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6-Aminonicotinamide Induces G_1 Arrest by Elevating $p27^{kip1}$ as well as Inhibiting cdk2, Cyclin E and p-Rb in IMR32 Neuroblastoma Cell Line

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Abstract: The effects of 6-aminonicotinamide (6-AN) on viability of IMR32 neuroblastoma cells in the presence of ATP or NAD^+ have been investigated. 6-AN caused marked reduction in cell viability and similar observations were also made with cells treated with 6-AN+ATP. However, cells treated with 6-AN+NAD^+ showed cell viability similar to untreated cells. Morphologically, 6-AN and 6-AN+ATP treated cells showed loss of neurites, polyhedric shapes, shrinkage of cell bodies and formation of lysed cells, while 6-AN+NAD^+ cells did not show any such changes. The flow cytometry analysis demonstrated that 6-AN increased cell population in G_0/G_1 phase and decreased cell population in S and G_2/M phase following a 72 h exposure. Western blot analysis showed that 6-AN stimulated a substantial increase in the level of the cdk inhibitor $p27^{kip1}$, but lowered the levels of cdk2, cyclin E and p-Rb. However, cdc25A and p53R2 were not significantly affected. Immunofluorscence staining of $p27^{kip1}$, cdk2, cyclin E and p-Rb revealed close correlation between the signal observed in the Western blot analysis. 6-AN+ATP treated cells showed similar results obtained with 6-AN treated cells in expression of cdk2, cyclin E, p-Rb proteins and $p27^{kip1}$. 6-AN+NAD^+cells showed greater expression of cdk2, cyclin E and p-Rb than those in 6-AN and 6-AN+ATP treated cells. The results suggest that 6-AN induced the G_0/G_1 phase arrest in IMR32 neuroblastoma cell lines through the increase of $p27^{kip1}$ and the decrease of cdk2, cyclin E and p-Rb.

Key words: 6-Aminonicotinamide, IMR32 cells, ATP, NAD^+, G_1 arrest

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6-Aminonicotinamide (6-AN) decreases the synthesis of ATP (Griffiths et al., 1981), poly (ADP-ribose) (Hunting et al., 1985), γ-globulin (Lee and Park, 2001), and RNA (Knoll-Kohler et al., 1980) but increases the synthesis of prealbumin (Lee and Park, 2001) and glucose-regulated stress protein (Belfi et al., 1990). 6-ANAD or 6-ANADP adducts, derived from the incorporation of 6-AN into NAD^+ or $NADP^+$, have been known to inhibit glyceraldehyde-3-phosphate dehydrogenase and NAD glycohydrolase (Kim and Park, 1998), 6-phosphogluconate dehydrogenase (Herken et al., 1969), glutamic dehydrogenase (Bielicki and Krieglstein, 1976) and monoamine oxidase (Yang and Park, 2000) but increase acid phosphatase (Iglesias-Rozas and Iglesias, 1974), aspartate aminotransferase and creatine phosphokinase (Lee and Park, 2001). All these multiple biochemical aberrations triggered by 6-AN have been suggested to be primarily responsible for cellular lesions and cell death.

It has been shown that the anti-proliferation action of anti-tumor drugs and induction of G_1 phase accumulation are related to the modulation in cell cycle regulatory proteins. The G_1 phase accumulation was induced by anti-cancer drug UCN-01 whose effect was closely associated with decreases in cdk2 and p-Rb and upregulation of $p27^{kip1}$ in HCT116 human colon cancer cells, WI-38 human lung fibroblast and saos-2 human osteosarcoma cells (Tadakazu et al., 1999; Akiyama et al., 1997). Yang and Kerry (2003) reported that $1,25\text{-}(OH)_2D_3$ mediates an increase in $p27^{kip1}$ level, inhibition of cdk2 activity, hypophosphorylation of Rb protein, and accumulation of cells in G_1 phase in LN-Cap human prostate cancer cells. Recently, histone deacetylase inhibitor BL1521 was demonstrated to cause an increase in $p27^{kip1}$ protein and downregulate cdk4 in neuroblastoma cells (Ouwehand et al., 2005). Since no or little information is available on the effects of 6-AN on

regulation of cell cycle progression, we undertook experiments to look at basic underlying mechanisms of some key regulatory proteins involved in cell cycle.

In the current study we present that 6-AN induces G_1 arrest via upregulation of $p27^{kip1}$and downregulation of cdk2 and cyclin E proteins.

Materials and Methods

Chemicals

6-Aminonicotinamide (6-AN), ATP, NAD^+, goat anti-rabbit, goat anti-mouse, and mouse anti-goat IgG-FITC conjugated secondary antibodies were obtained from Sigma Chemical Co. Inc. (St. Louis, MO). RPMI 1640, antibiotic-antimycotic solution (100 × penicillin-streptomycin) and trypsin-EDTA were from Gibco Laboratories (Grand Island, NY). Goat anti-rabbit IgG, goat anti-mouse IgG, and rabbit anti-goat IgG horseradish peroxidase conjugated secondary antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-cdk2, anti-$p27^{kip1}$, anti-cyclin E, anti-cdc25A, and anti-p53R2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PVDF membrane (Hybond-p) and enhanced chemiluminescence (ECL) reagents were purchased from Amersham (Arlington Heights, IL). All other reagents used were of analytical grade.

Cell culture treatments

IMR32 human neuroblastoma cell line was a generous gift from Professor Mi Young Kim at Asan Medical Center which was originally obtained from American Type Culture Collection. IMR32 were cultured using collagen coated dishes in RPMI 1640 media supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin-G and 100 μg/ml streptomycin). Cultures were maintained in a humidified atmosphere of 95% air, and 5% CO_2 at 37°C.

Cells were divided into four groups with about 6×10^6 cells in 100 mm diameter collagen coated dishes and were treated one day after plating. The control group was cultured in 0.5 FBS medium without any treatments, whereas 6-AN group, 6-AN+ATP group and 6-AN+NAD^+ group were cultured in the 0.5 FBS medium containing 0.5 mM 6-AN, 0.5 mM 6-AN+1 mM ATP, and 0.5 mM 6-AN+1 mM NAD^+, respectively. After 72 h, cells were harvested by multiple pipetting and centrifuged at 1300 rpm for 5 min at 4°C, and processed for biochemical assays.

Analysis of cell viability

The growth inhibition effect of 6-AN on IMR32 cells was determined by measuring MTT dye absorbance of living cells. Cells (2×10^5 cells per well) were seeded in 96-well microtitre plates (Nunc, Roskilde, Denmark). After exposure to the drug for 72 h, the medium was removed, and cells were incubated with MTT (5 mg/ml) in growth medium for 4 h at 37°C. After the medium had been aspired carefully, the precipitated formazan was solublized with DMSO and quantified spectrophotometrically at wavelength of 570 nm by using a microplate reader (Molecular Devices). Results were expressed as a percentage of the solution absorbance in the vehicle-treated control wells.

Morphological assessment

The effect of 6-AN on morphological changes in IMR32 cells was examined. IMR32 neuroblastoma cells were seeded in 6-well microtitre plates, and were exposed to 6-AN, 6-AN + ATP, and 6-AN + NAD^+ for 72 h and photos were taken under inverted phase-contrast microscope (Leica, Germany).

Cell cycle analysis

Cell cycle distribution was determined by staining DNA with propidium iodide (PI). Briefly, cells were incubated without or with treatment for 72 h (Park et al., 2002), after which cells were washed in PBS and fixed in ice-cold 100% ethanol at 4°C for 1 h. The resultant cells were again washed with PBS, and then incubated with PI agent (0.15 Triton-X 100, 0.1 mM EDTA, 50 μg/ml RNase A and 50 μg/ml PI) for 30 min. Percentage of cells in the different phases of cell cycle was measured with FACscan laser flow cytometer (Becton Dickinson, San Jose, CA), analyzed by using Becton Dickinson software (Lysis II, Cellfit).

Western blot analysis

After 72 h of the indicated treatments, the cells were washed with PBS and harvested in lysis buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% of Nonidet P-40, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF). Protein concentrations were determined by Bradford Protein Assay and read at 595 nm. Equal amounts of protein were fractionated by electrophorsis (10% SDS-PAGE gel). Prestained molecular weight markers were used as a standard. Proteins were electrotransferred to PVDF membranes in transfer buffer (25 mM glycine, 25 mM ethanolamine and 20% methanol) and then blocked at room temperature for 1 h in blocking buffer (1x TBS-T, 5% w/v non-fat dry milk). Blots were then incubated with anti-cdk2, anti-$p27^{kip1}$, anti-cyclin E, anti p-Rb, anti-cdc25A or anti-p53R2 primary antibodies in approperiate concentrations (1:500), and then washed three times in TBS-T buffer. The membranes were incubated for 30 min with respective secondary antibodies. The proteins were visualized by chemiluminescence reagent and exposure to Kodak film for a few minutes.

Immunofluorescence staining and microscopy

For immunofluorescence microscopy, cells rinsed with

PBS were fixed with 3.7% formaldehyde in PBS for 3 min at room temperature and were permeabilized with 0.5% Triton-X 100 (Sigma Chemical Co.) in PBS for 30 min. All primary antibodies were used at an appropriate dilution (1 : 200) for 1 h in humid chamber. Afterwards, the stained samples were washed with washing solution for three times. All secondary antibodies were affinity-purified and tagged with fluorescein (FITC) and used at 1 : 400 dilution. Nuclear counterstaining was performed by using 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI, 2 μg/ml). Finally all samples were mounted in DAKO fluorescent mounting medium, and the cells were evaluated and photographed by fluorescence microscopy (Axiophot-Zeiss light microscope, Oberkoken, Germany).

Densitometry and statistical analysis

The immunoblots were scanned, and the mean density for each band was analyzed using TotalLab software (v2003. 03). Prior titration experiments had confirmed that image densities were linearly proportional to protein masses. Each protein signal was normalized against the β-actin signal in each sample before comparisons for fold changes. The statistical evaluation was conducted by one-way ANOVA and Duncan's test for comparisons among groups. $p < 0.05$ was considered to be significant.

Results

Cell viability after 6-AN exposure

A time course of cell viability following the exposure of cells to 0.5 mM 6-AN was carried out using the MTT cell survival assay. The ability of ATP or NAD^+ to attenuate cell death following 6-AN exposure was also assessed. In the treated cells, cell viability dropped quite sharply at 24 h to ~58% level of the untreated control cells (Fig. 1). The addition of ATP to these cells did not prevent the reduction of cell viability. By 72 h, the percentage of cell viability in 6-AN and 6-AN + ATP was 35% and 40% of control levels, respectively. There was no significant difference in the percentage of cell viability between the 6-AN and 6-AN + ATP treated cells. However, NAD^+ displayed a significantly increase in cell viability over 6-AN or 6-AN + ATP treated cells and the cell viability similar to the untreated cells at all time points tested.

Morphological evaluation of IMR32 cells

Figure 2 shows morphological changes of IMR32 cells exposed to 6-AN, 6-AN + ATP or 6-AN + NAD^+ by inverted phase-contrast microscopy. The untreated control cells (Fig. 2A) showed normal morphology of flat polygons cells forming pseudopodia of various shapes. Cultivation of cells in the presence of 6-AN was accompanied by characteristic morphological changes (Fig. 2B) such as irregular shape and arrangements, of cells lost neurites, round-shaped nucleus, cytoplasmic vacuolization, and shrunken cell bodies. The inclusion of ATP in the culture media appeared to produce no apparent effects on the restoration of cell morphology which was severely deformed by 6-AN (Fig. 2C). However, the addition of NAD^+ produced a significant restoration of cell morphology to the untreated control cells. Taken together, these observations suggest that NAD^+ was much more effective at protecting cells against 6-AN than ATP.

6-AN induces G_0/G_1 cell cycle arrest in IMR32 cells

To further characterize the anti-proliferative activity of 6-AN in IMR32 neuroblastoma cells, we examined the effect of this drug on cell cycle regulation. Figure 3 shows cell population distributions (Fig. 3A) and representative histograms of flow cytometry data (Fig. 3B) in the untreated control, 6-

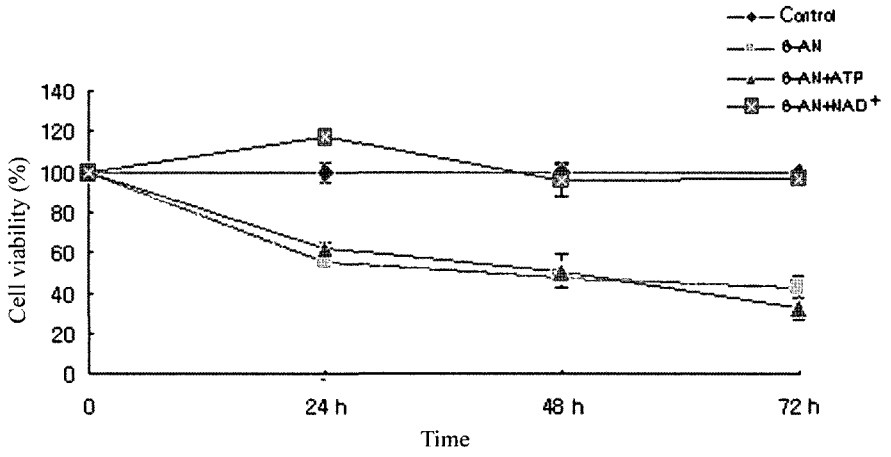

Fig. 1. Cell viability of IMR32 neuroblastoma cells after exposure to various treatments. Cells were exposed to 0.5 mM 6-AN, 0.5 mM 6-AN+1 mM ATP or 0.5 mM 6-AN+1 mM NAD^+ for 24 h, 48 h and 72 h. Cell viability was determined by MTT assay as described in Materials and Methods. Results represent the mean of at least four independent experiments; bars, standard deviation.

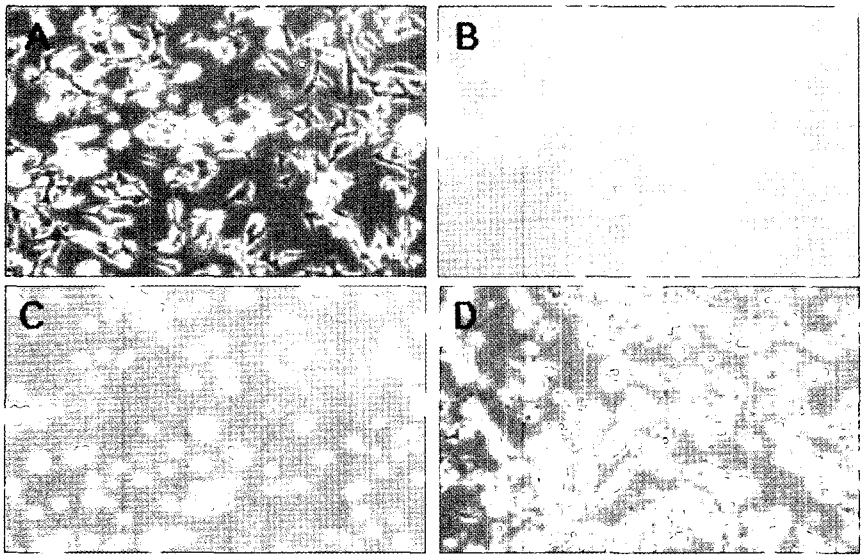

Fig. 2. Morphology of normal and various 6-AN treated IMR32 cells. Cells were incubated with various treatments for 72 h and then were observed by inverted phase-contrast microscopy. A, Untreated cells. B, 6-AN treated cells. C, 6-AN+ATP treated cells. D, 6-AN+NAD^+ treated cells.

AN, 6-AN+ATP and 6-AN+NAD^+ treated cells.

The cell cycle analysis revealed that cell population of 6-AN, 6-AN + ATP and 6-AN + NAD^+ treated cells in a G_0/G_1 phase was increased to 30%, 22% and 14%, respectively, as compared with the untreated control cells (Fig. 3A). In the S-phase, however, cell population of 6-AN, 6-AN + ATP and 6-AN + NAD^+ treated cells was decreased to 22%, 10% and 0%, respectively. The severity of reduction in cell population was more pronounced in G_2/M phase: 90% for 6-AN treated cells; 91% for 6-AN + ATP treated cells; 86% for 6-AN + NAD^+ treated cells as compared with the untreated control cells. The results indicate that 6-AN selectively affects the G_0/G_1 phase of the cell cycle.

6-AN downregulates cdk2, cyclin E and p-Rb, and upregulates cdk inhibitor $p27^{kip1}$

The results that 6-AN induced G_1 block in cell cycle progression prompted us to look at effects of 6-AN on regulation of activities of the G_1 cell cycle regulating components. As shown in Fig. 4A, 6-AN caused marked enhancement of expression of $p27^{kip1}$ but marked reduction of expression of cdk2 and cyclin E, and moderate reduction of expression of p-Rb, and no changes in the expression of cdc25A and p53R2. Similar observations were obtained with 6-AN + ATP treated cells in the expression of $p27^{kip1}$, cdk2, cyclin E and p-Rb proteins. In 6-AN + NAD^+ treated cells, there were moderate reductions of the expression in cdk2, cyclin E and p-Rb and virtually no changes in the expression of $p27^{kip1}$, cdc25A and p53R2. The results show that NAD^+ exerted more positive roles in ameliorating the effects by 6-AN than ATP.

Changes in nuclear staining of $p27^{kip1}$, cdk2, cyclin E and p-Rb proteins

The immunofluorescence staining revealed that $p27^{kip1}$ (Fig. 5A) was the most strongly expressed in 6-AN treated cells than untreated cells, ATP treated cells and NAD^+ treated cells. The expression levels of cdk2 in 6-AN and 6-AN+ATP treated cells were markedly lower than those in untreated and NAD^+ treated cells (Fig. 5B). Similar but slightly lower expression was observed with cyclin E and p-Rb (Fig. 5C and D). The results clearly demonstrated that the expression levels of these proteins revealed by indirect immunofluorescence images coincided well with western blot analysis (Fig. 4A).

Discussion

In this study, we investigated the underlying mechanism for the observed changes in cell viability and cell cycle progression in IMR32 neuroblastoma cells in response to the neurotoxin 6-AN. This drug was originally described as a niacin antagonist (Johnson and McColl, 1955), and was found subsequently to have anti-neoplastic activity in animal studies (Shapiro et al., 1956). It has been reported that 6-AN alone or in combination with N-(phosphoacetyl)-L-aspartate and 6-methylmercaptopurine riboside, enhances the therapeutic efficacy of bis(Chloro-ethyl) nitrosourea, 5-flourouracil, paclitaxel, doxorubicin and ionizing radiation in animal tumor models (Street et al., 1996; Martin, 1996). Furthermore, the addition of 6-AN (0.01 mg/ml) to C-1300 neuroblastoma cells caused significantly reduced cell division and morphological changes, and characteristic

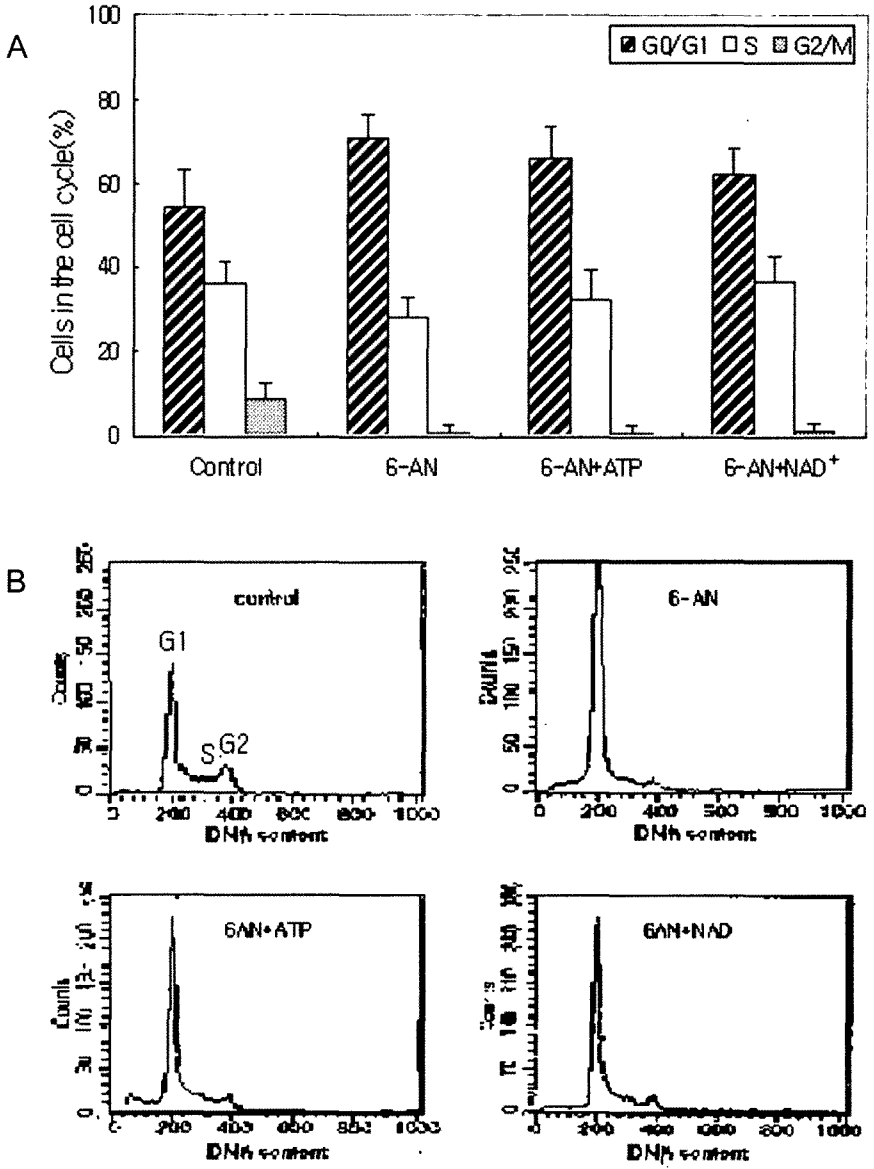

Fig. 3. 6-AN causes G_1 cell cycle arrest in IMR32 cells. A, Distribution of cell population in G_0/G_1, S and G_2/M phases of IMR32 cells treated with 0.5 mM 6-AN, 0.5 mM 6-AN+1 mM ATP and 0.5 mM 6-AN+1 mM NAD^+ for 72 h and stained with propidium iodide followed by flow cytometry analysis. B, Histograms of distribution of DNA content in IMR32 cells with or without treatments. Results are expressed as percentage of cells in G_0/G_1, S and G2/M phases of cell cycle. The data are representative of four independent experiments.

signs of differentiation (Zeitz et al., 1978).

As shown with 6-AN treatment in Fig. 1, 6-AN (0.5 mM) alone strongly retarded the growth of IMR32 and the addition of ATP to cultured cells didn't block the cytotoxic effects caused by 6-AN. Similar observations were also made with Ehrlich tumor cells treated with 1 mM ATP which led to marked reduction in glutathione levels and subsequent cell death (Estrela et al., 1995). ATP at varying concentrations has been suggested to act as both necrotic and apoptotic agents in various cell lines such as microglial cells (Ferrari et al., 1997), lymphocytes (Apasov et al., 1995), tumor and normal cells (Chow et al., 1997).

In contrast, NAD^+ addition served to retard the cell growth inhibition induced by 6-AN. Although the exact function and underlying mechanism of NAD^+ in the involvement in cytoprotection against 6-AN have not been established, NAD^+ served to minimize the deleterious consequences which may be fatal to the cell viability. NAD^+ has been known to function as a key element in cell viability through the regulation of the redox state in cells and proper maintenance of mitochondria (Ogata et al., 2000). Thus, in the current study, the external addition of NAD^+ may be mobilized to replenish the deficient levels of NAD^+, subsequently leading to the retardation of cell growth and cell death.

Our experimental findings showed that 6-AN is able to

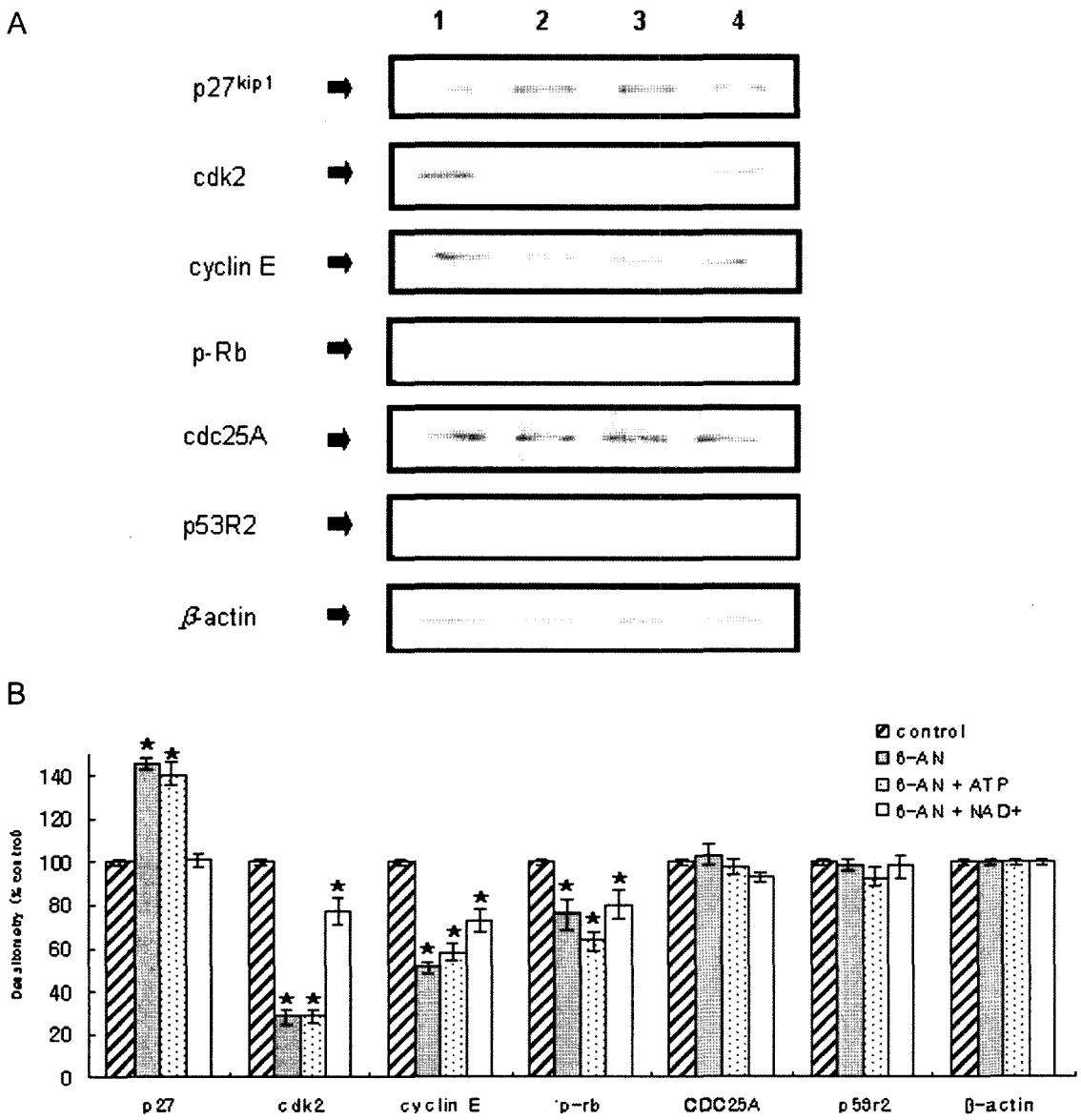

Fig. 4. Effect of 6-AN on cell cycle-related proteins in IMR32 cells. Cells were harvested at 72 h after incubation with 6-AN, 6-AN+ATP and 6-AN+NAD$^+$. Cells were then lysed, and the supernatants were subjected to Western blot analysis. Aliquots of 30 μg of protein extracts were analyzed by 10% SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with the indicated antibodies. A, p27, cdk2, cyclin E, p-Rb, cdc25A and p53R2. Where: Lane 1, untreated cells; Lane 2, 6-AN; Lane 3, 6-AN+ATP and Lane 4, 6-AN+NAD$^+$. B, Each value was measured by densitometric analysis of the immunoblots based on the density of the band in the vehicle control as 100%. β-actin were used as a control of protein loading ($P < 0.01$ *).

block cell cycle progression through G_1 arrest which is initially originated from both downregulation of cdk2, cyclin E and p-Rb proteins, and upregulation of $p27^{kip1}$. There is a strong possibility that the accumulation of nuclear $p27^{kip1}$ protein by 6-AN treatment may exhibit inhibitory effect on cdk2-cyclin E complex, thereby leading to a consequents inhibition of the cell cycle progression. In particular, the nuclear localization of $p27^{kip1}$ is essential for its growth-inhibiting function (Singh et al., 1998; Jian-Ying et al., 2003) and cdk2-cyclin E complex inhibition (Genevieve et al., 2001).

This has been well documented in other studies such that tangertin and related compounds (Pan et al., 2002) induced G_1 arrest through the inhibition of cdk2 and cdk4 and increased the expression of $p27^{kip1}$ and $p21^{waf1}$ proteins in colorectal cells. $p27^{kip1}$ has been believed to play a crucial role in the negative regulation of the cell division *in vivo* (Sherr, 1996; Fero et al., 1996). The control of proliferation in mammalian cells has also been known to be primarily associated with G_1 phase (Pardee, 1989; Sherr, 1993), and the passage from G_1 into S phases has been mainly regulated by activities of cyclin E-cdk2 complex and other

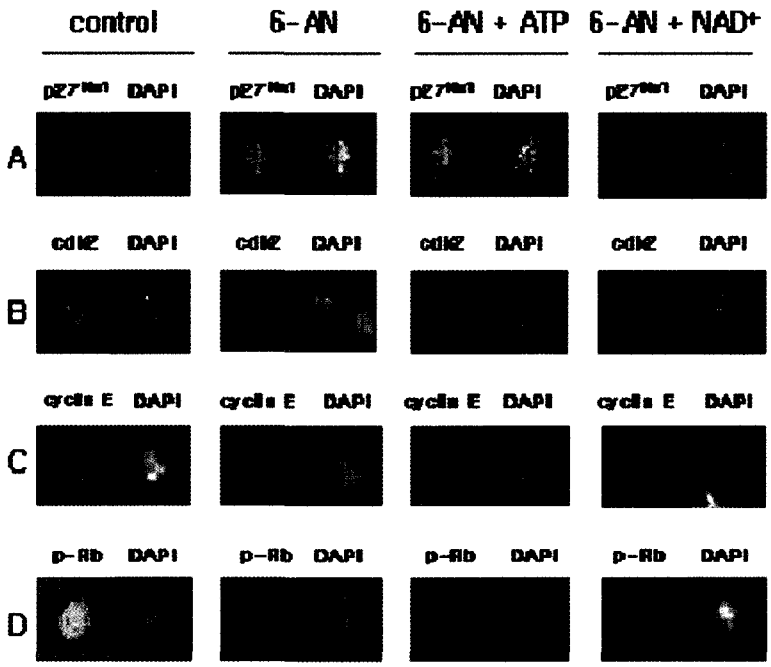

Fig. 5. Indirect immunofluorescence images of IMR32 cells. Cells were treated with 6-AN, 6-AN+ATP, or 6-AN+NAD^+ for 72 h to determine the intracellular distribution of p27 protein (A), cdk2 (B), cyclin E (C), and p-Rb (D). Cells were stained with primary antibodies and secondary FITC-conjugated antibodies (shown in green). Corresponding cell nuclei were visualized by DAPI (blue).

protein pathways (Lukas et al., 1997; Ohtsubo et al., 1995).

It is of great interest to note that ATP and NAD^+ produced distinct effects on the cell morphology, cell viability, expression of cell cycle regulatory proteins, and cell proliferation. One plausible explanation for these discrepancies is that there may exist different channels for the transport and utilization of these molecules. Recently, Kevin et al. (2004) reported that NAD^+ does not affect $p27^{waf1}$ induction so that it may not be involved in affecting the cell cycle arrest. In other studies it has been shown that ATP induces the suppression of the cell growth and upregulation of the cell cycle arrest protein $p21^{waf1}$ (Mina et al., 2002). Further studies will be required to elucidate the germane roles of ATP and NAD^+ in relation to the cell cycle progression in cells exposed to 6-AN.

In conclusion, 6-AN caused the upregulation of $p27^{kip1}$ and downregulation of cdk2, cyclin E and p-Rb, consequently contributing to the induction of G_0/G_1 phase arrests in IMR32 neuroblastoma cell lines.

Acknowledgments

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