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Cyclin-dependent Kinase 9: A Potential Anti-cancer Drug Target

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Abstract: Cyclin-dependent Kinase 9, CDK 9-cyclin T complex forms part of the positive transcription elongation factor b (P-TEFb) involved in the elongation phase of RNA polymerase II dependent transcription. CDK 9 target validation for drug development has been supported by a series of experimental observations including studies with non-specific CDK inhibitors such as flavopiridol and ongoing research to elucidate the mode-of-action-related toxicity of CDK 9 inhibition. Although the role of CDK 9 in transcription is in no doubt, there are conflicting reports on whether it is required globally for transcription. So, more robust target validation is necessary in order to understand its precise function and potential as a drug target. In the current study, CDK 9 knockdown (KD) A2780 cells were produced using lentivirus (495) ShRNA transfection. CDK 9 KD dramatically increased the sensitivity of A2780 cells to pan CDK inhibitors CDKI-77 and flavopiridol. Western blot characterisation also revealed significant down regulation of anti-apoptotic proteins, Mcl-1 and HDM2, and up-regulation of pro-apoptotic protein p53. CDK 9 KD also propelled A2780 cells to undergo apoptosis (with caspase 3/7 activation) in a time dependent manner, with minimal cell cycle perturbation. Down regulation of short-lived proteins (Mcl-1 & HDM2) further supports the role of CDK 9 in transcription; moreover, minimal effects on cell cycle confirm a non-central role for CDK 9 in cell cycle regulation. Furthermore, that CDK 9 KD appears sufficient to trigger apoptosis in A2780 cancer cells indicates that CDK 9 inhibition is a valid molecular target for development of anti-cancer drugs.

KEYWORDS: Cyclin dependent kinase 9; Anti-cancer drug; Anti-apoptotic proteins; Transcription; Cell cycle.

1.0 Introduction

CDK 9 is a cyclin-dependent serine-threonine protein kinase associated with positive transcription elongation factor b (P-TEFb) (Marshall and Grana, 2006; Wang and Fischer, 2008). It forms complexes with T and K-type cyclins that constitute the basal transcriptional factor P-TEFb, which phosphorylates the carboxy terminal domain (CTD) of the largest subunit (RPB1) of RNA polymerase II (RNAP II) leading to transcriptional elongation. P-TEFb is a 5, 6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) sensitive factor found to be needed for the synthesis of mature mRNA transcripts *in vitro* by RNAPII. It has been shown that DRB inhibits the production of mRNA when applied to mammalian cells and prevents transcriptional elongation by RNAPII *in vitro*. Subsequently, the role of CTD phosphorylation for RNAPII elongation was established and P-TEFb revealed a DRB-sensitive kinase activity that is involved in the phosphorylation of CTD (Marshall and Grana, 2006). In contrast to most other CDKs, CDK 9-cyclin T/K is not involved in cell cycle regulation but in RNA synthesis and processing (Wang and Fischer, 2008). CDK 9 appears to be required globally for transcription and is involved in several physiological processes. For example, it responds particularly to a number of cytokines such as tumour necrosis factor (MacLachlan *et al.*,1998) and interleukin-6 (De Falco *et al.*, 2002) demonstrating the involvement and a possible role in a variety of physiological processes like immune response,

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inflammation, cell activation, differentiation, growth and survival (Bagella *et al.*,1998; Wang and Fischer, 2008).

Fundamentally, the central role of CDK 9-cyclin T/K is in the elongation and RNA processing of RNAPII dependent mRNA transcripts. Subsequent to the action of CDK 7-CyclinH in the transcriptional cycle, CDK 9-Cyclin T/K resumes mRNA elongation by phosphorylating the Ser-2 heptad repeats of CTD as well as DRB Sensitivity Inducing Factor (DSIF) and Negative Elongation Factor (NELF) in order to remove the blockade and start the productive elongation process. The phosphorylation of DSIF and NELF is at SPT5 and RNA-binding RD subunits respectively and leads to de-repression of RNAPII that occurs after the initiation. The phosphorylated DSIF remain associated with RNAPII and in fact becomes a positive elongation factor. The phosphorylated NELF may or may not remain with the RNAPII (Marshall and Grana, 2006).

CDK 9-Cyclin T/K by virtue of its ability to phosphorylate CTD may play an additional role in RNA processing after completion of elongation as well as transcription termination. It may help in the recruitment of the splicing machinery that removes the introns from exons of the primary transcripts. This processing will allow mobilization of cleavage and polyadenylation factors. With the aid of phosphorylated CTD, mRNA is cleaved and a polyadenylic tail is attached at 3' end. When transcription reaches the termination point (in a rho-independent or rho-dependent manner) signalled by splicing, cleavage and polyadenylation, the RNA transcript (mRNA) will fall off the DNA and move to the cytosol for translation and protein production. The RNAP II complex becomes free for another round of transcription through dephosphorylation by FCPI CTD phosphatase. The activity of P-TEFb is dynamically and closely controlled. There are two forms of P-TEFb within the cell; the large inactive form and a small kinase-active form. The large inactive form with no kinase activity is made when P-TEFb complexes with a hexamethylene bisacetamide induced protein (HEXIM1 or HEXIM2), 7SK small nuclear RNA (SnRNA), P-TEFb-interacting protein for 7SK stability (PIP7S) and BCDIN3 domain containing protein. The kinase-active form contains the bromo domain protein Brd4 (Qintong *et al.*, 2005). The large inactive form may serve as a pool for the active free form (Haaland, 2005). The free form might be sufficient for transcriptional activity, however, a recent study has indicated that P-TEFb was recruited to initiation complexes by the bromo domain protein Brd4 and interaction is required for domain protein Brd4 and for P-TEFb to promote transcription (Jang *et al.*, 2005; Zhiyuan *et al.*, 2005).

Inhibition of transcription via CDK 9 inhibition might dramatically affect cancer cell proliferation since inhibition of transcription has greater effects on short-lived proteins such as anti-apoptotic proteins that are up-regulated in many cancers and confer on these cancers a survival advantage over normal cells (Chen, 2005). This makes CDK 9 inhibition a potential target in cancer drug development. CDK 9 validation for drug development has been supported by a series of experimental observations including studies with less specific CDK inhibitors like flavopiridol and research is ongoing to elucidate the mode-of-action-related toxicity of CDK 9 inhibition (Berth and Axel, 2006). Although the role of CDK 9 in transcription is not in doubt, there are conflicting reports on whether it is required globally for transcription. Some reports indicated that basal transcription can occur in the absence of RNAPII CTD phosphorylation by CDK 9, others show its indispensability (Serizawa *et al.*, 1993; Litingtung *et al.*, 1999).

To date, it has not been shown to what extent CDK 9 is redundant for transcription in general. So, more robust target validation is necessary in order to understand its precise function and potential as a drug target. This will reduce failure for any CDK9 inhibitor developed as drug candidate. Any effective target validation should involve the use of highly specific and potent inhibitors of the target protein. Both chemical and biological means can be used for such validation.

It is important to mention that a series of CDK 9 chemical inhibitors, such as CDKI-71 CDKI-77, CDKI-73 with nano-molar potencies, excellent selectivity and cellular efficacy have

been developed. However, results from biological evaluation of these compounds revealed that CDK 9 may play an important role in cancer cell proliferation, transcription, cell cycle and apoptosis (Liu *et al.*, 2012; Shao, *et al.*, 2012).

In the present study, more specific biological inhibition of CDK 9 using RNA silencing *via* short hairpin RNA (shRNA) was used in order to further assess its validity as potential target in cancer drug development.

2.0 Materials and Methods

2.1 Materials

2.1.1 Chemical

Flavopiridol was purchased from Selleckchem (UK) and all other chemicals used were of analytical grade.

2.1.2 Cell Culture

The A2780 (human ovarian carcinoma) cell line (WT) used in this study was obtained from the European Collection of Cell Cultures (ECACC, UK) and A2780 CDK 9 Knock down (KD) isogenic cells were generated at the Institute of Cancer & Genetics, School of Medicine, University of Cardiff. Cells were maintained in RPMI-1640 (Sigma-Aldrich, UK) with 10 % FBS.

2.2 Methods

2.2.1 Synthesis of CDKI-77

Synthesis of 2-((3-(1, 4-diazepan-1-yl) phenyl) amino)-4-(4-methyl-2-(methyl amino) thiozol-5-yl) pyrimidine-5-carbonitrile (CDKI-77) has been previously described (Wang *et al.*, 2009).

2.2.2 Proliferation Assay

MTT 3-(4, 5-dimethylthiozol-2-yl)-2, 5-diphenyl tetrazolium bromide, (Sigma-Aldrich, UK) cell proliferation assay was performed as previously reported (Mosmann, 1983). Cells were seeded at a density of 3000 cells per well in 96-well plates and incubated overnight to allow attachment. The cells were then treated with either CDKI-77 or flavopiridol for 48 h at various concentrations in order to generate a dose-response curve. GI_{50} (concentration of compound required to inhibit 50 % cell growth) values were calculated using nonlinear regression analysis.

2.2.3 Caspase-3/7 Assay

Caspase-3/7 activity was assayed according to the Apo-ONE homogeneous Caspase-3/7 assay kit instructions (Promega [G7790], UK). Cells were seeded ($5x10^3$ - $2x10^4$ cells per well) in 96 well plates and incubated at 37 ^{0}C, 5 % CO_2 for 24, 48 and 72 hours. Fluorescence was measured after 24, 48 and 72 hours incubation at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using EnVision Multilabel Plate Reader (Perkin Elmer). The amount of fluorescent product generated is proportional to the activity of caspase-3/7 present.

2.2.4 Annexin V/PI Double Staining

A2780 (WT and CDK 9 KD) cells (seeded at $1x10^5$ - $5 x 10^5$ density per well) in 6 well plates were incubated for 24, 48 and 72 hours. Apoptotic analysis (after the expiration of 24, 48 and 72 hours) was done using fluorescein isothiocynate (FITC) annexin V/Propidium iodide (PI) double staining according to the protocols provided by BD Bioscience, UK. Annexin V/PI-positive apoptotic cells were evaluated using flow cytometry (Beckman Coulter EPICS-XL MCL™) and data analysed using EXPO32™ software. The percentage of cells undergoing apoptosis was defined as the sum of early apoptosis (annexin V-positive cells) and late apoptosis (annexin V-positive and PI-positive).

2.2.5 Cell Cycle Analysis

A2780 (WT and CDK 9 KD) cells were seeded at $1x10^5$ - $5 x 10^5$ density per 6-well plates and incubated for 24, 48 or 72 hours. Cells were then harvested, washed (with cold phosphate buffered saline, PBS) and centrifuged

(1200 rpm; 5 minutes; 4°C). Cells were re-suspended in hypotonic fluorochrome solution (50 µg/ml propidium iodide, 0.1 % triton-X-100, 0.1 mg/ml ribonuclease A, 0.1 % sodium citrate). Samples were transferred to facs tubes, protected from light and stored at 4 °C overnight. Cellular DNA content was determined using a Beckman Coulter EPICS-XL MCL™ flow cytometer and data analysed using EXPO32™ software.

2.2.6 Western Blot Analysis

A2780 cells (WT and CDK 9 KD) were lysed in RIPA lysis buffer (Santa Cruz Biotechnology, CA). Protein content in the lysate was determined using the Bio-Rad DC protein assay kit according to manufacturer's instructions (Bio-Rad, Hercules, CA). Cell-lysate proteins (50-100 µg) were separated by SDS-polyacrylamide gel electrophoresis and then electrotransferred to an immun-Blot™ PVDF membrane (Bio-Rad laboratories, Hercules, CA). The membranes were blocked for 1 hour in TBST containing 10 % non-fat dried milk, incubated with primary antibody overnight and later followed by incubation with secondary antibody conjugated to horseradish peroxidase for 1hour. Blots were visualised by enhanced chemiluminescence (Amersham™ ECL™ Western blotting detection reagent) according to the manufacturer's instructions (GE healthcare, UK) and exposed to X-ray films (Amersham Hyperfilm™ ECL, GE Health care, UK). Antibodies used included RNAP-II Ser-2 and Ser-5 (Active Motif, UK), p53 (Dako, Denmark), HDM2 (Millipore, UK), β-actin (Sigma-Aldrich, UK), Mcl-1 and Bax (Cell Signaling Technologies, UK). Anti-mouse and anti-rabbit immunoglobulin G (IgG) horseradish peroxidase-conjugated secondary antibodies were obtained from Dako, Denmark.

2.2.7 CDK 9 Knockdown

Lentivirus was first produced by transfecting the 293T cells with mixture containing 1 µg lentiviral shRNA plasmid (495, EV or SCR), 0.75 µg P8.91 plasmid and 0.5 µg pMD2G plasmid (Sigma-Aldrich, UK) using transfection reagent Effectene from Qiagen according to manufacturer's instructions. The transfected 293T cells were then grown for 48 hours after which the resulting lentiviral particles were harvested using Lenti-X concentrator kit from Clontech. The concentrated lentivirus was then added to A2780 (WT) cells and incubated for 48 h. The A2780 cells with tranduced lentivirus were selected through growing the cells in the presence of puromycin (1µg/mL). The selected cells were grown, harvested and the level of CDK 9 expression was assessed by Western blot.

2.2.8 Statistical Analysis

Experimental statistical significance was evaluated using GraphPad Prism version 6.0 for windows (GraphPad software, USA) with a minimal level of significance set at $p \leq 0.05$. All experiments were performed in triplicate and repeated $\geq$ 2x. Data are expressed as mean or mean ± SD. Representative Experiments were selected for Figure preparations.

3.0 Results

The result of the transfection (Figure 1A) indicates ~ 70% knockdown after CDK 9 shRNA transfection as revealed by protein expression pattern during Western blot analysis (Figure 1A). CDK 9 knockdown also resulted in reduced phosphorylation at Ser-2 of RNAPII CTD (the phosphorylation site of CDK 9) confirming further CDK 9 KD (Figure 1B); however, Ser-5 phosphorylation (the CDK 7 phosphorylation site) remained unchanged (Figure 1B). Both the CDKI-77 and flavopiridol reduced Ser-5 phosphorylation via chemical inhibition of CDK9.

Figure 2A shows that there was a dramatic increase in the sensitivity of A2780 cells to CDKI-77 and flavopiridol after CDK 9 knockdown. The GI_{50} value of CDKI-77 increased 4-fold from 320 nM to 87 nM in CDK 9 KD cells (Figure 2A). A similar result was obtained for flavopiridol; GI_{50} values increased 5-fold from 37 nm to 7 nM in CDK 9 KD cells (Figure 2A). These results reveal that anti-proliferative activity of these compounds appears to be (at least in part) CDK 9-dependent

as CDK 9 down regulation enhances cellular potency.

Figure 2B indicates time-dependent caspase 3/7 activation in the transgenic (CDK 9 KD) cells with little change in the wild type (WT A2780) cells. Caspase activation in CDK 9 KD cells was significant ≥24 hour and continued to rise through 48 and 72 hours (Figure 2B). As with chemical inhibition, in CDK 9 KD cells, caspase activation was accompanied by significant time-dependent apoptosis (Figure 2B). The percentage apoptosis rose from ~ 3 % in the WT to about 20 % in the CDK 9 KD after 24 hours. This trend continued as the percentage cells undergoing apoptosis after 48 and 72 hours rose from ~ 5 % to 21 % and from ~ 5 % to 27 % in the CDK 9 KD cells respectively (Figure 2B).

Figure 2B revealed significant down-regulation of anti-apoptotic proteins, Mcl-1 and HDM2. For pro-apoptotic proteins, up-regulation of p53 protein was detected but Bax protein remained stable (Figure 2B).

Figure 3A presents the summarized minimal cell cycle effects when the percentages of cells at different phases of the cell cycle were compared in WT and CDK 9 KD cells. This appears to be the case at all time points examined. The only significant change observed was for the pre-G1 sub-diploid population (indicative of apoptotic cells). The percentage sub-G1 rose from 0.8 % in WT after 24 hours to about 8 % in CDK 9 KD cells after 72 hours. The increase appears to be time-dependent. Figure 3B presents a model for possible mechanisms of apoptosis following CDK 9 KD inhibition.

Discussion

The reduction in phosphorylation at Ser-2 of RNAPII CTD (the phosphorylation site of CDK 9) by the CDK 9 knockdown as well as the Ser-5 phosphorylation (the CDK 7 phosphorylation site) which remained unchanged indicated that a superior level of selectivity was achieved with biological inhibition (shRNA). This contrast to chemical inhibition of CDK9 in which both CDKI-77 and flavopiridol reduced Ser-5 phosphorylation (Shao *et al.*, 2012). These findings formed the basis that some level of certainty observed were solely due to CDK 9 inhibition.

One of the main attractions of targeting transcription in cancer is that short half-life proteins tend to be very sensitive to transcription inhibition and that many genes encoding proteins involved in cell growth; proliferation and survival are characterised by short-lived mRNA and proteins (Chen *et al.*, 2005). CDK 9, unlike other CDKs, appears to play its major role in transcription and transcriptional regulation. However, like other CDKs, it is considered a therapeutic target for the treatment of many diseases including cancer (Wang and Fischer, 2008). Its evaluation as a valid cancer target remains debatable and this study is a step towards validation of CDK 9 inhibition in cancer drug development. Earlier studies (Liu *et al.*, 2012; Shao, *et al.*, 2012) revealed through chemical inhibition by small molecule inhibitors that CDK 9 inhibition is a promising target in cancer chemotherapy. However, as chemical inhibition tends to be less selective; we report herein the use of more selective biological inhibition using RNA silencing to evaluate the potential and validity of CDK 9 inhibition in cancer chemotherapy.

In this study, ~ 70 % knockdown of CDK 9 in A2780 cells as seen from Western blot analysis. We have also shown that CDK 9 KD resulted in reduced phosphorylation of Ser-2 of RNAPII CTD, the site for CDK 9 phosphorylation, further confirming down-regulation of CDK 9. As the Ser-5 site on RNAPII CTD remains unchanged, the selectivity of biological inhibition via RNA silencing in contrast to chemical inhibition (Shao *et al.*, 2012) where both Ser-2 (CDK 9) and Ser-5 (CDK 7) sites were affected was reported in the present investigation. Hence, subsequent observations can be securely attributed to CDK 9 inhibition.

This study has also revealed that CDK 9 KD can sensitise A2780 cells to small molecule kinase inhibitors like CDKI-77 and flavopiridol. Hence, the present investigation have shown that CDK 9 KD dramatically increases sensitivity (4-5 fold) of A2780 cells to CDKI-77 or flavopiridol. This result revealed that the anti-proliferative activity of these compounds is influenced by the CDK 9 status of the cells

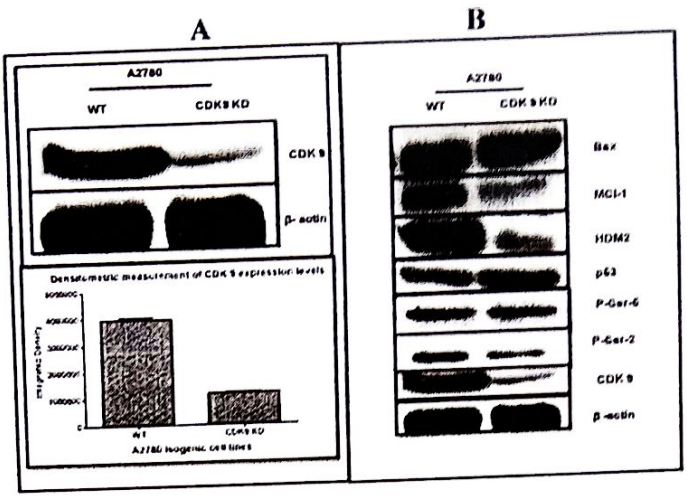

Figure 1: Characterisation of A2780 CDK 9 KD

(A) CDK 9 protein levels and integrated density measurements using Image J software (Version 1.46) in WT and KD A2780 cells; **(B)** Some key cellular protein levels in CDK 9 KD A2780 cells. WT and CDK 9 KD A2780 cells were grown to ~ 70 % confluence and then harvested for Western blot analyses. A representative blot was chosen from at least two independent experiments. β-actin was detected as an internal control.

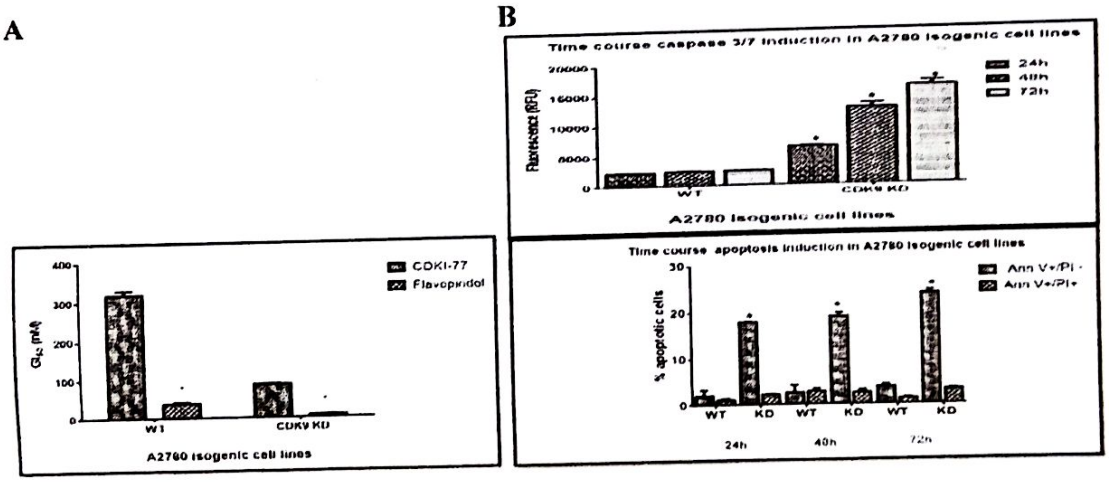

Figure 2: (A) Anti-proliferative activities of CDKI-77 and flavopiridol in A2780 isogenic cell lines The isogenic cells were treated with CDKI-77 and flavopiridol for 48 h and analysed by MTT assay. Vertical bars represent the mean ± SD of three independent experiments. Values significantly different ($p \leq 0.05$) from WT control are marked with an asterisk (*); **(B) Apoptosis and Caspase-3/7 activation in A2780 CDK 9 KD cells.** The WT and CDK 9 KD cells were cultured for 24, 48 and 72 hours harvested and used for both caspase 3/7 assays as well as apoptosis detection. The percentage of cells undergoing apoptosis was defined as the total of early apoptosis (annexin V- positive cells) and late apoptosis (annexin V- positive and PI-positive cells). Vertical bars represent the mean ± SD of three independent experiments. Values significantly different ($p \leq 0.05$) from WT control are marked with an asterisk (*).

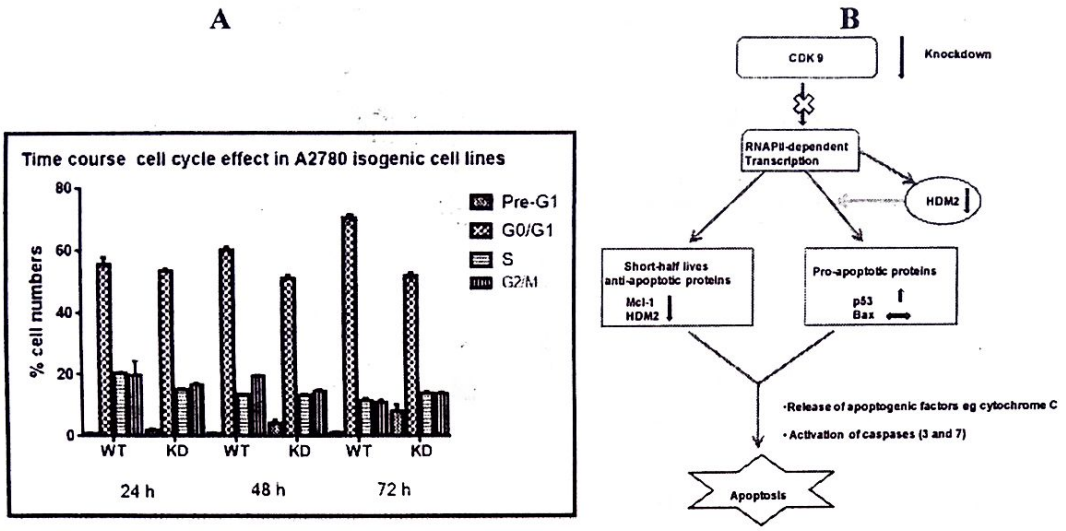

Figure 3: (A) Cell cycle effects in A2780 CDK 9 KD cells. WT and CDK 9 KD cells were cultured for 24, 48 and 72 hours harvested and analysed by PI DNA staining. There were minimal effects on G1, S and G2/M phases of the cycle. The percentage of cells in sub-G1 indicates death and late apoptotic cells vertical bars represent the mean ± SD of three independent experiments; **(B) Model for possible mechanisms of apoptosis following CDK 9 KD.** CDK 9 KD leads to transcription inhibition as a result of Ser-2 RNAPII CTD phosphorylation inhibition. Short half-life proteins such as Mcl-1 and HDM2 are sensitive to transcription inhibition and become down-regulated. Down-regulated HDM2 stabilised p53 which in turn acted on Bax protein. Suppression of survival protein Mcl-1 expression, down-regulation of p53-regulator HDM2 combined with subsequent pro-apoptotic properties of p53 and Bax promoted the release of apoptogenic proteins from mitochondria (such as cytochrome C) leading to apoptosis.

further indicating potential of CDK 9 in cancer growth inhibition.

CDK 9 KD in A2780 cells over time has resulted in caspase 3/7 activation with concomitant apoptosis induction. Little apoptosis was observed in the WT A2780 cells over time from 24 h-72 h. However, in CDK 9 KD cells, significant apoptosis was observed ≥24 hours of incubation accompanied by, during the same time period significant caspase 3/7 activation in A2780 CDK 9 KD cells. As down-regulation of Mcl-1 anti-apoptotic protein was observed, it is possible that the release of cytochrome C facilitated the activation of caspase 3/7 and consequently apoptosis execution (Liu *et al.*, 2012). That CDK 9 KD appears sufficient to trigger apoptosis in A2780 cells indicates that CDK 9 inhibition is a valid molecular target for anti-cancer drug development.

The present study has shown that CDK 9 KD resulted in the down-regulation of anti-apoptotic proteins, Mcl-1 and HDM2, accompanied by stabilisation of pro-apoptotic proteins p53 and Bax. The same period (24 hours) of incubation showing caspase 3/7 activation and significant apoptosis also showed down-regulation of anti-apoptotic proteins (Mcl-1 and HDM2) and stabilisation of the pro-apoptotic proteins (p53 and Bax). Down-regulation of both Mcl-1 and HDM2 after CDK 9 knockdown suggests a link between CTD phosphorylation and apoptosis. Earlier reports (Liu *et al.*, 2012; Shao, *et al.*, 2012) in the case of chemical inhibition indicated that transcription especially at the level of elongation, splicing and mRNA processing requires CTD phosphorylation by CDK 9, and inhibition of CDK 9 (whether chemically or biologically) led to transcription inhibition. Therefore, proteins such as Mcl-1 and HDM2 with short half-life are sensitive and become

depleted or down-regulated. Down-regulation will allow the otherwise suppressed apoptotic pathway in cancer cells to become active and the cells start to die (Chen *et al.*, 2005; Wang and Fischer, 2008). Up-regulation of p53 also suggests a link between CTD phosphorylation by CDK 9 and p53 protein. As reported earlier (Liu *et al.*, 2012), up-regulation of p53 could be a result of down-regulation of its negative modulator HDM2 rather than a direct effect on p53. This was confirmed as CDK 9 KD also down-regulated HDM2 concurrently with p53 up-regulation. As Bax pro-apoptotic protein is positively controlled by p53, its stabilisation could be a result of p53 up-regulation. As p53 stimulates the production of Bax, this Bax in turn acts on the mitochondria to promote the release of apoptogenic proteins that will eventually result in apoptosis (Kuwana *et al.*, 2005).

In cell cycle analysis, the present study have shown that CDK 9 is purely a transcriptional CDK with minimal role in cell cycle regulation. CDK 9 KD in A2780 cells did not lead to significant change in cell cycle profiles of the CDK 9 KD cells when compared with the WT cells. The present finding suggests that targeting transcription alone (via CDK 9 inhibition) affects cell survival and apoptosis without targeting the cell cycle.

Therefore, the current study has demonstrated that CDK 9 knockdown in A2780 cells appears sufficient to trigger apoptosis in A2780 cancer cells via down-regulation of short-lived proteins (Mcl-1 and HDM2). This finding further supports its role in transcription, and validate CDK9 as a molecular target for anti-cancer drug development.

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