

Research Article

## Better Therapeutic Target to Enhance Cisplatin Sensitivity in Cervical Cancer: PARP-1 or $\beta$ -catenin

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### Abstract

Development of resistance to cisplatin (CDDP) is a major bottleneck to treat cancer with CDDP, including cervical cancer (CC). We have recently shown that PARP-1 inhibition targets  $\beta$ -catenin signaling and enhance CDDP sensitivity in cervical cancer. This indicates that  $\beta$ -catenin itself could serve as an absolute target to potentiate CDDP

cytotoxicity. So, we determined the effect of  $\beta$ -catenin inhibition using JW74 either alone or in combination CDDP using CC cell lines on cell viability, apoptosis, cell cycle progression, proliferation, invasion and metastasis, clonogenicity. However, unexpectedly  $\beta$ -catenin inhibitor JW74 failed to significantly enhance CDDP- cytotoxicity in

both the cell lines. Since PARP-1 inhibitors inhibited  $\beta$ -catenin also, as determined through our previous work, so, we evaluated effect of JW74 mediated  $\beta$ -catenin inhibition on PARP-1 expression level inside cell. Intriguingly, we found that JW74 treatment enhanced PARP-1 expression as determined through western blotting. Increased PARP-1 serves responsible for CDDP resistance, thereby this seems stands responsible limiting our hypothesis. Further to confirm this, PARP-1 inhibitor PJ34 was added to a combination of JW74+CDDP and this significantly reduced cell survival and proliferation, enhanced cell death and decreased invasion and migration, hence enhanced CDDP sensitivity in CC cells. PJ34 with CDDP served as a better combination to increase caspase-3/7 cleavage, hence apoptosis, than combination of JW74 and CDDP; with PJ34 itself being less toxic to cells. Also PARP-1 expression was determined in human cervical cancer tissue sample at base level and compared to normal tissue sample. PARP-1 expression was significantly enhanced in human cervix cancer tumor samples. In summary,  $\beta$ -catenin inhibition increases PARP-1 expression, thereby, limits ability of  $\beta$ -catenin inhibitors to enhance CDDP cytotoxicity. Enhanced PARP-1 expression in cervix cancer tissue samples shows feasibility of using PARP-1 inhibitors in cervical cancer treatment.

**Keywords:** CDDP; Drug sensitivity; Wnt signaling; PJ34; JW74

## 1. Introduction

Globally, cervical cancer (CC) ranks as fourth common cancer among females with 5,69,847 new cases and 3,11,365 death reported in 2018 [1]. Approximately 90% of the CC cases occur in countries with low socio-economic status [2]. In India, CC ranks as commonest gynaecological cancer constituting 10% of all cancer-related mortality [1]. Persistent infection by high-risk subtype of human

papilloma virus (HPV) and subsequent malignant transformation accounts for 95% cases of CC. HPV 16 and 18 are the main high-risk subtypes causing  $\approx 80\%$  of all CC [3]. CC is majorly treated with concurrent radiation and cisplatin (CDDP)-based chemotherapy. CDDP mediates its cytotoxic effect by interacting with DNA to form DNA-protein and DNA-DNA adducts that activate signaling mechanisms leading to apoptosis [4]. Efficacy of CDDP is often limited due to innate/acquired CDDP resistance among cancer cells [5] leading to lower response to CDDP with an expected overall survival ranging from 10 to 17.5 months [6]. CDDP resistance has been classified into four types: (i) pre-target resistance, prevents CDDP binding to DNA by reducing CDDP accumulation, (ii) on-target resistance, by increased repair of CDDP-DNA adducts formed, (iii) post-target resistance, by errors in signal transduction in response to damaged DNA by CDDP, and (iv) off-target resistance, by interfering signal cross-talk required for CDDP-mediated cell death [7]. Poly (ADP-ribosyl) polymerase (PARP-1) is a nuclear DNA binding protein involved in DNA damage repair by nucleotide excision repair (NER) pathway. PARP-1 has been linked with CDDP resistance in various solid cancers [8] and use of PARP-1 inhibitors in BRCA mutated tumors leads to synthetic lethality and sensitize cancer cell to CDDP [9, 10]. We have recently shown that PARP-1 inhibitors downregulates  $\beta$ -catenin signaling to enhance CDDP sensitivity in CC cells [11]. Since  $\beta$ -catenin is involved in various malignancy and associated with increased cell survival, invasion, advanced stage and poor prognosis [12-17], we hypothesized that inhibition of  $\beta$ -catenin might enhance CDDP cytotoxicity in CC. Pharmacological abrogation was used for  $\beta$ -catenin inhibition through JW74, which is a tankyrase inhibitor. In this study, efficacy of JW74 to enhance CDDP-mediated cytotoxicity was determined and also compared with combination of PJ34 plus CDDP-mediated cytotoxicity.

## 2. Materials and Methods

### 2.1 Chemicals and cell culture

Both HeLa and SiHa used in the study were cultivated in DMEM high glucose supplemented with 10% FBS and 1% penicillin/streptomycin. KEMOPLAT (1mg/ml CDDP Injection) was used for CDDP treatment and JW74 ( $\beta$ -catenin inhibitor) was purchased from (Sigma-Aldrich). All chemicals and antibodies used in the study were purchased from Sigma-Aldrich and Santa Cruz, respectively.

### 2.2 Assessment of drug dose response by cell growth

Through MTT-based cytotoxicity assay, concentration as well as time-dependent response of JW4 was analyzed at 48h and 72h for both HeLa and SiHa. IC<sub>50</sub> value was determined and used to decide drug concentration for further experiments. For CDDP treatment, 2.5 $\mu$ M and 5 $\mu$ M doses were chosen and for  $\beta$ -catenin inhibition, we chose low doses of JW74 i.e. 5 $\mu$ M and 10 $\mu$ M.

### 2.3 MTT cytotoxicity assay

5 $\times$ 10<sup>3</sup> cells/well were plated in 96-well plate and treated with JW74 and CDDP alone or in combination for 48h and 72h. Following treatment, cells were subsequently incubated with 0.5% MTT at 37°C for 4h. The medium was then removed and 100 $\mu$ l DMSO was added to dissolve the formazan. The resulted absorbance was measured at 570 nm using a multi-well spectrophotometer (Bio-Tek, USA).

### 2.4 Immunoblotting

Whole cell lysate was prepared in RIPA lysis buffer and was resolved on 10% SDS-gel. Protein expression was analyzed using methodology described in our previous study [11]. Briefly, the protein was transferred to PVDF membrane at 40V overnight. After blocking with 3% BSA for 2 h, the target protein was detected by antibodies directed against  $\beta$ -catenin (Cat. No Sc-7963, 1:500) and PARP-1 (Cat. No. Sc-8007, 1:500). Quantification of the

target protein levels was conducted with Image J software (NIH,USA).

### 2.5 Determination of cell cycle progression and cell death

Cell cycle progression and cell death were analyzed through PI staining accordingly to previously described method [11]. Briefly, cells were permeabilized in 70% ethanol. Next day, cells were treated with 200  $\mu$ g/ml RNase A and stained with 50  $\mu$ g/ml PI in dark. Cell cycle distribution was determined through FACS Calibur flow cytometer (Becton Dickinson).

### 2.6 Clonogenic survival assay

Cells were seeded in a very low density (500 for untreated and 1000 for treated) in PD<sub>35</sub> and incubated overnight. Following PBS wash, cells were treated with CDDP, JW74 and PJ34 alone or as mentioned combinations for 2 h. Following PBS wash, cells were continuously cultured for next 10 days. Resulted colonies (atleast 30 cells/colony in untreated cells) were fixed with fixing solution (methanol:glacial acetic acid; 3:1) and stained with 0.5% crystal violet and counted under microscope.

### 2.7 Cell invasion assay

The invasion ability was ascertained using 6-well matrigel invasion plates (8- $\mu$ m pore size, Corning,) following manufacturers' protocol. Briefly, 2X10<sup>5</sup> (HeLa) or 4X10<sup>5</sup> cells (SiHa) cells in basal DMEM-HG, with/without drug were seeded in the upper chamber. Complete DMEM-HG (with 10% FBS) was added in the lower chamber for chemo-attractant. The culture was incubated at 37°C in 5% CO<sub>2</sub> atmosphere for 24h. The medium was then removed and cells were washed twice with PBS. Cells were fixed with 4% paraformaldehyde for 30 min and stained with hematoxylin and counterstained with eosin. Non-invaded cells were removed with a cotton bud. The number of cells in 5 different fields of view was counted under microscope.

## 2.8 Monolayer wound healing assay

The cells were seeded in high density in 6-well plate and resulted monolayer was scratched parallel with a sterile 10µL micropipette tip. Cells were washed with PBS and treated with indicated concentrations of CDDP, JW74 and PJ34 either alone or in combination for 22h (HeLa) and 36h (SiHa). The closure of the wounded area was observed under inverted microscope at 4X at 0 h and time point when approx. 50% of wound was closed in untreated cells and images were captured. The inhibition effect of treatment was expressed after normalization with control.

## 2.9 Detection of apoptosis

Cleaved caspase-3/7 is the active form of caspase-3/7, which are dedicated marker for apoptotic cell death and to assess apoptosis, the Caspase Glo<sup>®</sup> 3/7 assay (Promega) was used. Caspase-3/7 activity was measured in 96-well flat-bottomed culture plates.  $5 \times 10^3$  cells were seeded in 100 µl of drug free complete DMEM-HG medium and incubated overnight before drug treatment. Next day, spent media with unadhered cells was discarded, adhered cells were washed with PBS and 150 µl of DMEM-HG containing indicated drug concentration was added and incubated in 5% CO<sub>2</sub> incubator at 37°C for respective time points. After treatment, used media was removed and fresh DMEM-HG was added to each well. 100µl of freshly prepared Caspase-Glo<sup>®</sup> 3/7 reagent was added to each well containing 100µl of blank reaction (vehicle and cell culture medium), negative control (vehicle treated cells) or assay (treated cells in culture medium) and mixed gently and incubated at RT for 1 h. Following incubation, content was transferred carefully to pre labeled white-walled 96-well plate and luminescence of each sample was measured on luminometer (Synergy<sup>™</sup> HT Multi-detection, microplate reader, BioTek<sup>®</sup>). The data obtained were normalized to the control value of each cell line and fold change was plotted as bar graph.

## 2.10 Immunohistochemistry

Medical record files of CC patients registered during time period 2014-2015 were analyzed. Depending upon the availability of formalin fixed paraffin embedded (FFPE) tissue blocks, 44 cases were selected. Also, 4 non-cancer cervix tissue samples i.e. patients who underwent surgery for non-cancer indications, were used to compare PARP-1 expression in cancer and non-cancerous cervix tissue. For IHC, paraffin sections (4 µm) were deparaffinized in 100% xylene and re-hydrated in series of graded alcohol concentrations and water according to standard protocols. Heat-induced antigen retrieval was performed in 10 mM citrate buffer for 20 min at 100°C. IHC was performed with mouse anti-human PARP-1 antibody (Cat. No. Sc-8007, 1:200). Tissue sections were visualized with diaminobenzidine and counterstained with hematoxylin, using a bright field microscope (vanoxah-fl-2; Olympus Inc., Tokyo, Japan) followed by analysis by pathologist.

## 2.11 Statistical evaluation

All data are expressed as the mean  $\pm$  standard deviation from at least three replicates. Comparisons between groups were performed using the two-tailed Student's t-test with a significance cut-off at  $< 0.05$ . Statistical significance in difference in PARP-1 expression in normal cervix tissue vs. cervix tumor tissue was evaluated using student t-test.

## 3. Results

### 3.1 $\beta$ -catenin abrogation by JW74 is dose dependent

JW74 efficiently decreased the expression of  $\beta$ -catenin in dose dependent manner as shown by immunoblots in both the cell lines (Figure 1A and B). 5 µM and 10 µM of JW74 efficiently inhibited  $\beta$ -catenin expression and inhibition was more proficiently in SiHa cells than in HeLa cells.

### 3.2 JW74-mediated $\beta$ -catenin inhibition is associated with growth inhibition of CC cells

A dose- and time-dependent decrease in cell survival with

JW74 and CDDP was observed at both the time points. The  $IC_{50}$  values for JW74 were determined to be 10.5  $\mu$ M (48 h) and 9.1  $\mu$ M (72 h) for HeLa cells and 11.0  $\mu$ M (48 h) and 9.5  $\mu$ M (72 h) for SiHa cells (Figure 1C and D). Corresponding values for CDDP were 8.25  $\mu$ M (48 h) and 6.1  $\mu$ M (72 h) for HeLa cells, and 10.8  $\mu$ M (48 h) and 7.7  $\mu$ M (72 h) for SiHa cells [11]. These results indicate that CDDP is more cytotoxic than JW74 and sensitivity towards JW74 is comparable in both HeLa and SiHa cells (Figure 1C and D respectively).

### 3.3 $\beta$ -catenin inhibition enhances CDDP sensitivity in CC cells

Combining 5  $\mu$ M of JW74 with CDDP could not distinctly reduce the  $IC_{50}$  value of CDDP, however, combining 10  $\mu$ M of JW74 with CDDP significantly decreased the CDDP  $IC_{50}$  to 3.75 fold and 6.75 fold at 48 h and 4.06 fold and 4.27 fold at 72 h, respectively in HeLa and SiHa cells (Figure 1E-I). However, 10  $\mu$ M JW74 itself was highly toxic and survival reduced to  $\approx$  50%, whereas survival at 5  $\mu$ M of JW74 was greater than 75% in both the cell lines (Figure 1C and D); therefore, 5  $\mu$ M of JW74 was chosen as optimal concentration for further experiments.

### 3.4 Effect of $\beta$ -catenin inhibition on CDDP-induced cell cycle progression and cell death

We further analyzed the combinatorial effect of JW74 and CDDP treatment on CDDP-induced cell cycle progression and cell death. CDDP is not phase specific, however, cells are maximally sensitive in late-G1 phase, just prior to DNA replication [18]. CDDP causes S- or G2/M-phase block at low time point or low doses followed by cell death with increase in either concentration or time point [18, 19]. An increase in G2/M-population was observed in SiHa cells with CDDP treatment at both 48 and 72 h. In HeLa cells, CDDP treatment resulted in an increased S-phase population followed by increase in G2/M population. No

difference was observed in the % sub-G1 population as well as cell cycle progression in combinatorial treatment with JW74 and CDDP as compared to CDDP treatment alone in both the cell lines (Figure 2A-D).

### 3.5 Modulation of the PARP-1 expression through $\beta$ -catenin inhibition

We determined the effect of JW74 on PARP-1 expression and found increased PARP-1 expression in both the cell lines (Figure 2E and F). Therefore,  $\beta$ -catenin inhibition enhanced PARP-1 expression and this increased PARP-1 could be accountable for insignificant difference in cell death with combinatorial treatment of JW74 and CDDP compared to CDDP alone. Therefore, we next determined the effect of addition of PARP-1 inhibitor PJ34 (10  $\mu$ M) to combinatorial treatment of JW74 and CDDP and compared to CDDP alone.

### 3.6 Combined effect of $\beta$ -catenin and PARP-1 inhibition on CDDP-induced cell death

We determined the % sub-G1 population following PI staining to estimate the cell death. No difference in cell death at 48 h time point was observed in both the cell line following treatment with JW74+PJ34+CDDP as compared to CDDP alone (Figure 2A and 3C). We observed a slight increase in cell death after treatment of HeLa cells with JW74+PJ34+CDDP for 72 h; however, this was not significant as compared to treatment CDDP alone (Figure 2B). In SiHa cells, a marked and significant increase in cell death was observed when JW74 and PJ34 was combined with 5  $\mu$ M of CDDP as compared to only CDDP or CDDP+JW74 combination (Figure 2D).

### 3.7 Combined effect of $\beta$ -catenin and/or PARP-1 inhibition on anti-clonogenicity of CDDP

Colony formation assay was carried out to further examine the anti-proliferation effect of JW74 (and/or PJ34) and CDDP combined treatment vs. CDDP alone. We used 2.5



μM and 5 μM CDDP alone or with 5 μM JW74 and/or 10 μM PJ34. CDDP alone remarkably reduced the colony forming capacity of both the HeLa and SiHa cells (Figure 3A and C). Further, combining CDDP with JW74 reduced the clonogenicity by 1.07 and 1.15 fold in HeLa cells and 1.44 and 2.5 fold in SiHa cells at 2.5 μM and 5μM CDDP, respectively (Figure 3B and D). A combination of JW74+PJ34+CDDP was more effective than combination of JW74 and CDDP in inhibiting the colony forming capacity. Colony forming ability with JW74+PJ34+CDDP significantly decreased to 1.27 fold (HeLa) and 1.95 fold (SiHa) at 2.5 μM CDDP; and 3.87 fold (HeLa) and 5 fold (SiHa) at 5 μM CDDP (Figure 3B and D).

### **3.8 Combined treatment with β-catenin and/or PARP-1 inhibitor enhances anti-invasion/ migration effect of CDDP**

Wnt signaling regulates migration and invasion of cells, and thereby controls metastasis [20]. The combined effect of JW74 and/or PJ34 and CDDP on the migration was assessed through scratch assay. Combination treatment of JW74 and CDDP decreased cell migration in both the cell lines as compared to CDDP; however, the decrease was not significant (Figure 4A-D). Combination of JW74+PJ34+CDDP was more efficient and significantly reduced the cell migration in both the cell lines used. Migration reduced to 0.65 and 0.55 fold at 2.5 μM and 5μM CDDP, respectively in HeLa cells and to 0.83 and 0.68 fold at 2.5 μM and 5μM CDDP, respectively in SiHa cells (Figure 4B and D). We further determined the combined effect of JW74 and CDDP on cell invasion ability of HeLa and SiHa through transwell invasion assay. CDDP moderately reduced the invasion ability of both the cell lines and combined treatment with JW74 and CDDP further reduced the invasion ability of CC cells (Figure 4E-H). Like migration, cell invasion was significantly reduced to a greater extent upon co-treatment with JW74+PJ34+CDDP as compared to CDDP alone in both the cell lines (Figure 4E-H).

### **3.9 β-catenin inhibition increases CDDP-mediated apoptosis**

We determined the combined effect of 5 μM of JW74 and CDDP on CDDP-induced apoptosis through caspase 3/7 cleavage assay. A significant increase in caspase 3/7 cleavage in combination treatment as compared to CDDP alone was observed. However, apoptosis with JW74 alone was  $\geq$  apoptosis caused by 5 μM CDDP; higher concentration of CDDP used in the study (Figure 5A and B).

### **3.10 PARP-1 inhibition is more efficient than β-catenin inhibition in enhancing CDDP-mediated apoptosis**

We next determined the combined effect of PJ34 and CDDP on CDDP-mediated apoptosis and compared with apoptosis due to co-treatment with JW74 and CDDP. Apoptosis caused by PJ34 was lower than apoptosis caused by JW74 as well as CDDP alone (Figure 5A and B). Also, a significant increase in apoptosis was observed in co-treatment with PJ34 and CDDP as compared to CDDP alone. Co-treatment with PJ34 and CDDP caused more apoptosis than co-treatment with JW74 and CDDP.

### **3.11 Clinicopathological characteristics of cervix cancer patients**

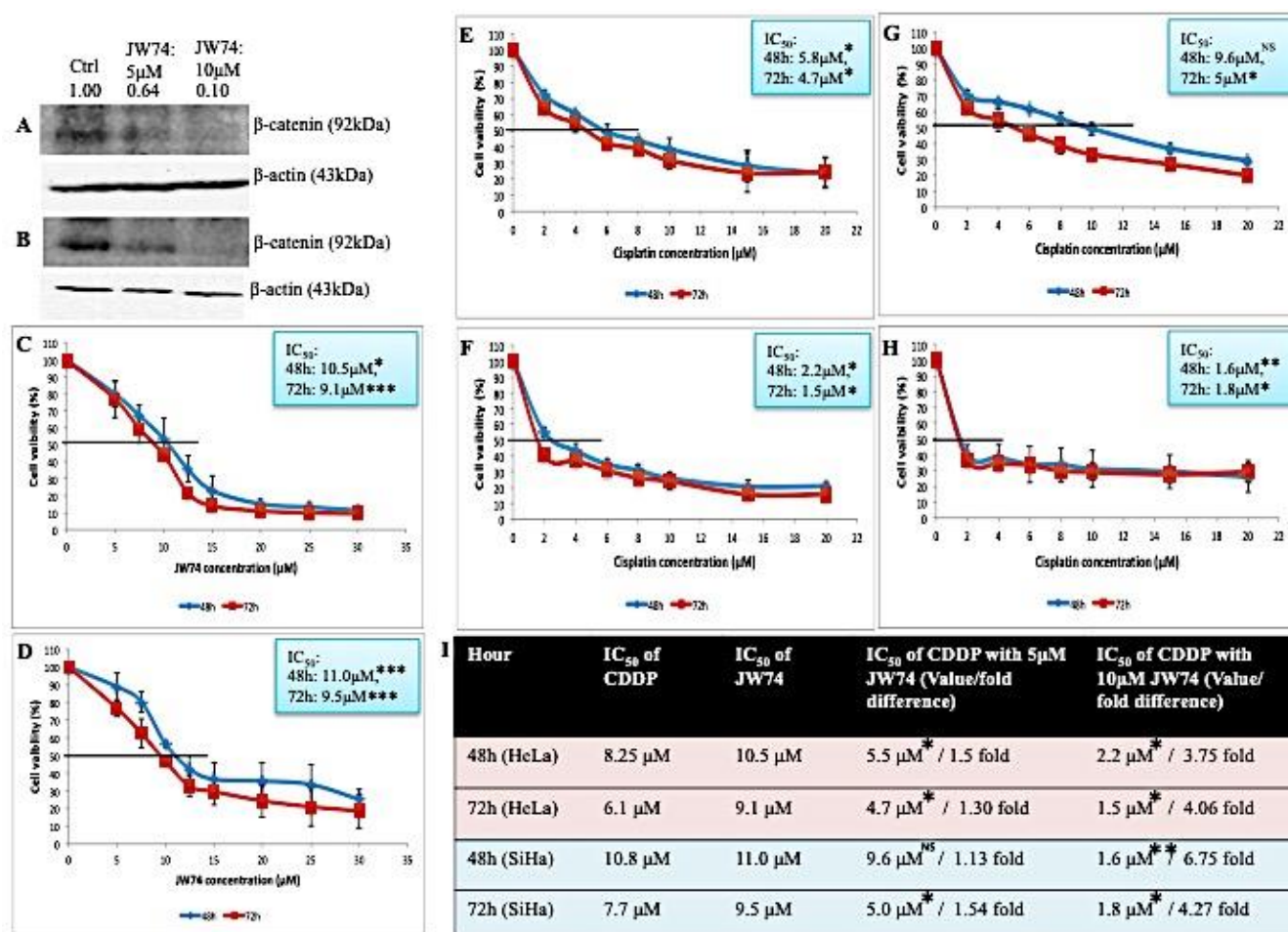
Figure 6G summarize clinicopathological characteristics of 44 cervix cancer patients. Majority of these patients were of squamous cell carcinoma histology (86.4 %) and were in locally advanced or advanced stage (stage III and IV: 56.8%).

### **3.12 Expression of PARP-1 in cervix tumor and non-tumor samples**

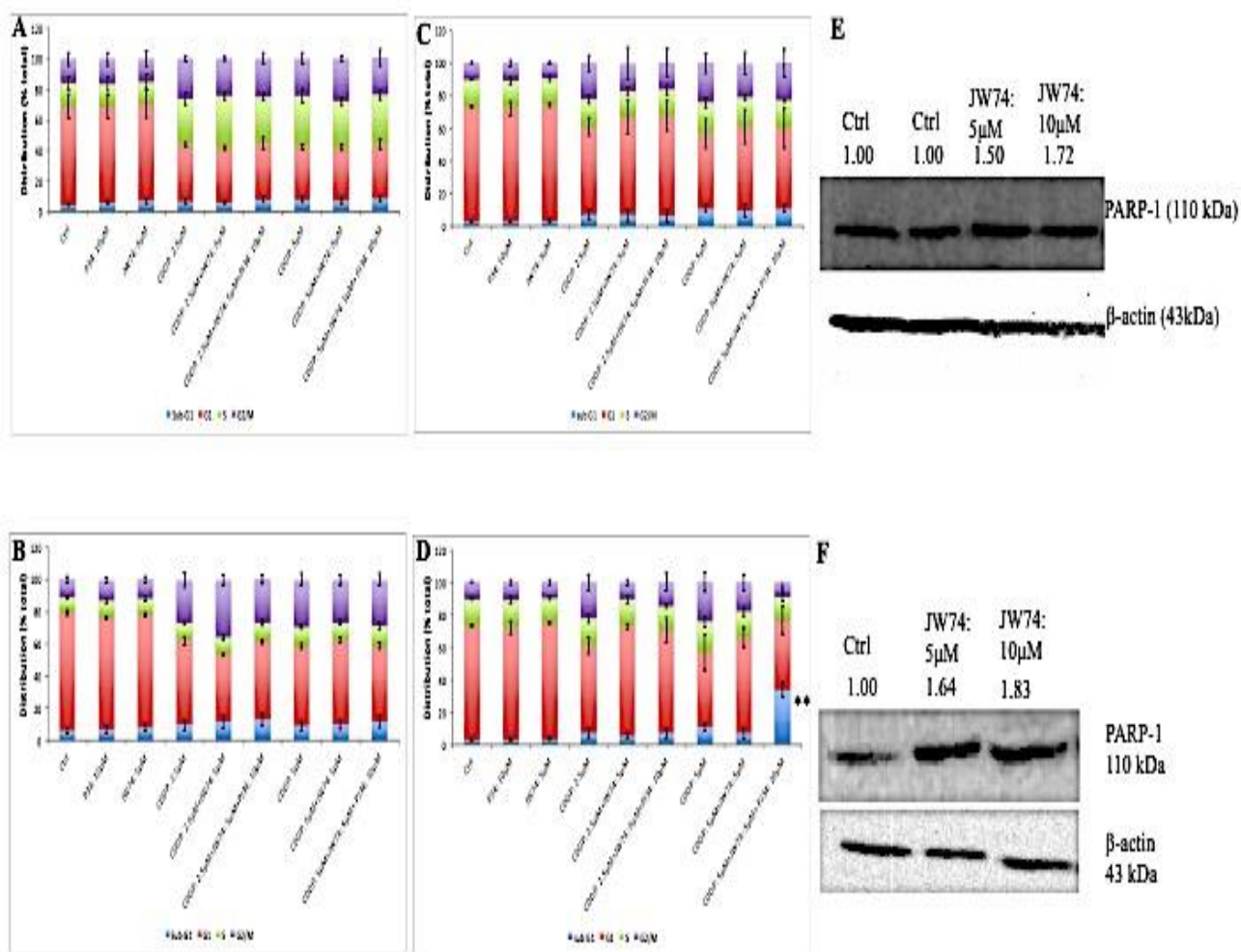
We correlated the expression of PARP-1 protein in non-tumor cervix tissue and CC samples (Figure 6). Non-tumor cervix samples did not show PARP-1 expression in the epithelial cells (intensity score: 0). Further, low immunoreactivity for PARP-1 was observed in the basal

cells but not in the mature cells of the cervix (Figure 6). However, the expression of PARP-1 was significantly

higher in CC tissue ( $p=0.0001$ , Figure 6) and was independent of tumor stage (Figure 6G-I).

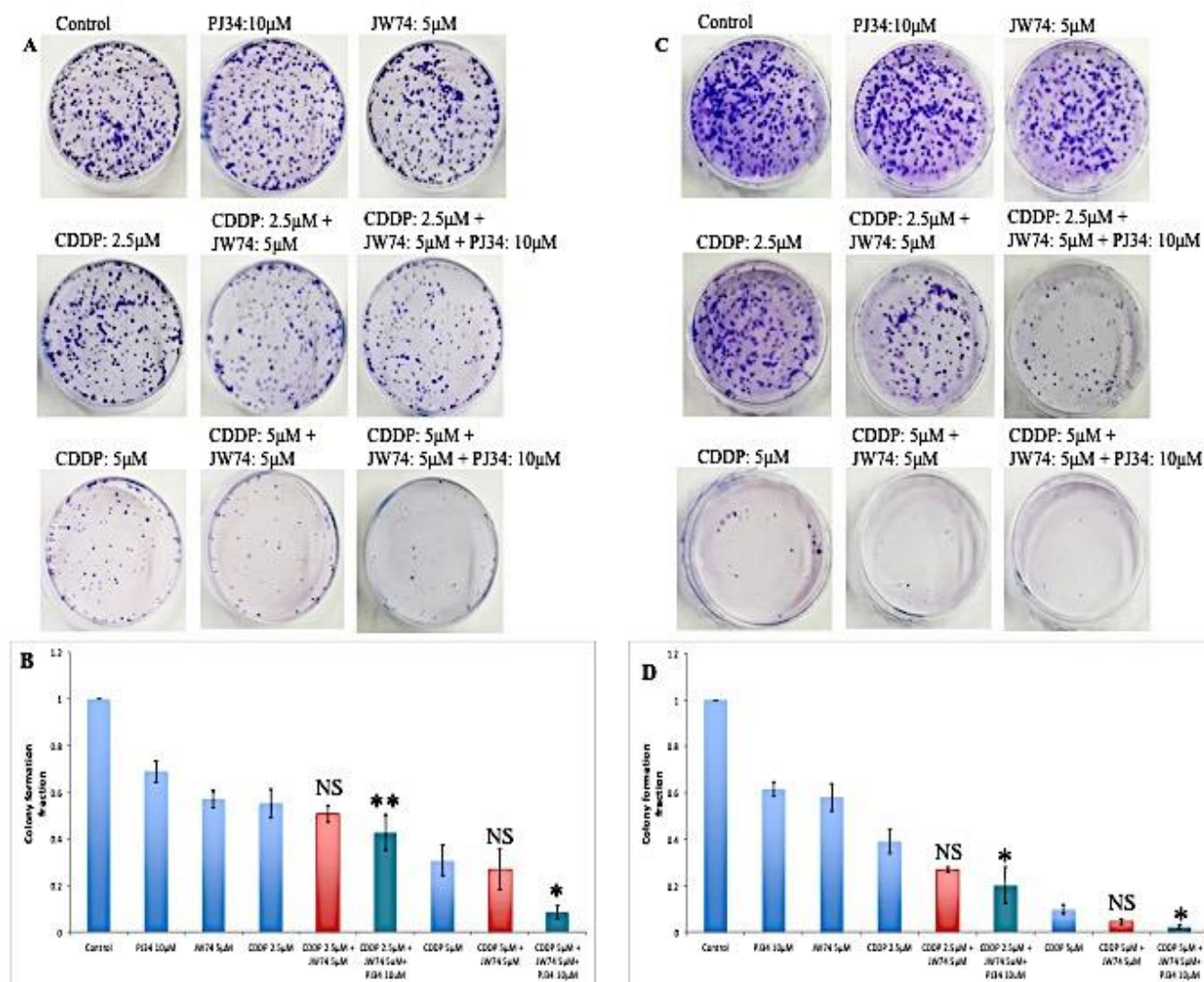


**Figure 1:** Representative immunoblots confirming  $\beta$ -catenin inhibition and dose-response effect of JW74 on cell viability and combined effect of CDDP and JW74 on  $IC_{50}$  value of CDDP. A and B, immunoblots showing endogenous level of  $\beta$ -catenin in HeLa (A) and SiHa (B) cells after treatment with JW74 (0, 5 and 10  $\mu$ M) for 48h. C and D, HeLa (C) and SiHa (D) cells were treated with indicated doses of JW74 for 48h and 72h, respectively and cell viability was determined by MTT assay. E-I, HeLa cells were treated with different doses of CDDP in combination with 5  $\mu$ M or 10  $\mu$ M of JW74 (E and F). SiHa cells were treated with different doses of CDDP in combination with 5  $\mu$ M or 10  $\mu$ M of JW74 (G and H). Table representing fold change in the  $IC_{50}$  value of CDDP upon combined treatment with JW74 and CDDP vs. CDDP alone in HeLa and SiHa cells at indicated time points (I). All the values are expressed relative to untreated cells (100% control value).  $\beta$ -actin was used as loading control. Error bars represent mean  $\pm$  SD ( $n \geq 3$  independent experiments).  $IC_{50}$  values for JW74 at different time points along with their p value is mentioned in the respective graph. NS: Non-significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

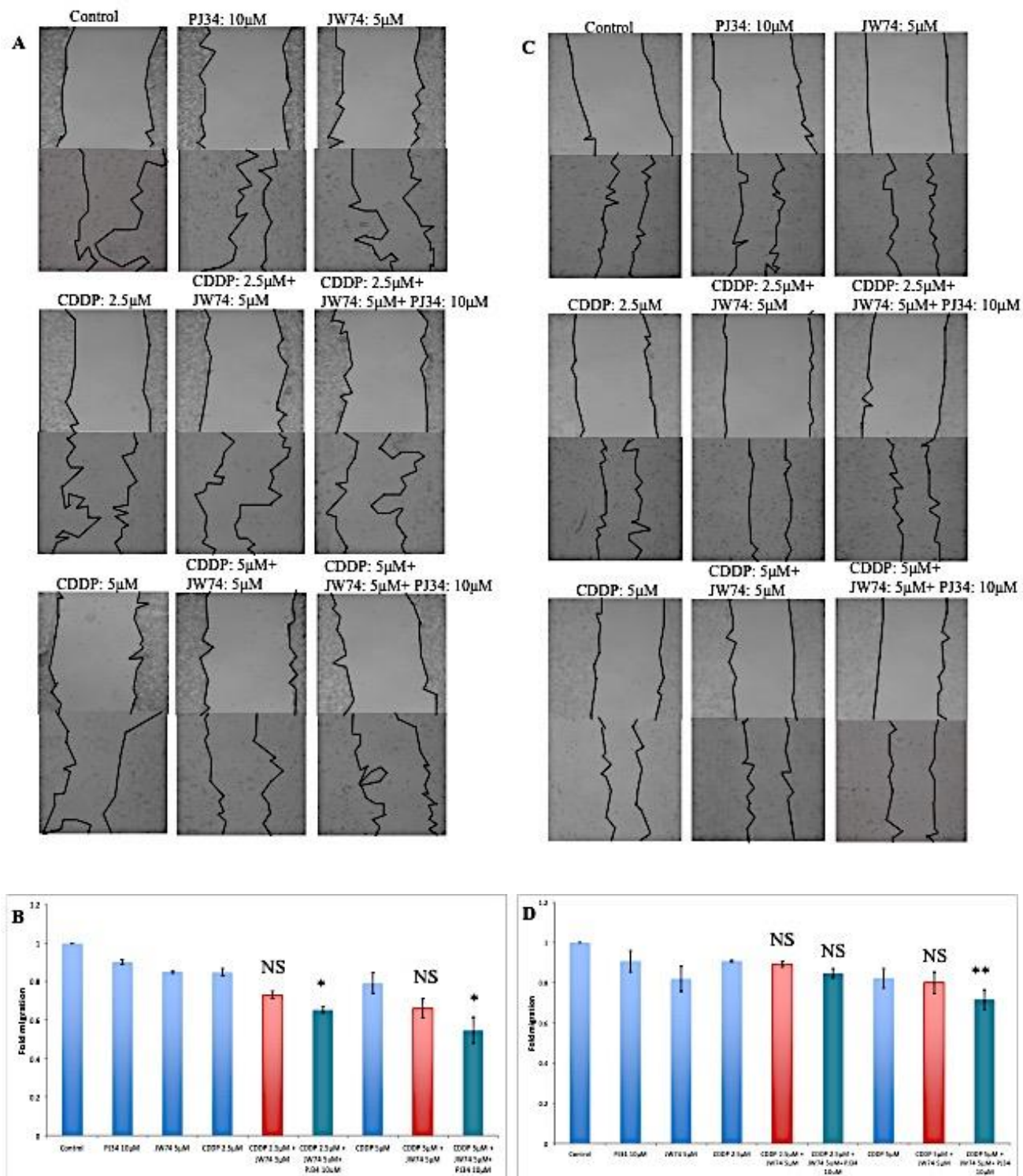


**Figure 2:** Combined effect of JW74 ± PJ34 & CDDP on cell cycle progression and representative immunoblots confirming effect of JW74 on PARP-1 expression. A-D, HeLa cells were treated with different doses of CDDP in combination with 5μM of JW74 ± 10μM PJ34 for 48h and 72h (A and B). SiHa cells were treated with different doses of CDDP in combination with 5μM of JW74 ± 10μM PJ34 for 48h and 72h (C and D). E and F, cells were treated with indicated doses of JW74 and resulted effect on PARP-1 expression was determined in HeLa (E) and SiHa cells (F), respectively. β-actin was used as loading control. Error bars represent mean ± SD (n ≥ 3 independent experiments). \*\*, p < 0.01.

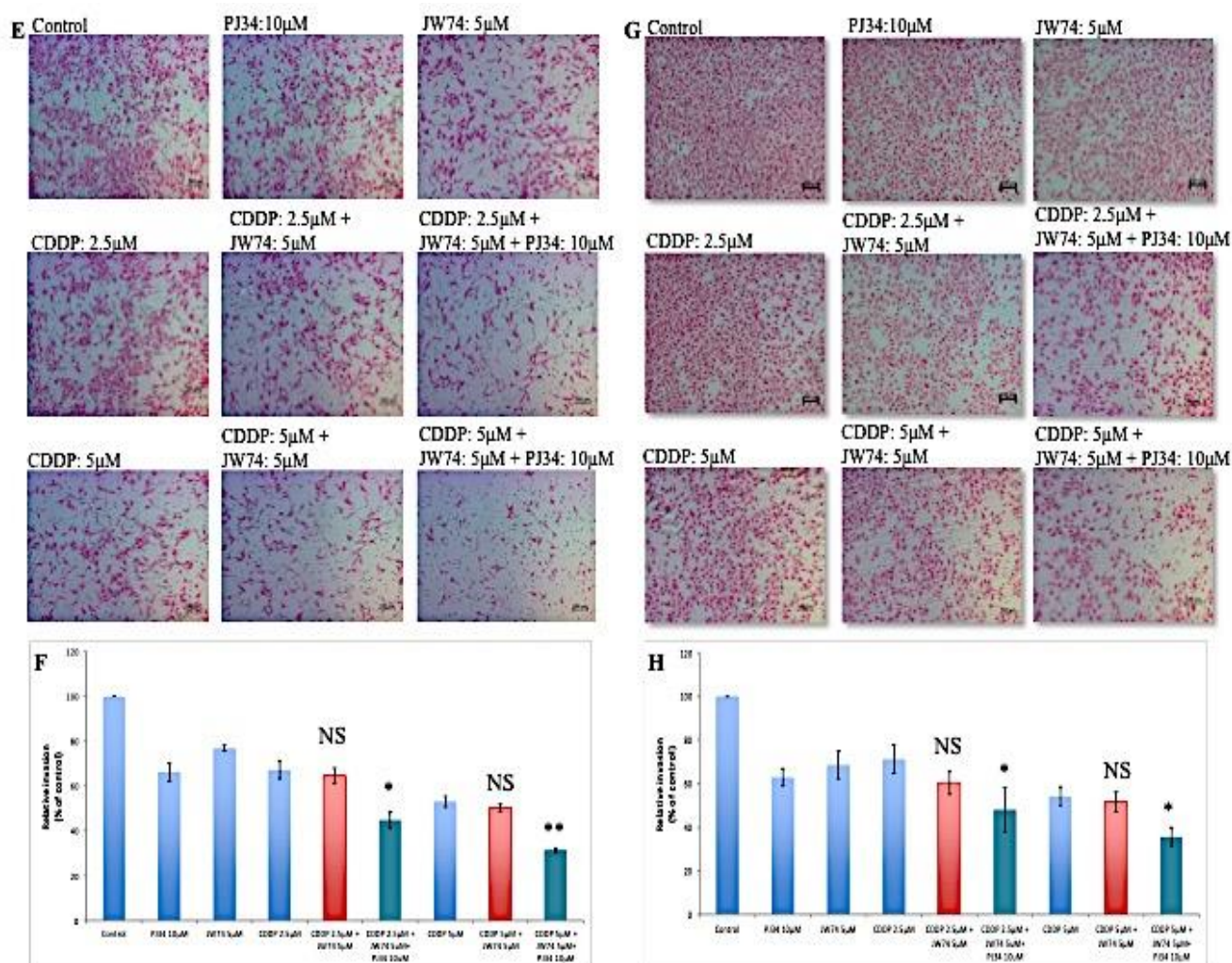




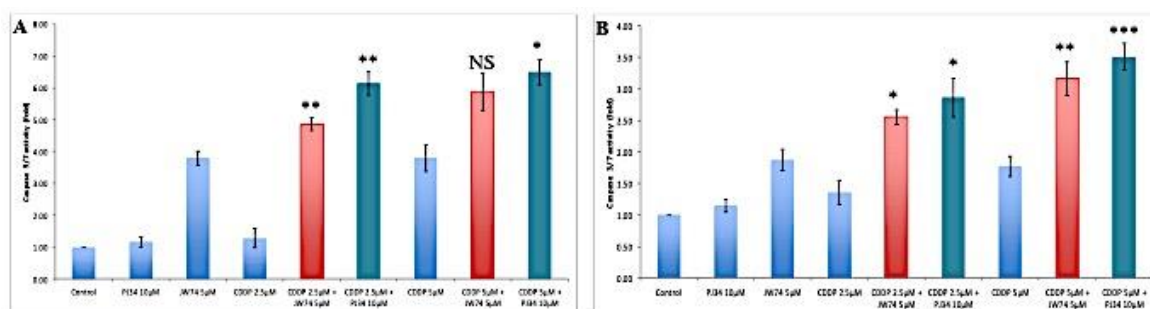
**Figure 3:** Combined effect of JW74  $\pm$  PJ34 & CDDP on colony formation assay. A-D, Representative images for HeLa (A) and SiHa (C) cells treated with 2.5 and 5  $\mu$ M CDDP, 10  $\mu$ M PJ34 and 5  $\mu$ M JW74 alone or combination of CDDP and JW74  $\pm$  PJ34 in indicated doses for 2h. Bar graphs showing colony forming ability with respect to control of each group in HeLa (B) and SiHa (D) cells. For each doses, three replicates were performed where the survival of untreated cells (control) was set to one. Error bars represent mean  $\pm$  SD ( $n \geq 3$  independent experiments). NS: Non-significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .





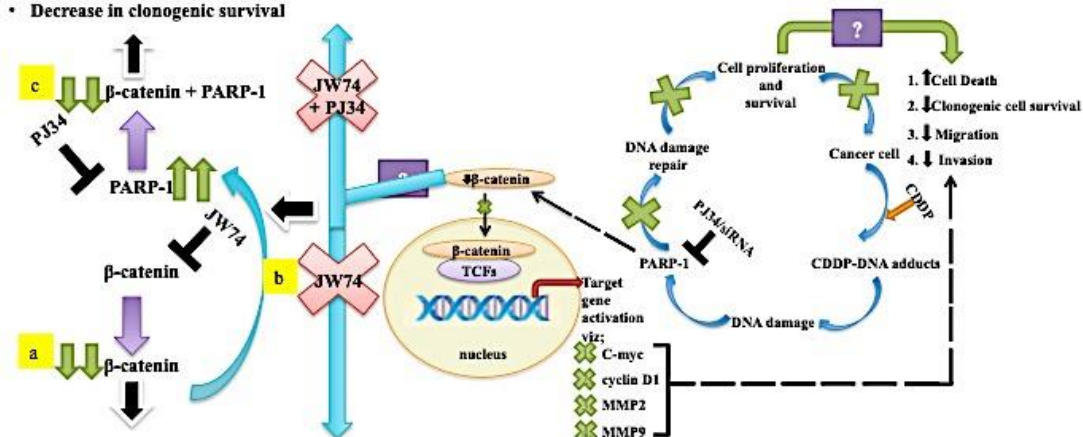


**Figure 4:** Combined effect of JW74 ± PJ34 & CDDP treatment on the invasion and migration. A-D, representative images (under 4X magnification) of scratch wound healing assay performed after treatment of HeLa (A) and SiHa (C) cells with 2.5 and 5μM CDDP, 10μM PJ34 and 5μM JW74 alone or combination of CDDP and JW74 ± PJ34 in indicated doses. Fold migration (with respect to control) bar graphs of each group in HeLa (B) and SiHa cells (D). E-H, representative images (under 10X magnification) of invaded cells after treatment with 2.5 and 5μM CDDP, 10μM PJ34 and 5μM JW74 alone or combination of CDDP and JW74 ± PJ34 in indicated doses in HeLa (E) and SiHa (G) cells. Fold change in invasion ability (with respect to control) bar graphs of each group in HeLa (F) and SiHa cells (H). Error bars represent mean ± SD ( $n \geq 3$  independent experiments). NS: Non-significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .



Combination JW74+PJ34 and CDDP significantly enhanced:

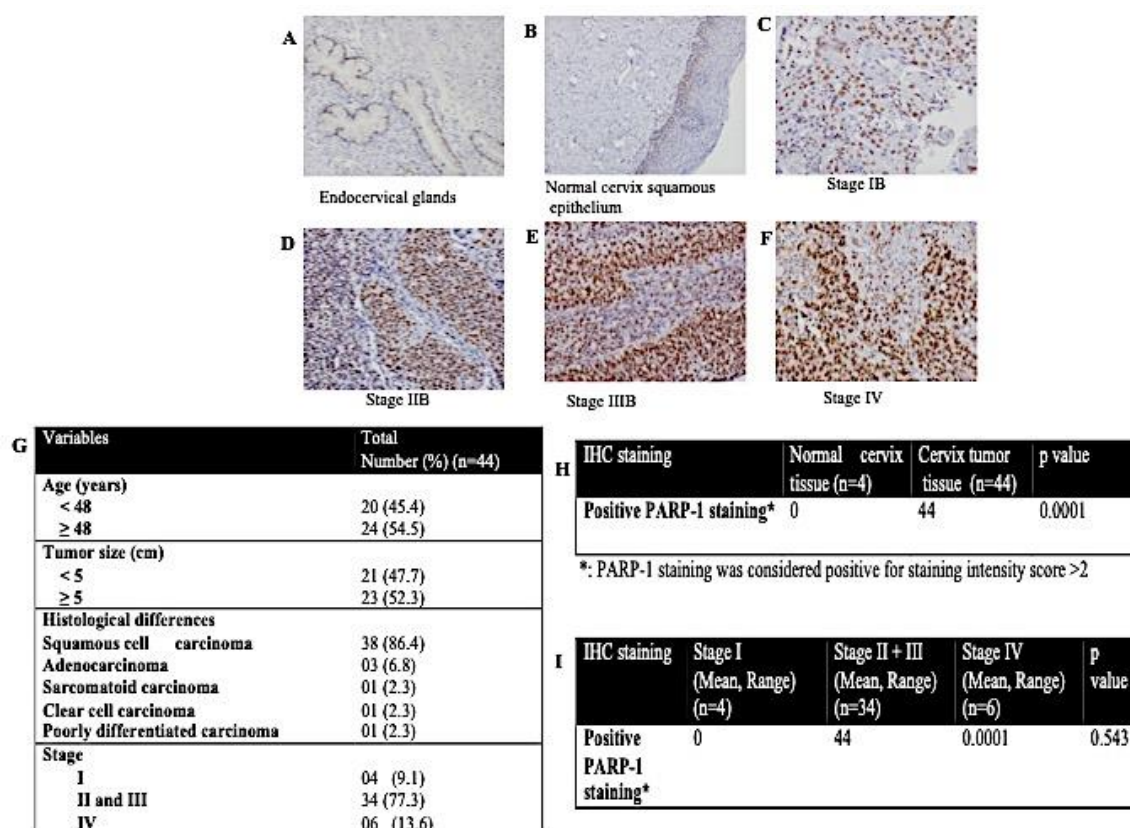
- CDDP mediated decrease in cell proliferation
- Cell cycle block/death
- Decrease in invasion and migration
- Decrease in clonogenic survival



Combination of JW74 and CDDP had no significant difference in CDDP mediated decrease in:

- Cell proliferation
- Cell cycle block/death
- Invasion and migration
- Clonogenic survival

**Figure 5:** Combined effect of JW74 & CDDP treatment on caspase-3/7 cleavage as compared to combination of PJ34 and CDDP. A-C, HeLa (A) and SiHa (B) cells treated with 2.5 and 5µM CDDP, 10µM PJ34 and 5µM JW74 alone or combination of either CDDP and JW74 or combination of CDDP and PJ34 in indicated doses for 24h. Bar graphs showing caspase-3/7 cleavage with respect to control of each group in HeLa (A) and SiHa (B) cells. C. Model for regulation of β-catenin signaling by PARP-1 inhibition and resulted effect on CDDP sensitivity. Also, showing probable mechanism (enhanced PARP-1 expression) for non-significant effect on augmenting CDDP sensitivity upon β-catenin inhibition through JW74 (a and b) and the resulted effect of PARP-1 inhibition in such condition via PJ34 (c) i.e. PJ34+JW74+CDDP (C) on CDDP efficacy. For each dose, three replicates were performed where the survival of untreated cells (control) was set to one. Error bars represent mean ± SD ( $n \geq 3$  independent experiments). NS: Non-significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .



**Figure 6:** Expression of PARP-1 protein in human normal cervix and cervical cancer samples as determined by IHC. (A-F) Expression of PARP-1 in human normal cervix tissue samples (A and B). Expression of PARP-1 in different stages of cervical cancer (C-F).

#### 4. Discussion

CDDP serves as basis for treatment of solid tumors, including CC [21]. CDDP interacts with N7 on purine base leading to CDDP-DNA adducts [22]. This causes activation of DNA damage repair pathways resulting into either repaired DNA and cell survival or activation of irreversible apoptotic signaling leads to cell death [23].  $\beta$ -catenin is involved in various human malignancies [24-27], and is actively involved in cell growth, adhesion and stemness [27-29]. Based on our previous findings that PARP-1 inhibition downregulates  $\beta$ -catenin expression and thereby, Wnt signaling [30], here hypothesized that inhibiting  $\beta$ -catenin could serve as a better approach to enhance CDDP sensitivity in CC. In the present study we tested the

efficiency of JW74 to enhance CDDP sensitivity using HeLa and SiHa cells and compared to PARP-1 inhibition. JW74 or 4-[4-(4-Methoxyphenyl)-5-[[[3-(4-methylphenyl)-1,2,4-oxadiazol-5-yl]methyl]thio]-4H-1,2,4-triazol-3-yl]pyridine acts by stabilizing AXIN2, a key component of the  $\beta$ -catenin destruction complex [31, 32]. JW74 efficiently reduced expression of  $\beta$ -catenin in both the cell lines and also, it reduced invasion, migration as well as clonogenic survival and enhanced cell death in CC cells used, however, JW74, hence  $\beta$ -catenin inhibition was less cytotoxic as compared to CDDP. We next determined the combinatorial effect of JW74 and CDDP on CDDP-mediated cytotoxicity either alone or in indicated combinations. JW74 was combined with sub-lethal doses of CDDP i.e. 2.5 $\mu$ M and



5 $\mu$ M. A remarkable decrease in the IC<sub>50</sub> value of CDDP was observed in combination with 10 $\mu$ M of JW74. But, being highly cytotoxic at 10 $\mu$ M a lower dose of JW74 (5 $\mu$ M) was used. A decrease in IC<sub>50</sub> value of CDDP was observed in both HeLa and SiHa at combinatorial treatment with 5 $\mu$ M JW74 and CDDP, however, decrease was not significant (Figure 1E-I). Other studies have also reported inhibition of  $\beta$ -catenin, insufficient to affect apoptosis in multiple myeloma [26, 33]. Studies show that,  $\beta$ -catenin level increases in S phase, accumulates during G2/M phase and decrease as cell enters G1 phase of cell cycle [34]. Also,  $\beta$ -catenin controls G1/S transition in MDCK cells [35]. Hence, we next determined the effect of JW74 either as single agent or in combination with CDDP on cell cycle progression. JW74 downregulates the expression of CCND1 and CDK6 gene, required for entry to G1 phase [12]; however, JW74 didn't effect CCNE1 and CDK2 gene, which regulates progression from G1 to S phase [36]. In our study also, JW74 alone didn't effect the cell cycle progression, whereas CDDP caused G2/M block in SiHa cells and S phase block population in HeLa cells at 48 h and 72 h, however, we could not find any marked increase in cell death or block in combination treatment with JW74 and CDDP (Figure 2A-D). Similarly,  $\beta$ -catenin inhibition didn't have marked effect on clonogenic cell proliferation and survival or even any significant differences in invasion and migration in combined treatment with JW74 and CDDP as compared to CDDP alone (Figure 3 and 4). We determined the possible reason for incompetence of JW74 to enhance CDDP cytotoxicity. With referral to our earlier finding; PARP-1 inhibition decreased  $\beta$ -catenin expression [11], we focused on the expression of PARP-1 upon  $\beta$ -catenin inhibition using JW74. Interestingly, we found an increased PARP-1 expression upon JW74 treatment (Figure 2E and F). Also, we found a significant increase in cell death at 72 h in SiHa cell when PJ34 was added to combination of JW74 and CDDP as compared to CDDP alone (Figure 2D).

A combination of JW74+PJ34+CDDP significantly reduced the clonogenic survival, cell invasion and migration ability in CC cells as compared to only CDDP (Figure 3 and 4). Indicating, it is enhanced PARP-1 expression upon  $\beta$ -catenin inhibition that limited efficacy of JW74 to enhance CDDP sensitivity in cervical cancer cells. Additionally, we compared the efficiency of JW74 and CDDP combination vs. combination of PJ34 and CDDP in enhancing CDDP-mediated apoptosis through caspase 3/7 cleavage assay. In combination, PJ34 and CDDP combinatorial treatment was more apoptotic than JW74 and CDDP combination. Also, JW74 itself was more apoptotic, whereas, PJ34 was less toxic than both the JW74 and CDDP doses used in the study. Combining these observations, PARP-1 inhibition was more efficient than  $\beta$ -catenin inhibition in enhancing CDDP-mediated cytotoxicity. Next, we observed constant high expression of PARP-1 protein in CC tissue sample while the expression in normal cervix tissue samples was non-evaluable (Figure 6H). PARP-1 overexpression has been associated with carcinogenesis in malignant melanomas, colorectal, breast, testicular and lymphangioleiomyomatosis [37-41]. Elevated expression of PARP-1 in cervix cancer creates a possibility for the use of PARP-1 inhibitors in CC treatment despite the absence of synthetic lethality in these HPV-associated CC. PARP-1 has been associated with invasiveness and higher tumor grade [42]. However, we didn't find any correlation between PARP-1 expression and tumor staging, which is line with other reports in gastric, colorectal, ovarian and breast cancer [42-45].

## 5. Conclusion

$\beta$ -catenin inhibition enhances PARP-1 expression and, hence, fails to significantly enhance CDDP sensitivity in CC cells. Significant increase in CDDP cytotoxicity uenin inhibitor plus CDDP cocktail further confirms it,. Combination of PARP-1 inhibitor with CDDP is a better

approach to increase CDDP sensitivity in CC cells than a combinatorial treatment with  $\beta$ -catenin inhibitor and CDDP. PARP-1 inhibitors can be efficiently used in treating cervix cancer patients both as base level as well as relapse state. However, further preclinical and *in vivo* studies are warranted to validate these findings and before the clinical utility of such therapeutic approach can be exactly determined.

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### Disclosure

None

### Conflicts of Interest

The authors declare no conflict of interest.

### Authors' Contribution

MM performed the experiments and data acquisition. LK and MM designed the study and drafted the manuscript. SC, NB, SK, SB, RG and LK supervised the study. SM was responsible for analysis of IHC slides. All authors read and approved the final manuscript.

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