer's instructions. RT-PCR was conducted according to Arora et al. [25]. In brief, cDNA was prepared using RNA sample (3–5 μg) adding 1 μg oligo$(dT)_{18}$, 0.5 mM dNTP, and 200 U of Revert Aid^{TM} H Minus M-MuLV RT enzyme (MBI Fermentas, Hanover, MD, USA). PCR was performed using selective human primers along with endogenous control gene (*β-actin*) (synthesized at Integrated DNA Technologies, Inc., Coralville, IA) on 2 μl of RT product incubated with 1 U of *Taq* DNA polymerase in a 50 μl reaction mixture containing 1 mM dNTP, 1.5 mM $MgCl_2$ (MBI Fermentas). The amplified fragments were detected in 2% (w/v) agarose gel and visualized on image analysis system IS1000 (Alpha Innotech, San Leandro, CA). The intensity of the bands was measured using software UNSCAN-IT automated digital system version 5.1 (Orem, USA) and then given in terms of relative pixel density for each band normalized to the band of β-actin.

2.4. Cell cycle analysis

For cell cycle analysis, the cells were prepared as described earlier [25]. Briefly, cells were washed with PBS and centrifuged at 200 × *g* for 10 min at 4 °C. The pellet was fixed in 1 ml of 70% ice-cold ethanol for 30 min and resuspended in 50 μg/ml PI with RNase A (100 μg/ml) followed by incubation for 30 min in dark. The samples were acquired and analyzed on flow cytometer using 'CellQuest' software.

2.5. Western blotting

To assess the protein expression Western blotting was performed as described by Towbin et al. [26]. Protein lysates from A431 cells were prepared and protein concentration was estimated by the method of Lowry et al. [27]. Proteins (50 μg) were resolved on 10–18% SDS-polyacrylamide gels followed by electro-transfer onto an immobile PVDF membrane. The blots were blocked

overnight with 5% nonfat dry milk, probed with respective primary antibodies (Plk1, p-Cdc25 C, Cyclin B1, p-Cdc2, p73α and β-actin) and detected by respective horseradish peroxidase conjugated secondary antibodies using chemiluminescence kit from Millipore (Bedford, MA, USA) and visualized by Versa Doc Imaging System (BioRad Model 4000, Hercules, CA). The intensity of the bands was measured using software UNSCAN-IT automated digital system version 5.1 (Orem, USA) and then given in terms of relative pixel density for each band normalized to the band of β-actin.

2.6. Assessment of cell viability by MTT assay

Cells were seeded into 96-well plates at 1×10^3 cells per well overnight and then transfected with siPlk1 as mentioned above with accordingly reduced concentrations of all the reagents. 20 μl MTT dye solution (5 mg/ml) was added to each well 72 h after the transfection and samples were incubated at 37 °C for 4 h. The formazan product was dissolved by adding 200 μl of DMSO to each well. The plates were read at 570 nm.

For cell chemosensitivity assay, first, the effect of CDDP on A431 cells' viability was evaluated by MTT analysis; then, 24 h after siPlk1 transfection, the cells in the 96 wells were treated with various concentrations of CDDP (at 0, 5, 10, 20, 30, 40, 50, 60 and 70 ng/ml). Cell viability was detected 48 h later, as described above.

2.7. Caspase-3 activity assay

Caspase-3 enzyme activity was measured using Caspase-3/CPP32 Fluorometric Assay kit (Biovision, CA, USA). 48 h after the transfection, 1×10^6 cells were lysed and incubated with 50 μM substrate, DEVD-AFC {Asp-Glu-Val-Asp}-{7-amino-4-trifluoromethyl coumarin} at 37 °C for 2 h. The samples were read on ELISA-plate reader.

2.8. DNA fragmentation assay

The DNA fragmentation pattern (DNA ladder) was studied using agarose gel electrophoresis as described earlier [28]. Briefly, 48 h after the transfection cells were pelleted by centrifugation at $200 \times g$ for 10 min, and the pellet was lysed with 0.5 ml lysis buffer (10 mM Tris–HCl, pH 7.5, 20 mM EDTA, 0.5% Triton-X-100) on ice for 30 min. The DNA in lysis solution was extracted with phenol/chloroform and precipitated with 3 M sodium acetate (pH 5.2) and cold ethanol. The purity of DNA at 260 and 280 nm absorbance was detected, and the ratio (A260/280) obtained was between 1.7 and 1.9. DNA (2 μg) was then loaded on a 0.8% agarose gel, and electrophoresis was carried out. The bands were visualized by ethidium bromide staining under UV light and analyzed on image analysis system IS1000 (Alpha Innotech, San Leandro, CA).

2.9. Immunofluorescence staining

Immunofluorescence of p73α and Plk1 proteins was carried out by fixing with 4% paraformaldehyde in PBS for 7 min, permeabilization with 0.1% Triton-X-100 in PBS for 7 min, and blocking with 3% BSA in PBS for 1 h with gentle agitation. After blocking, cells were washed in PBS and incubated with anti-p73α antibody and anti-Plk1 antibody for 1 h at room temperature, followed by the incubation with FITC-conjugated anti-rabbit IgG and Alexa Fluor-568 labeled goat anti-mouse IgG for 1 h at room temperature. Cell nuclei were stained with DAPI. Fluorescence microscopy was performed using Olympus IX51 (Olympus America Inc., Center Valley, PA, USA) and images acquired with the help of software Image Pro Express.

2.10. Statistical analysis

The data were analyzed as mean values ± SD for all treated and untreated controls. For the statistical analysis of all data, Student's *t*-test was used and $p < 0.05$ was considered as significant.

3. Results

3.1. Specific inhibition of Plk1 mRNA and protein by RNAi

To study the effects of Plk1 knockdown on the biological characteristics of A431 cells, A431 cells were transfected with siPlk1. After 48 h, cells were harvested following 100 nM siPlk1 transfection to measure Plk1 gene and protein expression. This time point and siPlk1 amount was chosen for maximum knockdown after carrying out preliminary experiments with 30, 50, 75 and 100 nM siPlk1 concentrations at different time durations of 12, 24, 36 and 48 h after transfection (data not shown). Relative to scrambled siRNA-transfected cells, siPlk1 transfection in A431 cells induced significant 70–80 and 80–90% decrease in Plk1 mRNA (Fig. 1A) and protein levels (Fig. 1B), respectively ($p < 0.05$). Concurrently, p-Cdc25c protein levels followed a similar pattern of reduction while on the contrary, Cyclin B1 and p-Cdc 2 protein levels were increased (Fig. 1B).

3.2. Plk1 knockdown by siPlk1 transfection inhibited cell proliferation

To determine the effects of siPlk1 transfection on A431 cells, cell proliferation by MTT assay was performed. Significant 40% decrease in cell proliferation as compared to the control (Fig. 1C) was seen on Plk1 knockdown ($p < 0.05$).

3.3. Plk1 knockdown induced growth arrest and cell death

Our next aim was to determine the functional consequences of Plk1 knockdown on the cell cycle characteristics and cell death of A431 cells. 48 h after transfection Plk1 depleted cells did show a strong G2/M arrest ($p < 0.05$) up to 39.61% as compared to control cells (15.24%) (Fig. 2A and C). Moreover, the cells with sub-G1 DNA content also increased noticeably from 13.10% at 48 h to 29.58% at 72 h, compared to control cells (0.74%) (Fig. 2B and C). To determine whether the cell death observed after Plk1 knockdown was mediated through caspase activation, caspase-3 activity was measured (Fig. 2D), exhibiting a significant increase by 24% in siPlk1-treated A431 cells, compared to control cells ($p < 0.05$). This increase in caspase-3 activity suggested the substantial contribution of apoptosis in total cell death. The induction of cell death by Plk1 knockdown was further confirmed by marked DNA fragmentation in Plk1 depleted cells, whereas there was no fragmentation in control cells (Fig. 2E).

3.4. Sensitization of A431 cells to chemotherapy by Plk1 knockdown

Combinatorial drug therapy is a treatment paradigm that has proved effective in cancer. But the key, of course, is to identify combinations that yield a benefit at low doses to limit unwanted toxicity. Thus, we tested CDDP, which is already an established antineoplastic drug in the clinic, in combination with Plk1-specific siRNA to study the effect on A431 cell proliferation and induction of cell death. siPlk1 significantly enhanced the chemosensitivity of CDDP, decreasing the 50% inhibitory concentration of CDDP by 5-fold (Fig. 3A) ($p < 0.05$). After treatment with CDDP in siPlk1 transfected cells, we observed a synergistic effect of siPlk1 and CDDP treatment on anti-proliferative response, caspase-3 activity and DNA fragmentation assay 48 h post-transfection. In flow cytometric analysis dose dependent increase in sub-G1 fraction of

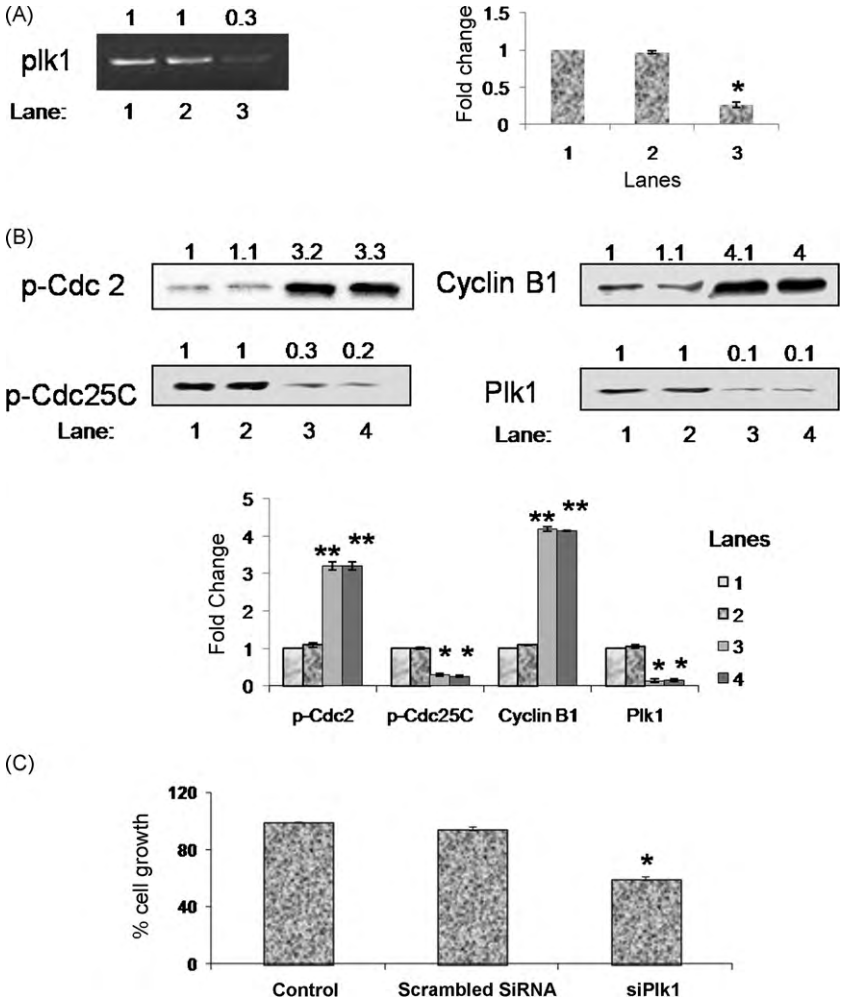

Fig. 1. (A) RT-PCR analysis of *plk1* gene. Lane 1: nontransfected control, lane 2: scrambled siRNA-transfected cells, lane 3: siPlk1 transfected cells. Right panel shows fold change calculated with respect to control on the basis of pixel density measured by UNSCAN-IT software. (B) Western blot analysis of Plk1, Cyclin B1, p-Cdc2 and p-Cdc25C proteins. Lane1: untreated control, lane 2: cisplatin treated, lane 3: siPlk1 transfected, lane 4: siPlk1/cisplatin both treated cells. Lower panel shows fold change calculated with respect to control on the basis of pixel density measured by UNSCAN-IT software. (C) MTT cell proliferation assay after siPlk1 transfection. Data shown is the result of three different experiments with similar results; represented as the relative density of bands normalized to β-actin. The data were significant at $p < 0.05$. *Significant decrease over control. **Significant increase over control.

cells was seen when CDDP doses 7.5, 10, 30 and 50 ng/ml were given in Plk1 depleted cells. At CDDP dose 10 ng/ml this fraction of apoptotic cells synergistically increased to 54.98% over 14.45% and 13.10% of CDDP alone and siPlk1 alone treated cells, respectively (Fig. 3B and C). Activity of caspase-3 enzyme was augmented by 3.24- and 3.06-fold over that in siPlk1 alone and CDDP alone treated cells, respectively (Fig. 2D). Concomitantly marked increase in DNA fragmentation was also seen (Fig. 2E). These results imply that Plk1 knockdown contributes to enhancing the chemosensitivity.

3.5. *Plk1 knockdown induces p73α and its downstream pro-apoptotic targets in p53-deficient cancer cells*

To understand the mechanism of CDDP chemosensitization of p53 mutant A431 cells by Plk1 depletion, levels of p73α protein expression were measured. p73α protein levels increased after Plk1 knockdown and it further enhanced synergistically on combining Plk1 depletion with CDDP treatment (Fig. 4A). The RT-PCR analysis demonstrated mRNA up-regulation of p73α downstream targets (bax and gadd 45a) following knockdown of Plk1 which further augmented on combining siPlk1 and CDDP treatment (Fig. 4B). Interestingly, the p73α mRNA expression remained unchanged, following Plk1 knockdown (data not shown). Taken together, these results confirm that Plk1 inhibition by siPlk1 treatment specifically induces p73α, at the protein and not at the mRNA level, and its downstream pro-apoptotic targets. Moreover, all these modulations of p73α protein and thereby its downstream targets are enhanced synergistically when CDDP treatment was given to Plk1 depleted cells.

To address whether Plk1 could associate with p73α in cells, subcellular distributions in response to CDDP were studied. Immunofluorescence microscopy showed that the endogenous p73α is detectable in cell nucleus regardless of CDDP treatment. It was worth noting that the endogenous Plk1 localizes majorly in the cytoplasm in the absence of CDDP, whereas CDDP treatment induces the nuclear accumulation of Plk1. Merged images revealed that Plk1 is largely co-localized with p73α in the cell nucleus in response to CDDP. Under our experimental conditions, immunofluorescence staining without primary antibodies did not show any positive signals. These observations suggest that Plk1 might interact with p73α in cells exposed to CDDP. On Plk1 knockdown expression level of Plk1 reduced with an increase in that of p73α. When CDDP treatment was given to siPlk1 transfected cells, synergistic amplification of p73α expression was seen with considerable reduction in nuclear translocated Plk1 (Fig. 5).

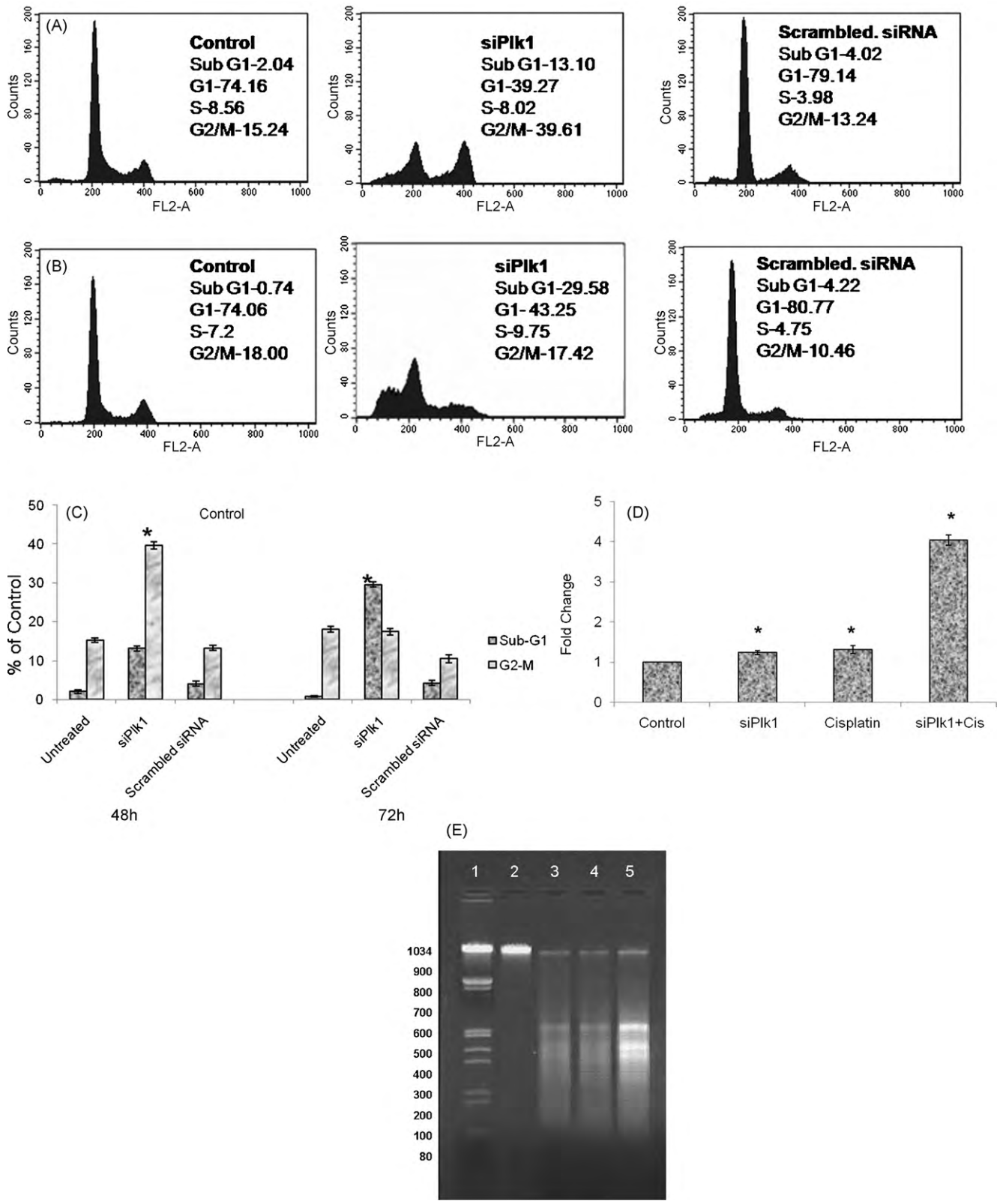

Fig. 2. Cell cycle assay (A) 48 h and (B) 72 h after siPlk1 transfection showing percent cells in each phase. (C) Bar diagram showing the G2-M and sub-G1 fractions of cells obtained in above-mentioned cell cycle assay. Cell death assessment through (D) caspase activity assay and (E) DNA fragmentation assay—lane 1: DNA ladder, lane 2: untreated control, lane 3: siPlk1 transfected, lane 4: cisplatin treated, lane 5: siPlk1/cisplatin both treated cells. Data shown is the result of three independent experiments with similar results; representative figures shown. The data were significant at $p < 0.05$. *Significant increase over control.

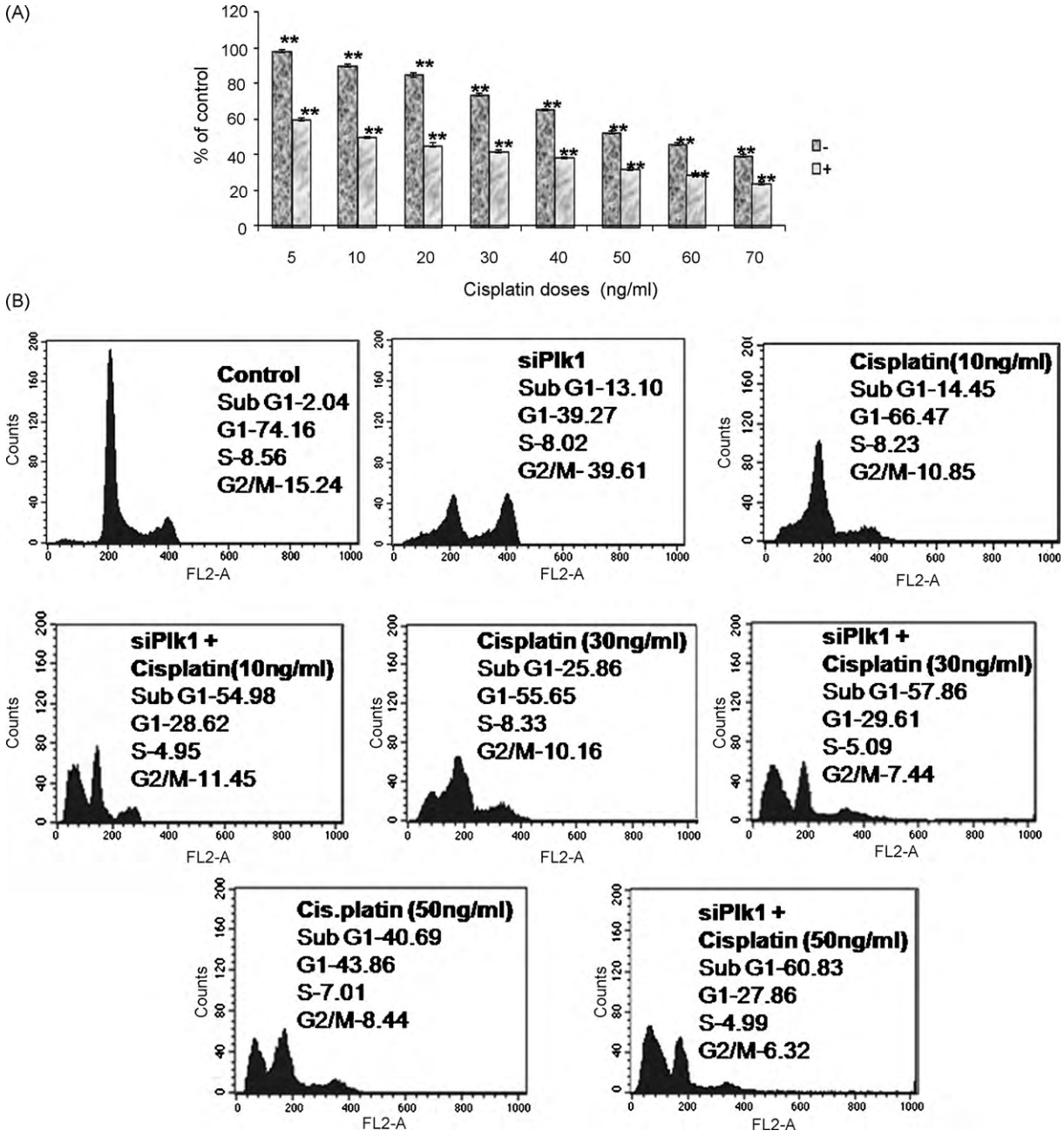

Fig. 3. A Cisplatin chemosensitivity assessment through MTT assay in (−) cisplatin and (+) siPlk1/cisplatin both treated cells. (B) Cell cycle assay in siPlk1/cisplatin both treated cells. (C) Bar diagram showing the sub-G1 fraction of cells obtained in above-mentioned cell cycle assay. Data shown is the result of three independent experiments with similar results; representative figures shown. The data were significant at $p < 0.05$. *Significant increase over control, **significant decrease over control.

4. Discussion

Now, it is well-established that Plk1 plays an important role in cell cycle regulation by functioning in centrosome maturation, spindle formation, mitotic entry, and cytokinesis. The significance of Plk1 has been demonstrated in a variety of tumors [7] and its knockdown through RNA interference has shown promise as a way to intervene in cancer progression [11–13]. Although like other cancers Plk1 expression is found to be significantly upregulated in SCC [6], the significance of Plk1 in the pathogenesis and management of this cancer is not well-understood. In this study we have explored the possibility of Plk1 as an effective therapeutic target in SCC A431 cells, by employing RNA interference technique to silence endogenous Plk1 expression and then analyzing the phenotypic changes. We achieved substantial reduction in Plk1 at both mRNA and protein levels by using a siRNA treatment strategy in A431 cell line. Plk1 downregulation led to significant inhibition of proliferation, accumulation of cells in G2/M phase, and finally cell death of A431 cells ($p < 0.05$). These results suggest that Plk1 can make an excellent molecular target for SCC therapy. Modulation of downstream pathways important in cell proliferation, with up-regulation of proteins Cyclin B1 and p-Cdc2 and reduction of p-Cdc25C, was observed.

CDDP is one of the most important classes of anticancer agents. Despite its relevant contribution in ameliorating the quality of life and overall survival of cancer patients, drug resistance and side-effects hamper its wide usage [4,5]. Therefore, it is desirable to find new ways of lowering drug dosage without losing effectiveness to limit side-effects and also to slow down drug resistance. Thus to achieve this we combined Plk1 knockdown with CDDP in A431

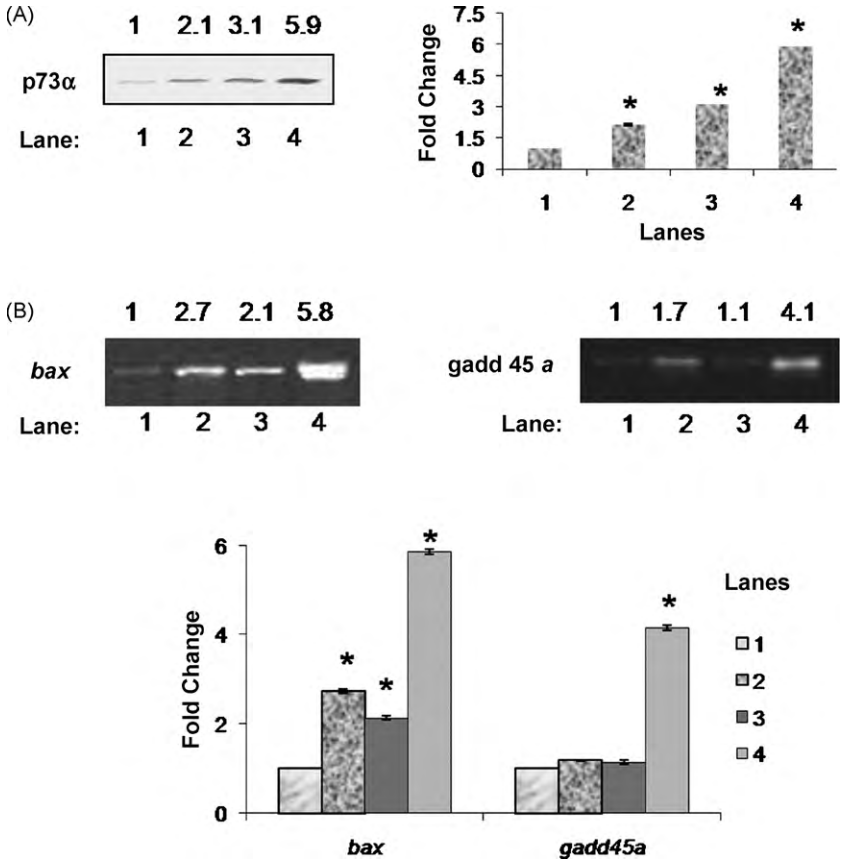

Fig. 4. (A) Western blot analysis of p73α protein. Right panel shows fold change calculated with respect to control on the basis of pixel density measured by UNSCAN-IT software. (B) RT-PCR analysis of *bax and gadd 45a* genes. Lane1. Untreated control, lane 2: cisplatin treated, lane 3: siPlk1 transfected, lane 4: siPlk1/cisplatin both treated cells. Lower panel shows fold change calculated with respect to control on the basis of pixel density measured by UNSCAN-IT software. Data shown is the result of three independent experiments with similar results; representative figures shown. The data were significant at $p < 0.05$.

cells. On combination, significant cell death could be achieved at doses even 5 times lower than that of CDDP alone and this effect was amplified synergistically ($p < 0.05$). The cytotoxic effect of CDDP is thought to be mediated via DNA damage by formation of DNA adducts and intrastrand crosslink adducts. In response to DNA damage, a p53 proficient cell with functional p53 will halt cell cycle progression until DNA is repaired or the cell undergoes apoptosis. Aside from being a crucial regulator in multiple points of mitosis, Plk1 is also required for checkpoint recovery after G2/M DNA damage arrest [18] and in response to DNA damage Plk1 transcriptional levels are negatively controlled by p53. However, with the loss of functional p53, the suppression of Plk1 transcription may not occur thereby leading to insufficient DNA damage response. In fact p53 mutation prevalent in 50% of the human skin cancer cases is the suspected reason for over-expression of Plk1 in BCC and SCC clinical samples as well as in SCC cell lines, viz. A253 and A431 [6]. Thus, one of the possible explanations for the synergistic apoptotic effect recorded on combining Plk1 knockdown with CDDP treatment in p53-deficient A431 cells is a more stringent G2/M arrest imposed on treatment with DNA-damaging-agents in Plk1 depleted cells, the phase most susceptible to DNA damage, which ultimately led to chemosensitization. A large body of evidence has validated the G2/M checkpoint as an attractive pathway for targeting and sensitizing tumor cells to cancer treatment [29–31]. Of late few studies have shown chemosensitization by targeting Plk1 in various cancer cells [15,32]. Although Degenhardt and Lampkin have recently reported the activity of Plk1 inhibitors against TP53 mutant tumor cells, yet, combinatorial potential with known anticancer therapeutic drugs had not been evaluated [14].

In addition, studies have also reported that Plk1 protects p53-deficient cells from p73-mediated apoptosis [33]. The identification of p53 family member, p73, has added another dimension to our understanding of tumorigenesis and cell response to cancer therapy. Recently, an important linkage between p73 and chemosensitivity has been established in SCC cell lines including A431 where CDDP and many other chemotherapeutic drugs were seen to increase the endogenous p73 levels [21]. In our study, Plk1 knockdown led to a significant increase in the protein level of p73α ($p < 0.05$). The mRNA levels of p73α transcription targets *bax* and *gadd 45a* increased while the p73α mRNA levels remained unchanged suggesting that the increase in the protein level was unlikely a transcriptional effect. CDDP treatment when combined with Plk1 knockdown increased p73α protein levels synergistically. Nuclear translocation and co-localization of Plk1 with p73α on CDDP treatment and then synergistic up-regulation of p73α on CDDP treatment in siPlk1 transfected cells indicate towards regulatory role of Plk1 in p73α protein level but the exact mechanism remains unknown. Other studies have also shown Plk1 mediated inhibition of p73's pro-apoptotic function [33]. Studies have reported that p73α plays a crucial role in the induction of massive apoptosis in p53-deficient cells [34]. Here also up-regulation of p73α underlies behind amplification of cell death with apoptosis being a significant contributor.

Conclusively, siPlk1 in combination with CDDP demonstrated a high efficacy in inhibiting proliferation of A431 cells by blocking cells in DNA damage sensitive G2-M phase and upregulating pro-apoptotic p73α protein. The data suggests that specific targeting of Plk1 can sensitize p53 mutant A431 cells to conventional

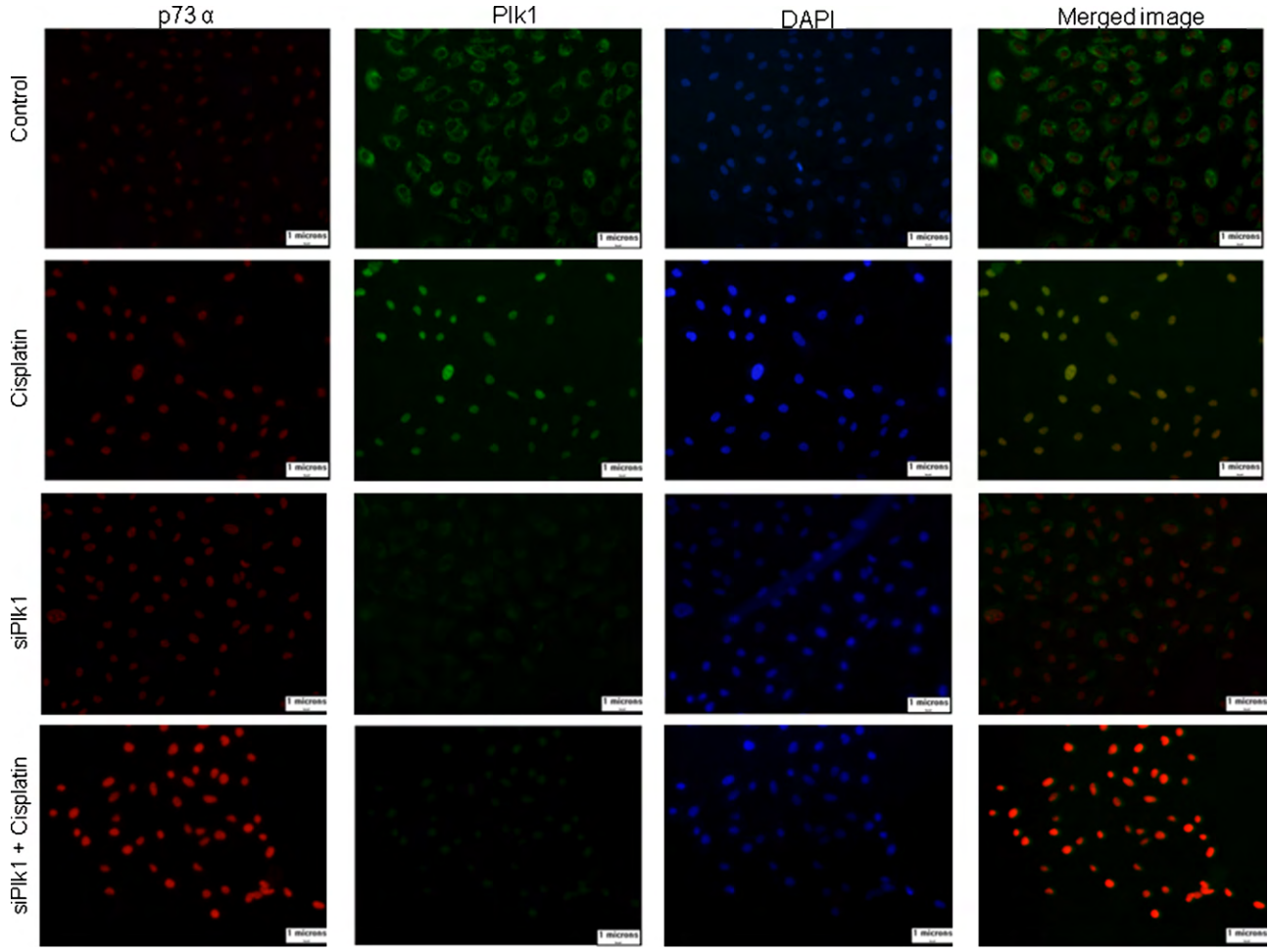

Fig. 5. Immunofluorescence microscopy for subcellular localization of Plk1 (green) and p73α (red) proteins after siPlk1/cisplatin both treatment. Data shown is the result of three independent experiments with similar results; representative figures shown.

chemotherapeutic agent CDDP, thereby reducing its side-effects by lowering the dosage.

Conflict of interest statement

None declared.

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