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Induction of Apoptosis by Cisplatin in multiple Cell Lines: A Comparative Study

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ABSTRACT

Since the discovery of cisplatin 35 years ago, it has enjoyed the status of a therapeutic agent of choice for treatment of various cancers including cervical cancer. Cisplatin is a DNA damaging platinum drug. Several studies are under way to elucidate its role in inducing apoptosis and inhibiting the pathways involved in cancer. The present study was undertaken to find out the inhibitory effect of cisplatin on survival pathways focusing on Erk 1/2, JNK, P53, Bcl-2, BAX, cytochrome-c and caspase-9 in HeLa and Vero cell lines. Results obtained showed that cisplatin was effective at a dose of 5-10μM in MTT assay conducted on the three cell lines namely, HeLa, Vero and BHK. The apoptotic activity was measured by using LDH assay, caspase-9 assay and western blot analysis of cytochrome-c. There was increase in cytochrome-c, caspase-9 and LDH showing thereby that it acts through the intrinsic pathway involving mitochondria. There was depletion of total Erk and JNK. This further demonstrates that the drug is involved in inhibiting the proliferative pathway. We are inclined to conclude that the drug is very effective at moderate doses for inducing apoptosis in cancer cells. Its collateral damages however, as reported by others, may restrict its use in treatment of cancer.

Key words: Cisplatin, Apoptosis, Therapeutic agent, Cancer

INTRODUCTION

The most common mechanism by which body eliminates damaged or unneeded cells without local inflammation is known as apoptosis or programmed cell death (Fadeel and Orrenius, 2005). The process of apoptosis is of great biological importance as it is involved in regulating normal tissue homeostasis (Norbury and Hickson, 2001). Survival and death signals work under a tightly controlled system and determine the fate of the cell. When the balance in cell growth and cell death is disturbed, a variety of pathological conditions can occur like autoimmune diseases, spreading of viral infections, neurodegenerative disorders, AIDS, ischemic heart diseases and cancer, among others (Fadeel *et al*., 1999).

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Cancer is a multi-factorial disease that refers to anomalous cell proliferation (Jailkhani *et al*., 2011). It is generally considered that it is an environmental disease; as studies show that 90-95% cases are due to environmental factors such as tobacco, diet, radiation, pollutants and certain viral infections while 5-10% cases are due to genetic dys-regulations and thus can be prevented by eliminating those factors which lead to this disorder (Anand *et al*., 2008).

It has been reported that in 2008 nearly 7.6 million deaths have been attributed to cancer, third most common cause of the death in developing countries (Ferlay *et al*., 2010). It is estimated that by 2030 there will be approximately 26 million new cases of cancer resulting in about 17 million deaths (Thun *et al*., 2010). Worldwide, cervical cancer is the second most common cancer in women (Chibwesha and Cu-Uvin, 2011), and fourth leading cause of deaths. Cervical cancer is one of the

globally preventable health problems. Although in last three decades its morbidity rate fall by 50% in US but even then it is a serious issue for women (Ferlay *et al*., 2010).

Intense research is underway to discover safe and effective drugs for the treatment of various cancers. Inhibition of growth and spread of cancer cells can be achieved by inducing apoptosis of cancer cells through less harmful therapeutic agents. In the body, apoptosis is induced by two types of signals. These are either extrinsic (toxins, hormones, growth factors, nitric oxide and/or cytokines (Popov *et al*., 2002: Brune and Zhou, 2003) or intrinsic (stress, heat radiations, nutrient deprivation, viral infections, hypoxia) (Cotran *et al.,* 1998) and increased calcium concentration (Marks*,* 2003).

Apoptosis remains suppressed by survival signals that are generated internally and through molecules derived from adjacent cells. The process is either initiated by inhibiting these essential survival signals or by direct activation of extrinsic or intrinsic pathways. Extrinsic pathway is induced by ligation of death receptor with death ligand (Hinz *et al*., 2000). For example, binding of tumor necrosis factor (TNF) with its receptor, TNFR1 causes the activation of adaptor molecules, FAS-associated death domain (FADD) and TNFR1-associated death domain (TRADD) (Mongiat *et al.,* 2007). Both activated molecules together cause the activation of caspase-8 (cysteine dependant aspartate specific proteases) and then executioner caspases lead to apoptosis (Chipuk and Green, 2006).

Other pathway of apoptosis called intrinsic pathway is regulated by Bcl-2 family of proteins through mitochondria (Spierings *et al*., 2005). Bcl-2 family of proteins consists of pro-apoptotic proteins Bax, Bad, Bim, Bid, Noxa and anti-apoptotic Bcl-2, Bcl-xL proteins which play a significant role in regulation of apoptosis (Adams and Cory, 1998). Multi-domain pro-apoptotic proteins Bax/Bak are up-regulated by some apoptotic signals such as DNA damage detected by p53 that causes activation of p53 transcription factor and consequently activation of Bax/Bak (Peixoto *et al*., 2011). It is known that Bax/Bak increase the permeability of mitochondrial outer membrane (OMM) by forming the pores within it (Mikhailov *et al*., 2003). As a result mitochondrial proteins cytochrome c and SMAC/Diablo are released in cytoplasm and make complex with apoptotic protease activating factor-1 (Apaf-1) (Youle and Strasser, 2008). This complex activates caspase-9 which subsequently activates caspase-3. Finally cell death is attained by the damage and cleavage of cytoskeletal proteins (Belizario *et al*., 2007; Han *et al*., 2008).

Apoptosis can be achieved in cancers by inhibiting one or the other proliferative signaling pathways. The important survival pathway that is aberrantly activated in many kinds of cancers is mitogen-activated protein kinase (MAPK) (Wu, 2007). Superfamily MAPK consists of three protein kinase families: Extracellular signal regulated protein kinases (Erk), c-Jun N-terminal kinases (JNK) and p38 kinases. These three kinases are activated under stress condition and regulate different cellular activities such as growth, differentiation, development, disease and apoptosis. When stress stimulus is initiated to these signals first pass on to MAPKKK which is usually GTP binding protein. Activated MAPKKK is phosphorylated which in turn activate MAPK kinase (MAPKK). MAPKK has dual characteristics and phosphorylate both Tyr and Ser/Thr sites on MAP kinase (MAPK). This activated MAPK can target numerous cellular/nuclear entities achieving their normal functions (Cowan and Storey, 2003).

Among 700 approved FDA drugs, cisplatin is considered a queen of chemotherapy (Boulikas, 2007). It has been demonstrated in different studies that cisplatin is the most effective than radiotherapy alone (Sahin *et al*., 2012). Platinum drugs have unique impact in the treatment of cancer (Kelland and Farrell, 2000). Cisplatin [cis-diammine-dichloroplatinum; CDDP] (Reed, 1999) is a platinum coordination complex which exhibits antitumor activities. Cisplatin has a broad spectrum (Zhang *et al*., 2009) and is widely used for the treatment of various types of cancers like bladder, cervix, head and neck, esophagus and small-cell lung cancer, it shows more effectiveness in case of testicular and ovarian cancers (Giaccone, 2000).

Cisplatin is a well-known DNA damaging drug (Sedletska *et al*., 2005), which forms inter- and intra-stand adducts with DNA (Chvalova *et al*., 2007) leading to apoptosis but the mechanism of

cisplatin DNA adducts inducing apoptosis is not yet clear (Reedijk and Lohman, 1985). One of the possible mechanisms to induce apoptosis through cisplatin could be by targeting specific kinases not the selective kinases as proposed by (Mansouri *et al*., 2003a).

The present study has been conducted to unveil the molecular pathway involved in induction of apoptosis with cisplatin as a therapeutic agent. The therapeutic efficacy has been investigated in two cell lines, namely, HeLa and Vero. Accordingly, this has been demonstrated by cell viability assay (MTT assay) and to ascertain whether this can be used as a biomarker. This has been further confirmed through western blot analysis of apoptotic proteins of intrinsic pathway p53, Bcl-2, BAX, cytochrome c and MAPK pathways involving Erk1/2 and JNK.

MATERIALS AND METHODS

Cell lines

HeLa cell line was provided by School of Biological Sciences, University of the Punjab, Lahore, Pakistan. BHK and Vero cells were attained by civility of University of Veterinary and Animal Sciences, Lahore, Pakistan.

Reagents

Cisplatin was purchased from Biovision (USA). RIPA lysis buffer was obtained from Santa Cruz Biotechnology (USA). Mouse monoclonal antibodies of P53, Anti-JNK, Anti-BAX and Rabbit (polyclonal) Anti-ERK were bought from Invitrogen (USA). Monoclonal Anti-β-Actin was purchased from Ambion (USA). All culture reagents like Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), antibiotic Pen- Strep and 2mM L-glutamine were purchased from Gibco, Life Technologies, USA.

Cell culture and treatment

HeLa, Vero and BHK cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml Pen- Strep antibiotic and 2mM L-glutamine were incubated at 37°C in 5% CO_2. In routine ~80 % confluent flasks were split 1:3 in 25ml flask as follows. The cells were washed with PBS, added trypsin 1x and incubated at 37°C for 5 min. After they were detached from the dishes; 1ml medium was added and transferred to falcon tube; centrifuge at 700g and pellet dissolved again in medium. Cells were transferred into new culture flask with fresh culture medium.

Stock solution of cisplatin

Stock solution of cisplatin (5mM) was prepared by dissolving 7.52 mg cisplatin in 5ml DMSO and stored at 4°C. Later on working dilution of 100µM was prepared from it.

Cytotoxicity assay

Viability of HeLa, BHK and Vero cells were assessed by MTT (methyl thiazoyltetrazolium) assay which was carried out in 96 well tissue culture plates with 5000 cells per well. Cells were treated with cisplatin at different concentrations (100nM, 500nM, 1µM, 5µM and 10µM) for 24 hours. After 24 hours of incubation, 0.025ml of MTT (5mg/ml) reagent was added in each well and incubated for 2 hours followed by addition of 100µl of extraction buffer (SDS 20%, DMSO 50%). The light absorbance was measured at 570nm after overnight incubation. Percent relative cell viability was calculated by the following relationship:

$$\text{Relative cell viability (\%)} = \frac{\text{A570 of sample}}{\text{A570 of control}} \times 100$$

Drug administration

Confluent cell culture plates (100mm) of HeLa and Vero cells were exposed to 1µM, 5µM and 10µM cisplatin and prepared for various experiment.

Caspase-9 assay

Caspase-9 assay was carried out according to the procedure given within caspase-9 colorimetric assay Kit (Biovision, USA).

Cell lysate preparation

In order to obtain whole cell lysate, cells were treated RIPA lysis buffer by following different steps. Initially, cultured cells were scrapped and shifted to falcon tubes for centrifugation. Following centrifuging at 4000 rpm for 5 minutes, supernatant was discarded and pellet was treated with 1ml RIPA lysis buffer containing Sodium Orthovandate and

phenylmethylsulphonyl fluoride (PMSF) and subjected to incubation for 30 minutes on ice. Then again the tubes were centrifuged at high speed for 10 minutes. This resulted in the separation of whole cell lysate, in the form of supernatant and debris, in the form of pellet. Then, supernatant was aspirated and stored at freezing temperature in a separate vial.

LDH test

Cells were cultured in 6 well culture plates and administered with different concentrations (1, 5 and 10µM) of cisplatin. Cells were separated from plates with the help of spatula and obtained supernatant after centrifugation at 4000rpm. LDH test was performed with the help of Synchron clinical system LDH reagent kit (Ireland) and reading was obtained on Beckman Coulter CX9 Pro.

Protein blotting

Protein obtained by cell lysis was quantified by using Qubit assay kit. Samples were denatured by boiling for 5 min with 2x sample buffer. These samples were loaded and separated on 12% SDS-PAGE at 90V. Resolved proteins were then transferred from gel onto nitrocellulose membranes (Bio-Rad, CA, USA) for 1.30 h at 90V. Membrane was blocked with 3% BSA in phosphate buffer saline with 0.1% (v/v) Tween-20 (PBST) at room temperature for 20 minutes. Blocked membranes were incubated in specific primary antibody diluted in (1:1000) PBST and HRP labeled specific secondary antibody (Rabbit or mouse) for 20 minutes each and subsequently washed with PBST by using SNAP id® 2.0 Protein Detection System. Finally, bands of desired protein were observed by using TMB substrate and recorded by gel documentation system (Bio-Rad).

RESULTS

The findings of MTT assay have been graphically presented in Figures 1a, b and c. It may be observed that cisplatin is most effective at doses of 5 and 10µM respectively. At these doses cell viability was reduced to less than 50%. MTT assay using various doses of cisplatin in Vero cells was conducted under similar conditions. It may be noted that in this cell type cisplatin becomes effective in

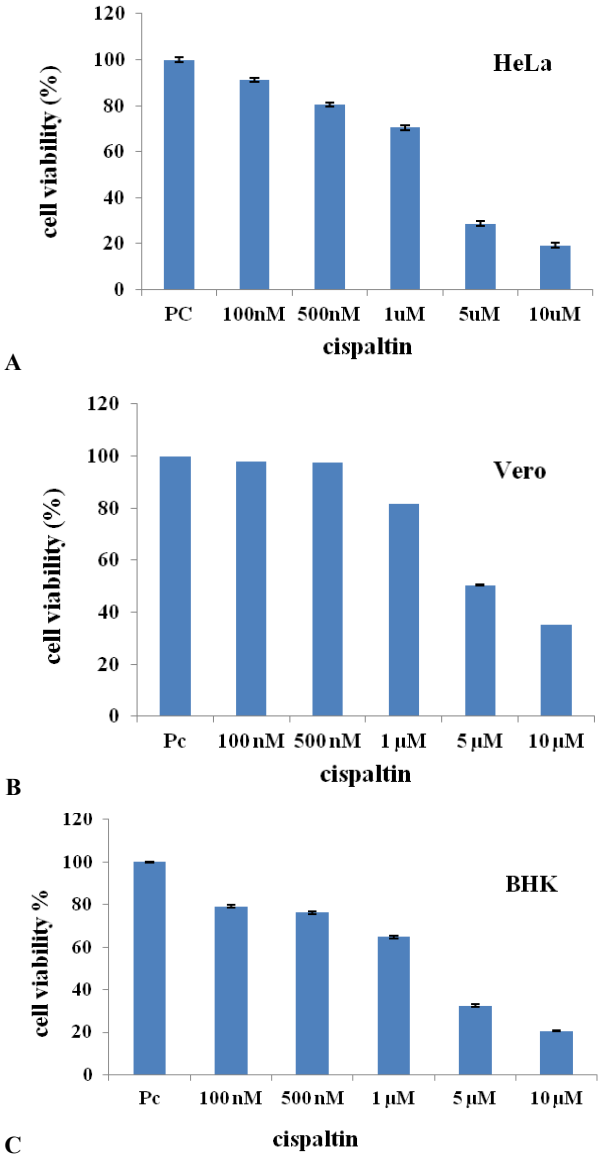

Fig. 1: MTT Assay, **A)**: Relative cell viability plotted against various doses of cisplatin in HeLa cells. Bar chart of collective three independent experiments in duplicates with their standard error of mean±SEM. **B):** Relative cell viability plotted against various doses of cisplatin in Vero cells. Bar chart of collective three independent experiments in duplicates with their standard error of mean±SEM. **C):** Relative cell viability plotted against various doses of cisplatin in BHK cells. Bar chart of collective three independent experiments in duplicates with their standard error of means ±SEM.

the same range as in case of HeLa cells. Similarly, in BHK cells the effective doses of cisplatin were also in the same range (Fig. 1c).

Illustrated data was obtained with LDH assay in two cell lines. There was a consistent increase of LDH at 1, 5 and10µM concentrations of cisplatin in each cell line. The data obtained in each case are recorded in Figures 2a and b.

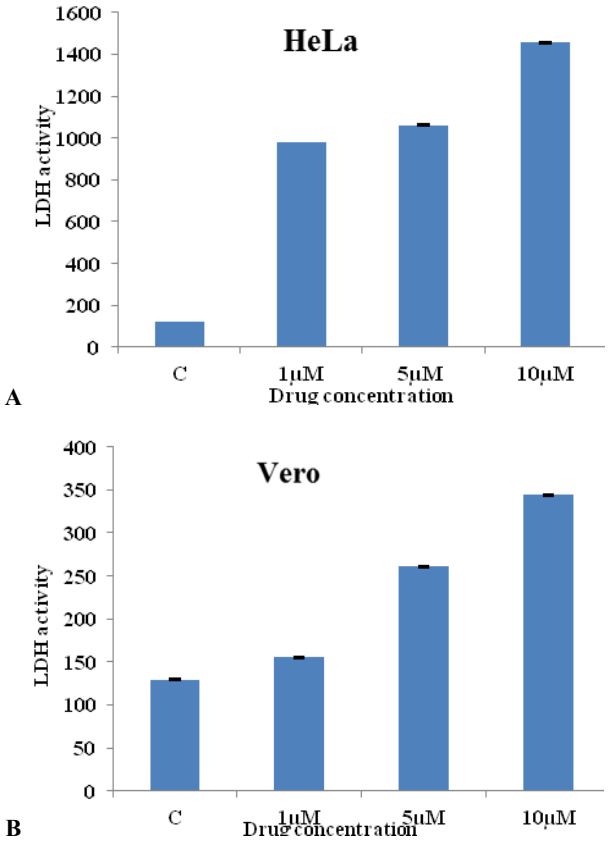

Fig. 2 **(A):** LDH activity with the help of bar chart plotted against various cisplatin concentrations on HeLa cells (n=3). **(B):** LDH activity with the help of line graph and bar chart plotted against various cisplatin concentrations on Vero cells (n=3).

In order to find out as to how cisplatin induces apoptosis, caspase-9 assay was conducted in Vero cells treated with various doses of cisplatin. The results obtained have been shown in Figure 3. It may be observed that cisplatin is effective in increasing the quantity of cleaved caspase (active) at a concentration as low as 1µM. There is a consistent increase of active caspase-9 at increasing concentrations of cisplatin. Caspase-9 as an apoptotic marker determines the ability of cisplatin to induce apoptosis as shown in our results.

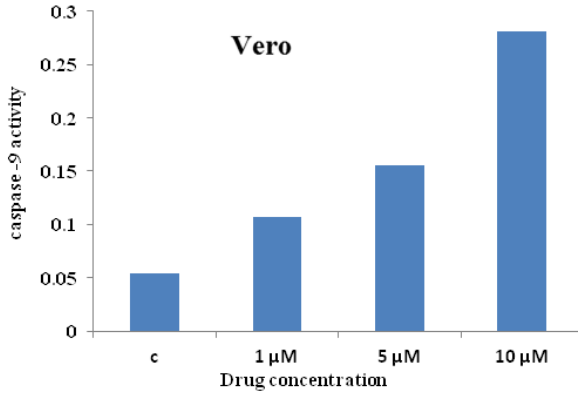

Fig. 3: Caspase-9 Assay. Bar chart of caspase-9 activity is plotted against various doses of cisplatin in Vero cell line.

In order to find out the pathway to which cisplatin effectively induces apoptosis, various proteins were blotted as described in materials and methods. The proteins which were subjected to blotting analyses included those of proliferative and apoptotic pathways. The proteins of apoptotic pathways were p53, BAX accompanied by cytochrome-c, increased quantity of which indicates the execution of apoptosis by BAD/BAX in Figure 4. In addition, the anti-apoptotic proteins blotted in this study included Bcl-2. Of the proliferative pathway blotting of Erk 1/2 and JNK was carried out. Blotting data has been shown in Figure 5.

The raised levels of caspase-9 and cytochrome-c clearly indicate the involvement of mitochondria in cisplatin based apoptosis induction. Conversely no change was observed in BAX levels as a result of immuno-blotting. These results help us to interpret that BAX protein is not involved in pore opening activity of mitochondria to release cytochrome-c. Furthermore, constant expression of p53 shows the involvement of p53 independent mechanism of action of cisplatin in both HeLa and Vero cells.

More prominent effects of cisplatin on proliferative proteins Erk 1/2and JNK have been observed in Vero cells as compare to Hela cells. A

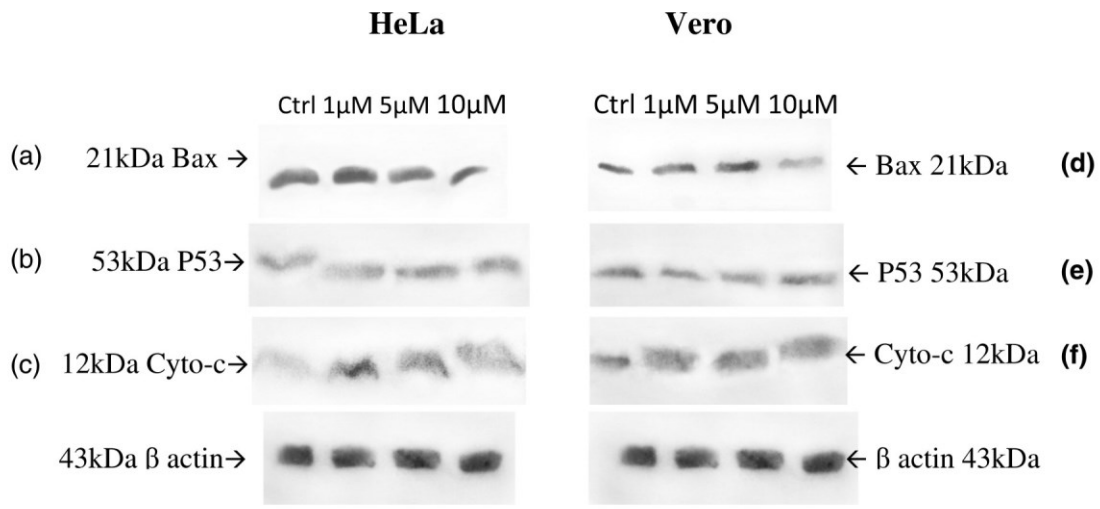

Fig. 4: Western blotting **(a)** shows bands of BAX **(b)** p53 and **(c)** cytochrome c at various concentrations of cisplatin administered on HeLa cells. **(d)** Shows band of BAX **(e)** p53 and **(f)** cytochrome c in cisplatin treated Vero cells on varying concentrations. This blotting experiment was normalized with β-actin.

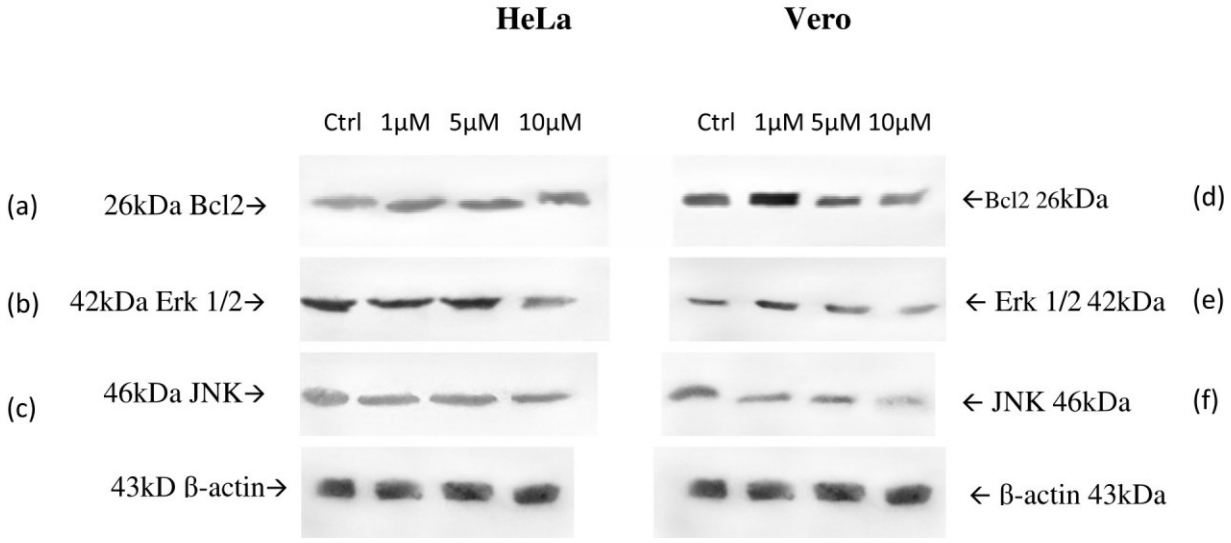

Fig. 5: Western blotting **(a)** shows bands of Bcl-2 **(b)** Erk 1/2 and **(c)** JNK at various concentrations of cisplatin administered on HeLa cells. **(d)** Shows band of Bcl-2 **(e)** Erk 1/2 and **(f)** JNK in cisplatin treated Vero cells. This blotting process was normalized with β-actin.

sudden decrease in Erk and JNK levels are found at 10μM concentration of cisplatin. However, anti-apoptotic Bcl-2 protein shows constant expression in both Hela and Vero cells. This may predict the involvement of any other anti-apoptotic protein like Bcl-xl or Mcl-1 in regulating the process of apoptosis through cisplatin.

DISCUSSION

The involvement of MAPK family of proteins in regulating apoptosis under stress condition has been widely investigated. Recent studies indicate that MAPK family of proteins Erk and JNK are important mediators of apoptosis induced by cisplatin (Perez *et al*., 1998; Eichhorst *et al*., 2000;

Germain *et al*., 2010). JNK and Erk are stress activated protein kinases. These kinases are activated under stress stimuli such as UV radiation and DNA damage. Previous studies have shown that cisplatin is a DNA damaging anti-tumor drug (Liu *et al*., 1996; Hayakawa *et al*., 2003).

A number of studies have shown that the activation of Erk pathway activates survival signal (Guyton *et al*., 1996) however, other studies have reported that the activation of Erk pathway is important in cisplatin induced apoptosis (Wang *et al*., 2000). In the present study, we have investigated the effect of various doses of cisplatin on the modulation of Erk in two cell lines: HeLa and Vero. Our results indicate the down regulation of total Erk at 10μM of the drug in both cell lines. Our results are in agreement with those reported by others (Wang *et al.,* 2000).

JNK, another MAPK has also been demonstrated to induce cell survival (Bogoyevitch and Kobe, 2006). Other studies have provided evidence about the role of cisplatin in apoptosis during the aging process (Lin and Dibling, 2002). This has been related to NF-kB mediated inhibition of JNK. It is possible that this phenomenon may be cell specific (Mansouri *et al*., 2003b; Nehme *et al*., 1999). In our present study using two different cell lines, Vero and HeLa, we have shown that total JNK expression is reduced at 10μM dose of cisplatin in both cell types. This aspect highlights varying effects on different cell types. By and large we believe that the role of JNK in apoptosis remains controversial, though in aging process it may be the major component involved in inducing apoptosis.

Members of Bcl-2 family play an important role in apoptosis. In fact a critical balance is maintained between Bcl-2 family antiapoptotic (Bcl-2, Bcl-xl and Mcl-1) and proapototic (BAX, BAD and BAK) proteins. We have observed no change in the levels of Bcl-2 as observed by western blotting. Surprisingly enough, a small decrease in BAX was noticed in cisplatin treated cells of both cell lines. We are inclined to suggest that cisplatin has little or no effect on Bcl-2 family proteins. These data are in agreement with previous studies (Burger *et al*., 1997; Tsuruta *et al*., 2001). It is possible that in cisplatin induced apoptosis Bcl-xl or Mcl-1 may be involved in regulating the process of apoptosis. We were unable to demonstrate any change in p53 which normally is anticipated as a proapoptotic phenomenon. This is so yet, increase in cytochrome-c observed in our experiments on both cell lines was indicative of the fact that cisplatin induces apoptosis through mitochondrial pathway involving caspase-9 and caspase-3, since cytochrome-c is known to activate caspase-9 in the apoptosome with subsequent activation of the executioner caspase-3. This is supported by rapid increase in cytochrome-c and caspase-9 as demonstrated in our data.

In essence, we conclude that cisplatin induces apoptosis through the release of cytochrome c from the inter-membranous mitochondrial matrix through proapoptotic BAX and BAD. This is possible that other anti-apoptotic proteins like Bcl-xl or Mcl-1 may be involved in regulating this process. However, not withstanding this fact, cisplatin, as demonstrated by others acts through damaging cellular DNA.

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