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# Involvement of apoptosis in 17-oxo-17a-aza-D-homo-5-androsten-3β-yl phenyl acetate induced toxicity in mouse macrophages

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## ABSTRACT

*The current study was undertaken to investigate the role of apoptosis in 17-oxo-17a-aza-D-homo-5-androsten-3β-yl phenyl acetate induced toxicity. Mouse macrophages were exposed to five different concentrations (0.01, 0.5, 1.0, 2.0 and 5.0 µg/mL). Post-exposure, cell viability was evaluated at 18 and 24h points by DPD–xylene. The data showed time and concentration dependent increase in apoptotic cells at all the tested concentrations. DNA fragmentation was measured 48 h post-exposure and results for apoptosis biomarkers showed the cleavage of DNA into smaller units , further suggests that apoptosis may be a contributing factor in acute 17-oxo-17a-aza-D-homo-5-androsten-3β-yl phenyl acetate induced toxicity.*

**Keywords** :- apoptosis, DNA fragmentation, toxicity, steroids, mouse macrophages

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## INTRODUCTION

Benign prostatic hyperplasia is the nonmalignant enlargement of the prostate gland with increase in numbers of both epithelial and stromal cells with in the periurethal transition zone of the prostate, resulting in the constriction of prostatic urethra **(Fig. 1)** [1]. The prevalence increases to 50% by the age of 60 years and to 90% by the age of 85 years [2].

Abnormal increase in the number of cells in prostate may result not only from increased cell proliferation but also from decreased level in programmed cell death

(apoptosis) [3]. Cells die in response to development signals, and the process is characterized by number of biochemical changes. Any influence between the physiological process of cell proliferation and cell death may lead to change in prostate size with the subsequent development of abnormalities in the gland [4]. So it is reasonable to assume that cytotoxic agents are able to induce apoptosis, cause significant decrease in proliferation rate and are useful for the treatment of disease that involve abnormal or uncontrolled cell proliferation.

Nature treasure has an abundant source of cytotoxic agents obtained from various plant sources like *Paclitaxel [5], Thapsia garganica [6]* and extract of Vitex agnus–Castus fruit [7]. Number of semi-synthetic derivatives like vinblastine [8], doxorubicin A [9], fluoroindolo carbazoles [10], certain derivatives of quinoline [11] have also been reported as therapeutic agents for the treatment of symptomatic BPH. Various synthetic derivatives of suberoylanilide hydroxamic acid [12], 2-arylthiazolidine-4-carboxylic acids [13] etc. has been reported to possess significant cytotoxic property.

Number of 17-oxo-17a-aza-D-homo-5-androsten-3β-yl esters have been synthesized in our lab [14]. Compounds were evaluated for their antiproliferative activity using prostate cancer cell lines DU-145. Previous studies have shown that compound 17-oxo-17a-aza-D-homo-5-androsten-3β-yl phenyl acetate **(Fig. 2A)** posses significant antiproliferative activity at higher concentrations [14], but cytotoxic agents however frequently exhibit-unspecific toxicity. Nevertheless the ability to selectively kill the target cell remains a highly desirable property of potential new therapeutic cytotoxic agents. Thus *in vitro* acute toxicity studies were carried out using mouse macrophages. Post-exposure, cell viability was evaluated at 24h by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction using five different concentrations. The results obtained from MTT assay were statistically significant ($P<0.001$) and acute toxicity of the compound **2A** was found to be comparable to reference drug finasteride **(Fig. 2B)** [14], but it is not known apoptosis is associated with 17-Oxo-17a-aza-D-homo-5-androsten-3β-yl phenyl acetate induced toxicity.

Apoptosis is a common form of programmed cell death in multicellular organisms, which is essential for normal biological processes such as morphogenesis, tissue homeostasis, and the elimination of damaged or virally infected cells, thus play a important role in various pathologic and toxicological process [15, 16]. It is characterized by specific morphologic and biochemical properties [17]. Morphologically, apoptosis is characterized by a series of structural changes in dying cells such as blebbing of the plasma membrane, condensation of the cytoplasm and nucleus, and cellular fragmentation into membrane apoptotic bodies [17, 18]. Biochemical events lead to characteristic degradation of chromatin, and chromosomal DNA fragmentation. [17, 19]
Although apoptosis is important for normal developmental processes, its aberrant regulation may contribute to a number of diseases such as cancer, autoimmune disorders, and viral infections [16]. Apoptosis is induced by various pathological conditions and by a variety of factors such as deprivation of trophic factors [20], heatshock [21] and various cytotoxic substances [22]. Because unscheduled apoptosis may be induced by various toxic chemicals resulting in organ specific pathologies, it is of utmost interest to investigate whether 17-oxo-17a-aza-D-homo-5-androsten-3β-yl phenyl acetate induces apoptosis in mouse macrophages. The present study

investigates the role of apoptosis in 17-Oxo-17a-aza-D-homo-5-androsten-3β-yl phenyl acetate induced acute toxicity.

## MATERIAL AND METHODS

### Chemical and biochemical

All the chemicals were of reagent grade and were used without purification. Phosphate buffer saline (PBS), potassium phosphate monobasic ($KH_2PO_4$), sodium chloride (NaCl), RPMI 1640 medium, fetal bovine serum (FBS), hydrochloric acid (HCl), antibiotic solution, sodium dihydrogen phosphate ($NaH_2PO_4$), disodium hydrogen phosphate ($Na_2HPO_4$), and dimethyl sulfoxide (DMSO) were purchased from Hi Media (Bombay).

### Animals

Albino mice (laca strain) weighing 20-25 g of either sex were procured from Central Animal House, Panjab University, Chandigarh. Animals were housed under standard conditions and allowed to free access to both food and water available *ad. libitium* until used.

### Samples

All steroids were dissolved in ethanol and diluted to appropriate concentration: 0.01, 0.5, 1.0, 2.0, 5.0 μg/mL from the two stock solutions of 1 mg/mL and 0.001μg/mL. Stocks were maintained at room temperature.

### Macrophages isolation and treatment

The apoptotic effect of the title compound **(2A)** was evaluated on mouse peritoneal macrophages as model system. Mouse macrophages were isolated surgically and cells were counted using hemocytometer. Cells were grown in RPMI with 10% fetal bovine serum and penicillin & streptomycin at $37^o$ C with or without test compound dissolved in DMSO. Experiments were conducted in triplicate. Cells were then washed with phosphate buffer, fixedin aceto-methanol and stained with giemsa. The number of apoptotic cells was counted under microscope after mounting the slide with DPD-Xylene.

### DNA fragmentation

DNA fragmentation assay also known as DNA laddering technique which is used to identify DNA cleavage that occurred during programmed cell death. It largely depends on large percentage of cells undergoing apoptosis at the same time. In this assay, cells were harvested for 48h with two different concentrations (10μ g & 5μg /mL) of the title compound and centrifuged at 10,000 rpm. The pellets with $1x10^6$ cells were suspended in DNA lysates buffer and incubated at $55^0$ C overnight. After which equal volume of phenol: chloroform was added and mixture was vortexed and centrifuged to separate out the layers. Following dilution of aqueous layer with equal volume of chloroform and isoamylalcohol, the final treatment was given with 1/ 10 3M sodium acetate and absolute ethanol and stored at control experiment were also performed in two sets without title compound . Finally two treated and two controls were the loaded onto 0.8% agarose gel and then run in tris acetate buffer at 20V until the dye had migrated 4-5cm. DNA was then observed using transilluminator and photographed.

## RESULTS AND DISCUSSION

Earlier experiments of MTT reduction assay were conducted to assess acute toxicity and post exposures results indicated the comparable toxicity to finasteride [14]. Further to study the involvement of apoptosis in **2A** induced toxicity, in present studies, mouse macrophages were exposed to five different concentrations for 18 and 24 h, fixed with aceto : methanol, and stained with giemsa. The assay quantifies the percentage apoptotic cells. The results obtained were statistically significant ($P<0.001$)**. Fig. 3, 4 & 5** demonstrated a direct and proportional relation between % apoptosis and concentration / time.

Qualitative characterization of apoptosis is further represented in **Fig. 6 & 7**. Arrows indicate the apoptotic cell. It could be observed that the control cells (DMSO as control vehicle) are normal in shape and big in size with intact nuclear membrane **(Fig. 6A, 7A).** However, the cells treated with continuous increasing concentrations of **2A** exhibited characteristics of apoptosis including cell size reduction, membrane shrinkage in shape (**Fig. 6B-6F** and **7B-7F**). Further in comparisons to that DMSO control treated cell, the size of the cell are two fold reduced.

DNA fragmentation is one of the hallmarks of the apoptosis. Macrophages were treated with 5 and 10µg/mL of **2A** for 48 h. The results (**Fig. 8**) showed that **2A** has produced marginal DNA fragmentation. Though exact mechanism of DNA fragmentation need to be elucidated, but this may be because of the activation of signal protein known as caspases. The caspases are a family of proteins that are one of the main executors of the apoptotic process. They belong to a group of enzymes known as cysteine proteases and exist within the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis [23]. These proteins breakdown or cleave key cellular substrates that are required for normal cellular function including structural proteins in the cytoskeleton and activate degradative enzyme DNases. The result of these biochemical changes is appearance of morphological changes in the cell.

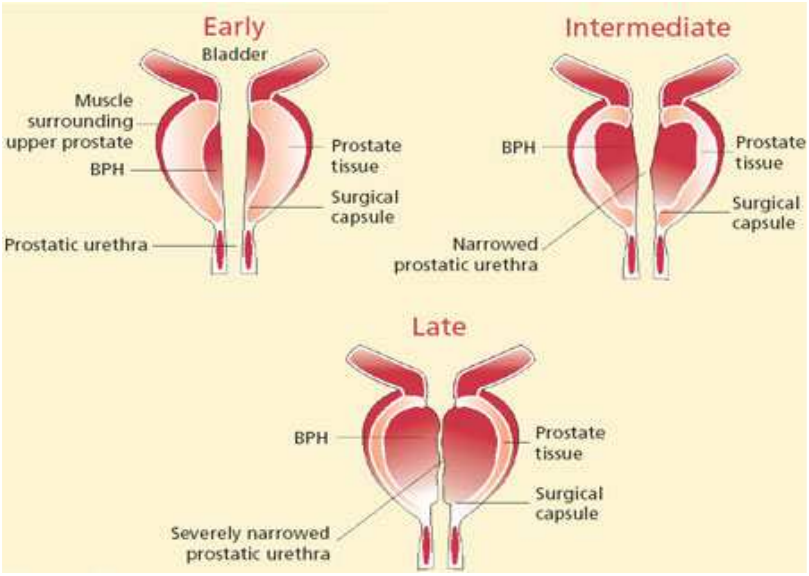


**Figure-1 Stages of benign prostatic hyperplasia**

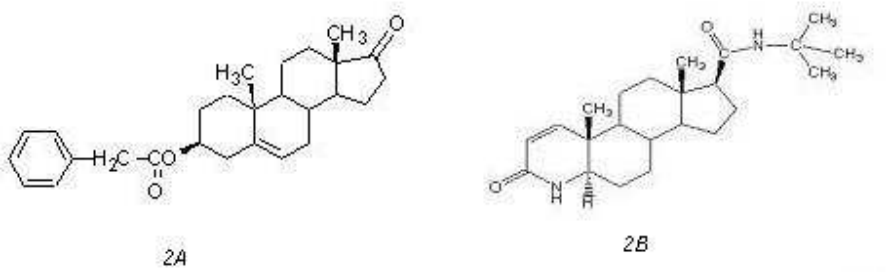


**Figure 2 Structures of (2A) - 17-Oxo-17a-aza-D-homo-5-androsten-3β-yl phenylacetate; (2B) –Finasteride**

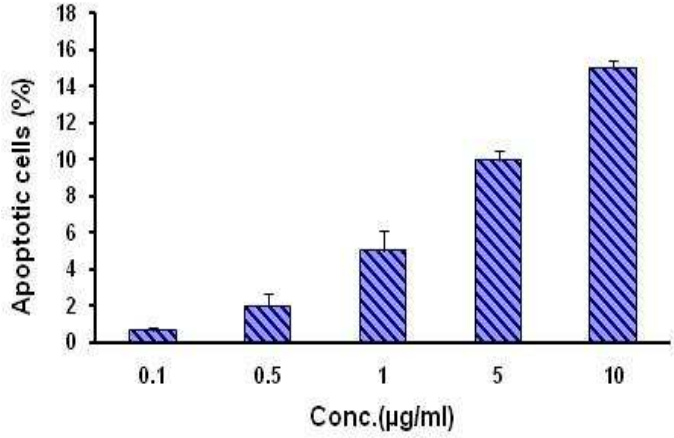


**Figure 3 Dose-response relationship with regard to toxicity of the compound (2A) on the number of apoptotic cells, after 18h treatment. Each point represents a mean±SEM of 5 independent experiments (each in triplicate).**

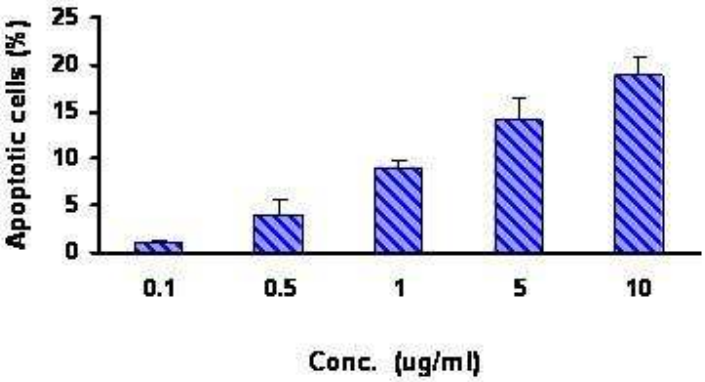


**Figure 4 Dose-response relationship with regard to toxicity of the compound (2A) on the number of apoptotic cells, after 24h treatment. Each point represents a mean±SEM of 5 independent experiments (each in triplicate).**

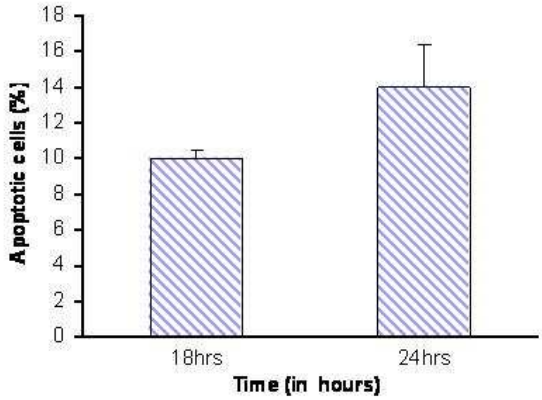


**Figure 5 Time dependent response towards apoptosis after 17-Oxo-17a-aza-D-homo-5-androsten-3β-yl phenylacetate (2A) treatment on macrophages**

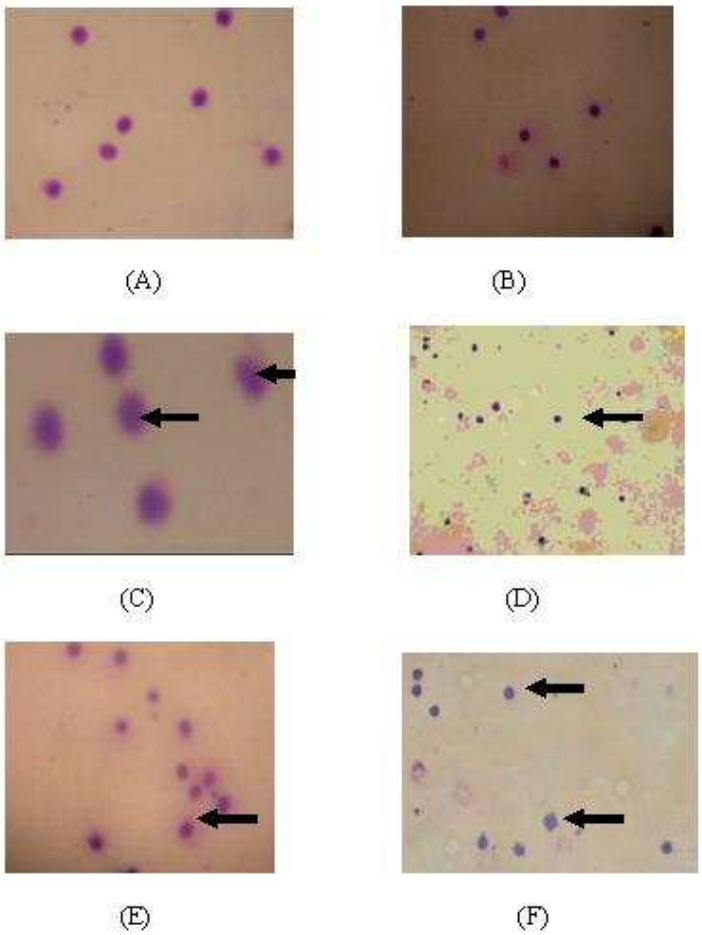


**Figure 6 Dose dependent response towards programmed cell death: macrophages treated with (2A) for 18 h, fixed with aceto: methanol, stained with giemsa, number of apoptotic cells were counted. Arrow in the fig showing apoptotic cell. A) Control B) 0.1µg/mL treated C) 0.5µg/mL treated D) 1µg/mL treated E) 5µg/mL treated F) treated 10µg/mL**

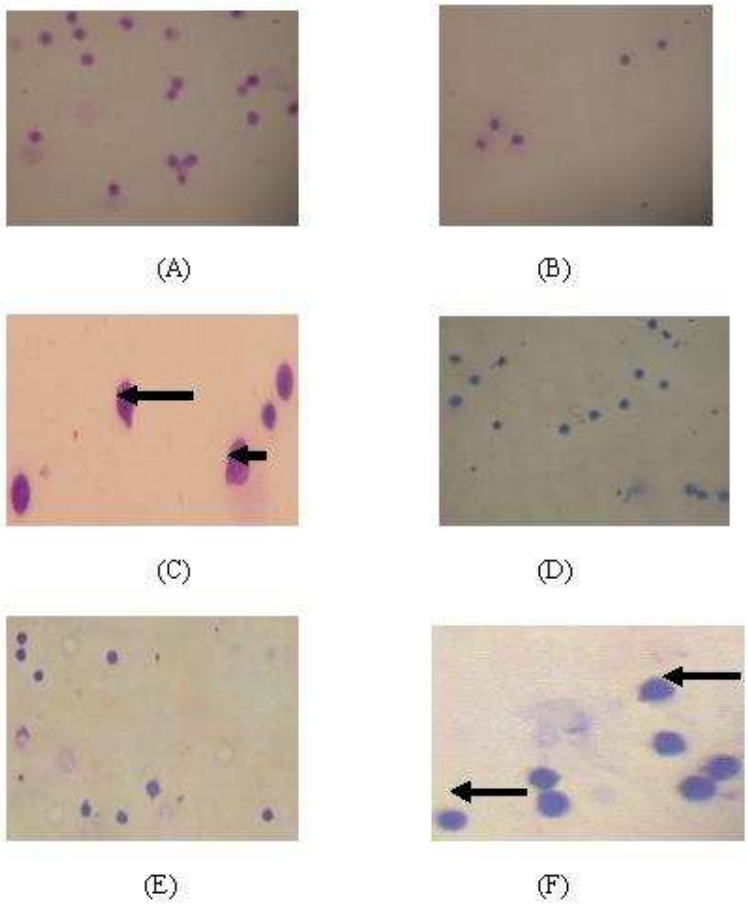


**Figure 7 Dose dependent response towards programmed cell death: macrophages cells treated with (2A) for 24 h, fixed with aceto: methanol, stained with giemsa, number of apoptotic cells were counted. Arrow in the fig showing apoptotic cell. A) Control B) 0.1µg/mL treated C) 0.5µg/mL treated D) 1µg/mL treated E) 5µg/mL treated F) treated 10µg/mL**

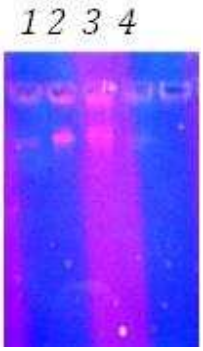


**Figure 8 Effect of 2A on DNA fragmentation in macrophages. Lanes: 1. Control; 2. treated 5µg/mL; 3. treated 10µg/mL; 4. Control**

## CONCLUSION

Compound **2A** belong to 17-oxo-17a-aza-D-homo-5-androsten-3β-yl esters series. Our earlier reported results clearly indicated the efficacy (antiproliferative activity) and toxicity profile of complete group [14] . Present study was designed to investigate the involvement of apoptosis in 17-oxo-17a-aza-D-homo-5-androsten-3β-yl phenyl acetate **2A** induced toxicity. Reduction in cell size and shrinkage in shape are the cellular indications of apoptosis. Marker of apoptosis (DNA fragmentation) through activation of caspases and other degradative enzymes, suggests that apoptosis may be involved in toxicity. Further, results from present studies i.e. the involvement of apoptosis in 2A induced toxicity could be utilized to predict the activity (antiproliferative ) against cancerous cell in future .

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