Induction of apoptosis and role of paclitaxel-loaded hyaluronic acid-crosslinked nanoparticles in the regulation of AKT and RhoA

ABSTRACT

Cancer is a complex multifactorial disease and leading causes of death worldwide. Despite the development of many anticancer drugs, there is a reduced survival rate due to severe side effects. The nontargeted approach of convention drugs is one of the leading players in context to toxicity. Hyaluronan is a versatile bio-polymer and ligand of the receptor (CD44) on cancer cells. The MCF-7 and HT-29 cancer cell lines treated with hyaluronic acid-paclitaxel (HA-PTX) showed the distinguishing morphological features of apoptosis. Flow cytometric analysis showed that HA-PTX induces apoptosis as a significant mode of cell death. The activation level of tumor suppressor protein (p53) increased after PTX treatment in MCF-7, but no changes observed in HT-29 might be due to hereditary mutations. The lack of suppression in AKT and Rho A protein suggest the use of possible inhibitors in future studies which might could play a role in increasing the sensitivity of drug towards mutated cells line and reducing the possibilities for cancer cell survival, migration, and metastasis.

Keywords: AKT, apoptosis, drug delivery, flow cytometry, hyaluronan, paclitaxel, Rho A

INTRODUCTION

Cancer is an uncontrolled growth of cells that occur due to rapid cell division. The unchecked multiplication of cells often leads to tumor formation, which further undergoes metastasis and cancer development – there an estimated 9.6 million cancer deaths, 18.1 million new cancer cases in 2018. Breast cancer is the second leading cause of death, followed by colorectal and lung cancer. By regular disease screening, along with improved prognosis, enhance the survival rate. There is a total decrease of 56% in the death rates from breast and colorectal cancer. Nanomedicine has been prepared as one of the approaches for combating diseases. The conventional method of cancer treatment involves chemotherapy and radiotherapy but causing severe side effects with a low survival rate. The poor solubility of potential anticancer drugs remains the major challenge in drug delivery.

Previous studies show that polymeric nanocarriers show potential as therapeutic carriers. The biopolymer,
hyaluronic acid (HA), a glycosaminoglycan present inside the human body and strong affinity towards CD44 receptors. The anticancer drug paclitaxel (PTX) is a powerful anti-mitotic agent shown remarkable results as an anticancer agent. However, it causes severe side effects and acquires resistance in various cells. The induction of cell death plays an essential role in the cytotoxicity of potential drugs. The most fundamental process of cell death and development is apoptosis. Apoptosis, a highly regulated mechanism of cell death, characterized by distinct morphological and biochemical features. The up-regulation and down-regulation of cancer progression pathways determine the fate of the cell. Any dysregulation of AKT results in cancer progression and various other autoimmune diseases. Similarly, RhoA and RhoC overexpressed in various human tumors. Furthermore, the downregulation of RhoB, activate the expression of RhoA in the liver, colon cancer, and skin tumor.

In our previous study, the PTX-loaded HA-crosslinked nanoparticles (NPs) cytotoxicity on MCF-7, HT-29, and A549 and drug release kinetics were studied. However, in the present study, the HA-PTX NPs induced apoptosis mode of cell death and regulation on AKT and Rho A as cell proliferation and cell survival proteins studied in MCF-7 and HT-29 cells.

RESULTS AND DISCUSSION

CELL CULTURE

The cell lines purchased from American-Tissue-Culture-Collection.org MCF-7 (ATCC HTB-22™) and colorectal HT-29 (ATCC HTB-38™). The Dulbecco’s Modified Eagle’s medium (DMEM) and Roswell Park Memorial Institute (RPMI) media and chemicals obtained from Sigma Aldrich (USA). The anticancer drug PTX purchased from Acros Organic (USA). The FITC-Annexin-V apoptosis detection kit, Platinum ELISA kit, adenosine diphosphate/adenosine triphosphate (ADP/ATP) kit, AKT ultra-sensitive kit, and Rho A assay kit acquired from BD Bioscience (USA), Abcam (UK), and Cytoskeleton (USA). All chemicals were of analytical grade.

Cell culture

The cell lines culture in DMEM and RPMI 1640 medium, containing 10% fetal bovine serum and 1% antibiotics. Cells were culture in 5% CO₂ at 37°C. Cell lines were grown in 25 cm² and 75 cm² tissue culture flasks in a humidified atmosphere, containing 5% CO₂ and 95% air, at 37°C. The cell culture procedure and guideline followed by Ethical Committee of the Faculty of Medicine and Health Sciences, UPM.

Inverted microscopy and flow cytometric analysis

Cell morphology studied under a Motic AE31 inverted microscope. FITC Annexin V Apoptosis Detection kit (USA) used to study the induction of apoptosis and necrosis. The cells were resuspended in binding buffer containing FITC-Annexin V and propidium iodide (5 μl each) and then analyzed by flow cytometer.

Adenosine diphosphate/adenosine triphosphate ratio

The ADP/ATP ratio performed by using ADP/ATP (bioluminescent) method. One hundred μl of reaction mix consisting of 5 μl reconstitute ATP monitoring enzyme and 95 μl nucleotide releasing buffer were added to each sample-treated well and read after 5–10 min. After reconstituted ADP converting enzyme 1 μl of ADP, converting enzyme was added, and the samples again read in 5–10 min in a luminometer.

Tumor suppressor protein (p53)

P53, activation level analyzed by Human p53 Platinum ELISA US kit. Briefly, the cells (2 × 10⁶ cells/ml) were collected and undergoes lysis using 1X Cell Lysis Buffer. One hundred μl p53 standard added to get the serial dilution ranging from 50 to 0.8 U/ml. Fifty microliter of each sample added in triplicate to all wells. One hundred μl of diluted streptavidin-horseradish peroxidase (HRP) was added to all wells, including the blank wells, and incubated at room temperature for 1 h. The results read using ELISA reader at 450 nm.

AKT and Rho A

AKT analyzed by AKT1 ultra-sensitive kit (ELISA). Concisely, after 24 h treatment, the cells were lysed and re-lysed by using cell extraction buffer and chilled 1× cell lysis buffer. The 50 μl of standard solution, treated samples, and control samples added to the microtiter wells. AKT1 detection antibody solution of 50 μl was added. After incubation, one hundred μl Anti-Rabbit immunoglobulin G HRP working solution was added to each well. Stop solution of 100 μl added to all well. Similarly, Rho A activity observed using Rho A assay kit. The experiment was performed according to manufacturer protocol. Briefly, 50 μl of anti-RhoA 44 primary and secondary antibody used. The mixed HRP-based detection reagent was used as detection system. The absorbance measured at 490 nm using a ELISA plate reader.

Statistical analysis

The data analyzed using a one-way analysis of variance also, Tukey honestly significant difference and least significant difference test (SPSS version 16.0, Chicago, SPSS Inc.). The P < 0.05 considered significant, whereas P < 0.01 as very significant. All experiments triplicated, and the data expressed as means with standard deviation.

RESULTS AND DISCUSSION

Morphological and flow cytometry

The morphological observation MCF-7 and HT-29 cancer cells was studied using a light microscope and flow
cytometry. The cells treated with HA-PTX NPs and PTX (1 and 100 ng/ml) treatment for 24 h [Figure 1a-j]. The treated cells showed morphological features of apoptotic cell death, nucleus becoming phase-dense, and condensed rounded cells. Flow cytometric analysis [Figure 2a-j] suggests that the cell treated with HA-PTX NPs more sensitive towards apoptosis (V/PI) as compared to free PTX. The induction of apoptosis observes in MCF-7 treated with low 1 ng/ml and 100 ng/ml concentration. In HT-29, the process of necrosis observes when cells treated with 1 ng/ml concentration of HA-PTX.

**Adenosine diphosphate/adenosine triphosphate ratio**

In breast (MCF-7), the ADP/ATP ratio is high at 10 ng/ml HA-PTX as compare to PTX [Figure 3]. However, HT-29 shows higher ADP/ATP as compared to the MCF-7 cancer cell lines after 10 ng/ml PTX and HA-PTX treatment. Similarly, at 100 ng/ml, colorectal cancer shows an increase in ADP/ATP ratio as compare to breast (MCF-7) cancer cell line.

**Tumor suppressor protein (p53)**

Figure 4 shows that MCF-7 showed dose-dependent effects up to 10 ng/ml HA-PTX with a 2–3-fold increase of p53.

![Figure 1: Morphology of breast cancer cell line (MCF-7) for (a) control; (b and c) paclitaxel and hyaluronic acid-paclitaxel treated at 1 ng/ml; (d and e) paclitaxel and hyaluronic acid-paclitaxel treated at 100 ng/ml; colorectal cancer cell line (HT-29) for (f) control; (g and h) paclitaxel and hyaluronic acid-paclitaxel treated at 1 ng/ml; (i and j) paclitaxel and hyaluronic acid-paclitaxel treated at 100 ng/ml after 24 h](image)

![Figure 2: Flow cytometric analysis of breast cancer cell line (MCF-7) for (a) control; (b and c) paclitaxel and hyaluronic acid-paclitaxel treated at 1 ng/ml; (d and e) paclitaxel and hyaluronic acid-paclitaxel treated at 100 ng/ml; colorectal cancer cell line (HT-29) for (f) control; (g and h) paclitaxel and hyaluronic acid-paclitaxel treated at 1 ng/ml; (i and j) paclitaxel and hyaluronic acid-paclitaxel treated at 100 ng/ml after 24 h](image)
level as compared to control. The better p53 responses at low concentrations of PTX and HA-PTX in MCF-7 were consistent with other reports. In HT-29 cells, elevated basal levels of p53 protein and no effect on p53 level with further HA-PTX treatment. However, this may suggest the presence of a p53 mutant, as the majority of colorectal cancer cell lines are p53 mutated, amino acids 273 Arginine mutates into Histidine results in dysregulation in cell-cycle checkpoints. In HT-29 cells, elevated basal levels of p53 protein and no effect on p53 level with further HA-PTX treatment. However, few studies show that inhibitor along with PTX may be produced antagonistic effects. Interestingly, the acquired up-regulation was also negligible after treated with HA-PTX. Similarly, Rho GTPases over-expressed in various malignancies. In our studies, the Rho A activity not found to be down-regulated after treatment with HA-PTX. However, we can propose that in our future studies that by using HA-PTX along with inhibitors might give positive outcomes. As previous studies show that Y27632 or by the silencing of RhoA against RhoA possibility increase the sensitivity in resistant cells. Taxol might not activate Rho A activator protein GEF-H1. HA-PTX also does not affect RhoA-dependent changes such as stress fiber formation, which explains the nonactivation of AKT and Rho A levels in our study. HA-PTX causes cell growth arrest might be via inhibition in depolymerization dynamic changes involve in cell growth and division.

AKT and Rho A
Both HA-PTX and PTX showed nonsignificant upregulation of AKT [Figures 5 and 6] activation at 1–100 ng/ml in A549, but lower activation (<0.4) observed in HT-29. The HA-PTX did not trigger the activation as well as lack of suppression of AKT and Rho A protein levels.

DISCUSSION
In our studies, HA-PTX inhibits cell growth via cell arrest and induction of apoptosis without up-regulating the survival proteins such as AKT and RhoA. The increase in ADP/ATP ratio suggested the induction of growth arrest as ATP molecules aided in the process of growth and cell division. Our results supported by previous studies that taxol can decrease fructose 1,6-bisphosphate which, reduce the facility of ATP molecules, mandatory for

**Figure 3:** Adenosine diphosphate/adenosine triphosphate ratio in breast and colorectal cancer cell lines treatment at 1 and 100 ng/ml treatment after 24 h

**Figure 4:** p53 activation in breast and colorectal cancer cell lines after paclitaxel and hyaluronic acid-paclitaxel treatment at 1 and 100 ng/ml treatment after 24 h

**Figure 5:** AKT activation in breast and colorectal cancer cell lines treatment at 1 and 100 ng/ml treatment after 24 h

**Figure 6:** Rho A activation in breast and colorectal cancer cell lines after paclitaxel and hyaluronic acid-paclitaxel treatment at 1 and 100 ng/ml treatment after 24 h

AKT is over-expressed in many human tumors. The lack of suppression in AKT was found in our studies. However, we could suggest that in future studies, various AKT inhibitors along PTX-HA could increase drugs sensitivity towards cancer cells. However, few studies show that inhibitor along with PTX may be produced antagonistic effects. Interestingly, the acquired up-regulation was also negligible after treated with HA-PTX. Similarly, Rho GTPases over-expressed in various malignancies. In our studies, the Rho A activity not found to be down-regulated after treatment with HA-PTX. However, we can propose that in our future studies that by using HA-PTX along with inhibitors might give positive outcomes. As previous studies show that Y27632 or by the silencing of RhoA against RhoA possibility increase the sensitivity in resistant cells. Taxol might not activate Rho A activator protein GEF-H1. HA-PTX also does not affect RhoA-dependent changes such as stress fiber formation, which explains the nonactivation of AKT and Rho A levels in our study. HA-PTX causes cell growth arrest might be via inhibition in depolymerization
of the microtubule, and induce apoptosis in MCF-7 and HT-29 cancer cells.

**CONCLUSION**

HA-PTX NPs induced apoptosis with the characteristic feature of apoptosis. The flow cytometry analysis showed a dose-dependent increase in apoptosis in cancer cell lines. The activation level of p53 increased in MCF-7 after treatment with HA-PTX NPs, but no changes observed in HT-29 might be due to colorectal cancer cell mutations. However, HA-PTX did not induce the activation of cancer progression pathway proteins AKT and Rho A in MCF-7 and HT-29. HA-PTX triggered growth arrest and apoptosis as the mode of cell death via p53 activation in MCF-7. Therefore, further in vivo study needs to done to explore the potential of HA-PTX in drug development.

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**Conflicts of interest**

There are no conflicts of interest.

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