**STUDY-3.1**

*Inhibition of hyperplasia formation by 2- [piperidinoethoxyphenyl]-3-[4-hydroxyphenyl]-2H-benzo(b)pyran in rat uterus*
Introduction

Estrogen hormones induce proliferative disorders and changes in the structure of tissues in the uterus resulting into the formation of hyperplasia (Martin et al., 1973; Gunin et al., 2001). The culmination of these estrogen-induced changes in proliferation and morphogenesis leads to atypical hyperplasia and subsequently the formation of cancer in the endometrium (Emons et al., 2000; Akhmedkhanov et al., 2001). Progestagens have been used widely in the treatment of endometrial hyperplasias, especially of the simple forms, with satisfactory results (Affinito et al., 1994; Saarikoski et al., 1990; Figueroa-Casas et al., 2001). Unfortunately, progestin treatment leads to depletion of progesterone receptor within the target tissue and thus causing response failure in adjuvant settings as is evident from the studies carried out in nude mice (Satyaswaroop et al., 1992) and in humans (Janne et al., 1979).

Estrogen has a variety of effects in the formation of endometrial hyperplasia including stimulation of cell proliferation, enhanced Akt activity which is a survival factor with an anti-apoptotic activity, and it also affects cell cycle by regulating the stability of cyclins (Simoncini et al., 2000; Bouskine et al., 2008; Zhang et al., 2009). Estrogens have been found to act as mitogens (Taylor et al., 2002), therefore, a logical approach to the treatment of estrogen-related hyperplasic endometrial growth is the use of anti-estrogens, which are thought to antagonize the action of estrogen by direct competition to estrogen receptor (ER) sites (Rochefort et al., 1983; MacGregor and Jordan, 1998). Different classes of synthetic compounds have been developed so far which are capable of antagonizing ER action. Among these, benzopyrans are the class of potent anti-estrogens and have high oral bio-availability (Dhar et al., 1991). These compounds show high affinity for ER in uterine cytosol and have no estrogen agonistic activity in human breast cancer models studied in vitro and in vivo (Gauthier et al., 1997; Couillard et al., 1998, Carlos and Antonio 2000). In a quest to design nonsteroidal pure antiestrogens, benzopyran derivatives synthesized at Central Drug Research Institute (CDRI), India, display significant anti-estrogenic activity and inhibit uterine growth (Sharma et al., 1990; Kharkwal et al., 2011). However, their
effects and therapeutic potential on endometrial disorders have not been explored as yet.

The potent antiestrogenic profile of 2-[piperidinoethoxyphenyl]-3-[4-hydroxyphenyl]-2H- benzo(b) pyran (K-1) encouraged us to evaluate its antiproliferative effects on estrogen-induced hyperplasia in rat uterus. The present study was, therefore, undertaken to determine the anti-proliferative action of compound in endometrial hyperplasia by studying the modulation of genes which are involved in cellular proliferation and survival.

Materials and methods
Compound

2-[piperidinoethoxyphenyl]-3-[4-hydroxyphenyl]-2H-benzo(b) pyran (K-1) was synthesized according to the methods as described earlier (Sharma et al., 1990; Kapil et al., 1990; Hajela et al., 1999)

Animal preparation and treatment schedule

Young adult rats (Sprague Dawley strain) of body weight of 150g of the Institute colony were used in this study. Animals were housed under uniform animal husbandry conditions (24±1°C) with free access to pelleted food and water.

All animal procedures were carried out as per guidelines provided by Institute’s Animal Ethics, Use and Care Committee. Prior approval was obtained from the Institutional Animal Ethics Committee, for animal experimentation.

Rats were ovariectomized (Zarrow, 1964) bilaterally under ether anesthesia and given rest period of 2 weeks. Following the rest period, rats were divided into various groups (6 to 8 rats per group): Group I received olive oil and gum acacia as vehicle, Group II received estradiol (20 µg/kg body weight, in olive oil, subcutaneously), Groups III and IV received in addition to estradiol, K-1 at 100 µg and at 200 µg/kg body weight doses, respectively (in gum acacia, orally). Groups V and VI received only K-1 at 100 µg/kg body weight and 200 µg /kg body weight, respectively. All treatments were given for 14 days. Animals were sacrificed 24 h after the last treatment.

Uteri were collected, weighed and stored at -80°C until analysis. For RNA isolation, tissue was collected in RNA later at room temperature for 24 h and then stored at -80°C. A mid portion of single horn of each uteri were preserved in 4% paraformaldehyde for histological and histomorphometric analysis.

Histomorphometric analysis

Formalin-fixed uterine tissues were sectioned, stained with hematoxylin/eosin and examined under light microscope (Nikon 80i, Japan). Images were captured with NIS-
Elements F 3.0 camera (Nikon, Japan) and analysed using Leica QWin software (Innocente et al., 2009; Lortlar et al., 2010). Endometrial area (EA), luminal area (LA), luminal epithelial cell height (LEH), gland number, glandular area (GA), stromal area and glandular/stromal area ratio (G/S) as an indicator for cellular hypertrophy, was calculated by averaging the measurements at three locations in three different hematoxylin and eosin--stained uterine tissue sections for each individual animal.

Western blot analysis

Uterine tissue was homogenized in an ice-cold phosphate buffer (50mM) containing 10mM sodium molybdate, 50mM sodium fluoride, 1mM EDTA, 400 mM sodium chloride, 12mM monothioglycerol, 2mM PMSF, leupeptin (2µg/ml) and protease inhibitor cocktail (50µg/g tissue) (Sigma Aldrich, USA) using ultraturrax homogenizer. The homogenate was incubated on ice for 1 h with occasional shaking and centrifuged at 16,000×g for 15 min at 4°C. Protein concentration in supernatant was determined by Bradford assay (Bradford, 1976). Sample containing 35µg protein was boiled for 10 min in denaturing sample buffer consisting of 10% glycerol, 1% SDS, 1% β-mercaptoethanol, 10 mM Tris-HCl (pH 6.8) and 0.01% bromophenol blue, separated on 12% acrylamide gels and transferred to Immuno-Blot™ PVDF membrane (Millipore). Non-specific sites were blocked with 5% skimmed milk for 2 h at room temperature and then incubated overnight at 4°C with primary antibody at 1:5,000 dilution for ER, PR, PCNA and cyclinD1 (Santa Cruz) or at 1:1,000 dilution for cleaved caspase-3, cleaved caspase-9, cleaved PARP, S437p-Akt, Akt, XIAP, c-Fos, c-Jun, c-erbB2, c-myc, β-catenin, Bax and Bcl-2 (Cell Signaling Technology). Subsequently, the blots were washed three times in 0.1% Tween-20 in tris-buffered saline (TBS) and then incubated with 1:10,000 dilution of secondary antibody (HRP conjugate) for 1h at 25°C. After extensive washing with 0.1% Tween-20 in TBS, substrate solution was added to the membrane, incubated for 5-15 sec and exposed at room temperature. The membranes were developed with enhanced chemiluminescence kit, following the manufacturer’s (GE Healthcare, USA) instructions. For normalization, the membranes were stripped using buffer containing
62.5 mM Tris, pH 6.8, 2% SDS, 100 mM β- mercaptoethanol and then reprobed with anti-β actin antibody (Santa Cruz). Quantitation of band intensity was performed by using Quantity One software (v. 4.5.1) (Bio-Rad).

**Real-time polymerase chain reaction**

Total RNA from the uterine tissue was extracted using the Tri-reagent by following the manufacturer’s instructions. cDNA was synthesized from 5µg of total RNA using first strand cDNA synthesis kit. The quantification of the selected genes by real time - PCR was performed with a LightCycler (Roche). The nucleotide sequences of the primers used have been given in table 3.1.1. Expressions of the investigated genes were compared to the steady expression of β-actin. The PCR system was programmed according to the manufacturer’s instructions. The experiments were repeated three times.

**Table: 3.1.1.** Sense & antisense primers for amplification of targeted messages with real-time PCR

<table>
<thead>
<tr>
<th>mRNA target</th>
<th>Sense (5') primers</th>
<th>Antisense (3') primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>5’ caaccagtggctggagt 3’</td>
<td>5’ catgggactcagatgtaatgactg 3’</td>
</tr>
<tr>
<td>PR</td>
<td>5’ ggccagctgccatgtaagttcc 3’</td>
<td>5’ ggtcatcgatgtgtaatgactg 3’</td>
</tr>
<tr>
<td>Bax</td>
<td>5’ ggtgagcggctgcttgtct 3’</td>
<td>5’ ggggatctgggtcaatgtaatgactg 3’</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>5’ tgtgagccctcctctcct 3’</td>
<td>5’ ggggatctgggtcaatgtaatgactg 3’</td>
</tr>
<tr>
<td>β-actin</td>
<td>5’ cccgctgctgcccaaccttc 3’</td>
<td>5’ cgtcatccatggcgaact 3’</td>
</tr>
</tbody>
</table>

**Terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL)**

To demonstrate the apoptosis in tissue cells, we carried out TUNEL assay by using In Situ Cell Death Detection Kit (Roche). Paraffinized uterine sections were taken for TUNEL assay. Sections were de-paraffinized by xylene (3 x 3’) and rehydrated by 100% ethanol (3 x 2’), 95%, 80%, 70% ethanol (1 x 2’each), 1x PBS (1 x 5’) followed by antigen retrieval in microwave oven (4 x 5’) (600 ml of 10mM sodium
citrate, pH 6) and cooled for 20 min then washed with milli Q water (3 x 5’) and 1x PBS (1 x 5’). For positive control, one section was incubated with DNase-I for 15 min. For negative control, enzyme was omitted in reaction buffer. After 60 min incubation at 37°C, slides were soaked in 1x SSC buffer (150 mM sodium chloride and 15 mM sodium citrate) for 15 min at room temperature to stop reaction. Finally, the slides were washed in 1x PBS (5 x 5’), mounted in 3:1 Vectashield : DAPI and sealed. Sections were examined under light microscope (Nikon 80i, Japan) and images were captured at 40X with the NIS-Elements F 3.0 camera (Nikon, Japan).

**Caspase-3 colorimetric assay**

One of the major steps in apoptotic cell death is the activation of caspases. Caspase-3 activity was measured using colorimetric Caspase-3 assay kit (Sigma- Aldrich) according to the manufacturer’s instructions. Briefly, whole uterine extract (~50 μg protein) was incubated for 2 h at 37 °C in the presence of 1 mM caspase-3 substrate (DEVD-pNA), and the O.D. was measured at 405 nm and activity was expressed as fold changes.

**Statistical Analysis**

Results are expressed as mean ± SEM for the number of experiments indicated. Statistical analysis was performed using Analysis of variance (ANOVA) followed by Newman Keul’s test. Differences were considered significant at p<0.05.

**Results**

**Effect of K-1 on uterine mass of overectomized rats**

The wet uterine mass of ovariectomized rats receiving estradiol showed significant increase (4.8 fold, p<0.001) as compared to control group. When K-1 was administered in rats receiving estradiol, a significant decrease in uterine weight was
observed (p<0.001). In rats receiving higher concentration i.e. 200 µg/kg of K-1, the uterine weight was comparable to that of control group (p>0.05) (Fig.3.1.1).

**Fig. 3.1.1.** Effect of K-1 treatment on uterine growth and proliferation marker proteins in rat. (A) Data represents the wet uterine weight of control, estradiol (E2) and E2+ compound treated groups. The results are represented as the mean ± SEM, of 3 independent experiments (6-8 rats per group in each experiment).

**Uterine histology and histomorphometry**

The haematoxylin-eosin -stained uterine sections and the histomorphometric analysis are represented in Fig 3.1.2 (A&B). The effects of estradiol on uterine histologic parameters showed extensive proliferation of endometrial epithelium (Fig 3.1.2A). As compared to control, in estradiol- treated rats, uterine sections showed an increase of nearly 5-fold in luminal epithelial cell height, 2.4-fold in endometrial area, 2.9-fold in luminal area, 4.6-fold in glandular area and 2.3-fold in the ratio of glandular vs. stromal area (Fig 3.1.2B). All these changes were statistically significant (p<0.05 to <0.001) and were indicative of the development of uterine hyperplasic conditions. But when compound K-1 was given in rats receiving estradiol, a significant decrease in each of these parameters was observed in comparison to estradiol-treated group (p<0.05 to 0.001). The effect was highly significant at 200 µg/kg dose.
Fig. 3.1.2 (A & B) Effect of K-1 compound on endometrial hyperplasia formation in rat uterus. Ovariectomized rats were treated with E\textsubscript{2} (20μg/ kg) or estradiol (E\textsubscript{2}) +compound K-1 at 100μg/kg or 200 μg/kg, for 14 days. (A) Cross sectional view of rat uteri showing histological changes at 4X (a-d) and 20X (e-h) magnification, obtained from control (a, e), estradiol (E\textsubscript{2}) (b, f) or E\textsubscript{2}+ compound K-1-100μg/kg (c, g) or E\textsubscript{2}+ compound K-1-200μg/kg (d, h) treated rats. (B) Histomorphometric analysis of formalin-fixed paraffin-embedded rat uterine sections of various groups. Values are expressed as mean ± SEM, n = 6. p values are a-p<0.001, b-p<0.01, c-p<0.05 and d-p>.05 vs. control and e-p<0.001, f-p<0.01, g-p<0.05 and h-p>.05 vs. estradiol.

Effect on expression of proliferation markers PCNA and cyclinD1
For analysis of proliferation markers, immunoblotting of PCNA and cyclinD1 was performed in uterine tissue. An increase of >1.8-fold was observed in estradiol-treated group in comparison to control, in both proteins (p<0.01 to 0.001). Whereas in K-1-treated group, significant reduction in PCNA (~55%) as well as in cyclinD1 (~65%) was observed in comparison to estradiol-treated group (p<0.001) (Fig.3.1.3 A & B).

**Fig 3.1.3 (A& B)** PCNA and cyclinD1 expression by Western blot analysis. The protein lysates from control, estradiol (E2) and K-1-treated groups were prepared and subjected to Western Blotting with anti-PCNA and anti-cyclinD1, as described in "Materials and Methods". Equal amounts of protein lysate (35μg) were analyzed in each lane. β-actin was used as an internal control to correct for loading. Densitometric quantitation of protein expression levels is shown as fold changes. Results are expressed as mean ± SEM, n = 3, p values are a-p<0.001, b-p<0.01, c-p<0.05 and d-p~.05 vs. control and e-p<0.001, f-p<0.01 vs. estradiol.

**Expression of ERE & AP-1 regulated proteins & proto-oncogenes in rat uterus**

To study the effect of compound K-1 on ERE (estrogen response element)- and AP-1 regulated genes, immunoblotting of ER, PR, c-Jun and c-Fos was performed in uterine tissue (Fig. 3.1.4 A). Down regulation of ER (p<0.001) whereas up-regulation
of PR, c-Fos and c-Jun (p<0.01) in estradiol-treated group was observed in comparison to control group. In K-1-treated group, the expression of ER, PR, c-Jun and c-Fos was significantly down regulated at 200 ug/kg dose in comparison to estradiol-treated group (Fig. 3.1.4 B).
The inhibitory effect of 2,3-diaryl-2h-1-benzopyran derivative on estrogen-induced uterine hyperplasia and endometrial cancer.

**Fig. 3.1.4.** (A & B) The expression level of ER, PR, c-Fos and c-Jun as determined by western blotting. A representative immunoblot from three independent experiments is shown. Equal amounts of protein lysate (35 μg) were analyzed in each lane. β-actin was used as a internal control to correct for loading. Densitometric quantitation of protein expression levels are shown as fold changes. Data are expressed as mean ± SEM, n = 3. p values are a-p<0.001, b-p<0.01, c-p<0.05 and d-p>.05 vs. Control and e-p<0.001, f-p<0.01, g-p<0.05 and h-p>.05 vs. estradiol.

**The quantitative mRNA expression of ER and PR**

The quantitative mRNA expression analysis clearly indicated the up-regulation of ER and PR (p<0.001) genes in estradiol-treated group in comparison to control group and was down-regulated (p<0.001) in K-1 treated group in dose-dependent manner (Fig. 3.1.5).

![mRNA expression graph](image)

**Fig. 3.1.5** Changes in the expression of ER and PR mRNA in control, estradiol and compound treated groups determined by Real Time-PCR analysis. Results are expressed as mean ± SEM, n = 3. p values are a-p<0.01, b-p>.05 vs. control and c-p<0.001, d-p<0.05 vs estradiol.
Western blot analysis of proto-oncogenes

Western blot analysis of proto-oncogenes such as β-catenin, c-erbB2 and c-myc was analysed to see the effect of compound K-1 on rat uterus (Fig 3.1.6 A). Results revealed that K-1 caused the significant inhibition of estadiol-induced expression of β-catenin (p<0.001), erbB2 (p<0.05) and c-myc (p<0.001) as compared to uteri of estradiol-treated animals (Fig. 3.1.6 B).

**Fig. 3.1.6 (A & B).** The expression level of β-catenin, c-erbB2 and c-myc as determined by western blotting. A representative immunoblot from three independent experiments is shown. Equal amounts of protein lysate (35 μg) were analyzed in each lane. β-actin was used as a internal control to correct for loading. B. Densitometric quantitation of protein expression levels are shown as fold changes. Data are
expressed as mean ± SEM, n = 3. p values are a-p<0.001, b-p<0.01, c-p<0.05 and d-p>.05 vs. Control and e-p<0.001, f-p<0.01, g-p<0.05 and h-p>.05 vs. estradiol.

**Apoptosis inducing activity in uterine hyperplasia**

The results of TUNEL staining performed on rat uterine sections of different groups are represented in Fig.3.1.7 A. Least number of positively stained cells were observed in control group whereas K-1-treated group showed significant number of TUNEL-positive cells in a dose-dependent manner. The TUNEL-positive cells clearly indicated the apoptotic potential of K-1.

Further to confirm apoptosis, caspase-3 activity was measured using colorimetric assay (Fig 3.1.7 B). Caspase-3 activity was significantly decreased in estradiol-treated group whereas the activity was significantly increased (p<0.001) in compound K-1 treated rat uteri. In the presence of caspase inhibitor, no significant activity of caspase -3 was observed.

![Image](image_url)  
**Fig. 3.1.7 A.** Detection of K-1 induced apoptosis in rat uteri. (A) Figure represents the apoptotic cells of formalin-fixed paraffin-embedded rat uterine sections, detected by TUNEL staining. TUNEL-positive cells were stained greenish yellow. The images were captured at 40x.
Caspase-3 proteolytic activity in uterine tissue measured by colorimetric Caspase-3 assay kit. Protein lysates were prepared from uterine tissue of control and treated groups. Proteolytic activity was measured by cleavage of the caspase-3 substrate DEVD-pNA as described in "Materials and Methods".

**Akt survival pathway in uterine hyperplasia**

Compound K-1 decreased the intracellular levels of pAkt, dose-dependently indicating that compound K-1 interfered with Akt phosphorylation in uterine hyperplasia (Fig. 3.1.8 A). The densitometric analysis of immunoblots showed significant increase in pAkt (p<0.01) as well as in XIAP expression (p<0.001) in estradiol-treated group whereas its down regulation was observed with compound K-1 in a dose-dependent manner (p<0.001 at 200 µg dose) (Fig. 3.1.8 B).
The expression level of phosphorylated Akt and XIAP proteins were determined by western blotting. A representative immunoblot from three independent experiments is shown. Equal amounts of protein lysate (35 μg) were analyzed in each lane. β-actin was used as an internal control to correct for loading. B. Densitometric quantitation of protein expression levels are shown as fold changes. Data are expressed as mean ± SEM, n = 3. p values are a-p<0.001, b-p<0.01, c-p<0.05 and d-p>.05 vs. Control and e-p<0.001, f-p<0.01, and g-p>.05 vs. estradiol.

Cleavage of caspase-9, -3, and PARP

To analyze the role of different caspases in K-1 induced cell death, processing of important caspases like caspase-9, -3, and -8 were analyzed by immunoblotting (Fig. 6A). Estradiol decreased the expression of active-form of caspase -9, -3 and cleaved PARP by ~45%, ~50% and ~40% respectively (p<0.01) whereas compound K-1 significantly increased the expression of cleaved caspase-9, -3 and cleaved PARP by 3.6-fold, 3.4-fold and 1.9-fold at 200μg/kg (p<0.001) after 14 days of treatment (Fig. 6 B). The expression of cleaved caspase-8 could not be obtained (data not
These results indicate that the apoptotic-signaling pathway activated by compound K-1 is likely to be mediated via the mitochondrial (intrinsic) pathway.

**Fig.3.1.9 (A & B).** Effect of compound K-1 on apoptotic markers (A) cleaved caspase-9, -3 and PARP and A representative immunoblot from three independent experiments is shown. Equal amounts of protein (35 μg) were analyzed in each lane. β-actin was used as a control to correct for loading. B. Densitometric quantitation of protein expression levels are shown as fold changes. Results are expressed as mean ± SEM, n = 3. p values are a-p<0.001, b-p<0.01, c-p<0.05 and d-p>.05 vs. control and e-p<0.001, f-p<0.01, g-p >0.05 vs. estradiol.

**Bax/Bcl-2 ratio**

We next investigated the expression levels of pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins. The expression level of Bax was found to be decreased by 50% in E2-treated rat uteri as compared to control group (p<0.001). However, it was increased by
1.7- and 2.6-fold in K-1-treated groups at 100 µg and 200 µg/kg dose respectively in comparison to estradiol-treated group (p<0.001) (Fig. 3.1.10 A). The expression level of Bcl-2 protein was increased by 1.6-fold in uteri of estradiol-treated group which was found to be decreased by 40% and 60% at 100 µg and 200 µg/kg doses, respectively, in K-1 treated rats (p<0.01 to <0.001) (Fig. 3.1.10 B).

**Fig.3.1.10(A & B).** Effect of compound K-1 on apoptotic Bax and Bcl-2 proteins expression as determined by western blot analysis. A representative immunoblot from three independent experiments is shown. Equal amounts of protein (35 µg) were analyzed in each lane. β-actin was used as a control to correct for loading. B. Densitometric quantitation of protein expression levels are shown as fold changes. Results are expressed as mean ± SEM, n = 3. p values are a-p<0.001, b-p<0.01, c-p<0.05 and d-p>.05 vs. control and e-p<0.001, f-p<0.01, g-p >0.05 vs. estradiol.

At mRNA level, the ratio of Bax/Bcl-2 was found to be increased by 2.6- and 5.3-fold in K-1 treated rats at 100 µg and 200 µg/kg doses respectively (Fig. 3.1.11).
Fig.3.1.11 Changes in the ratio of bax/ Bcl-2 mRNA in control, estradiol and compound treated groups determined by Real Time-PCR analysis. Results are expressed as mean ± SEM, n = 3. p values are a-p<0.01, b-p>0.05 vs. control and c-p<0.001, d-p<0.05 vs estradiol.

**Effect of K-1 treatment in ovariectomized rat uterus**

Results revealed that in ovx rats, K-1 did not induce uterine growth (Fig.3.1.12 A) and the expression of proliferation marker proteins i.e. ERα and PR and PCNA, as compared to that of control rats, at both the doses studied (p>0.05) (Fig.3.1.12 B & C). The expression of cleaved PARP, a marker for apoptosis also did not change (p>0.05) under the influence of K-1. These results indicated the lack of inherent estrogenecity in K-1.
Discussion

Endometrial hyperplasia is an overgrowth or thickening of endometrium which may involve part or all of the endometrium. Hyperplasia usually develops in the presence of continuous estrogen stimulation unopposed by progesterone (Epplein et al., 2008) and is characterized by increased gland to stroma ratio. Severe hyperplasia, called carcinoma in situ, is the earliest detectable stage of the endometrial cancer (Horn et
al., 2004; Sorosky, 2008; Chen et al., 2009). Our studies in rat uteri demonstrated that a physiological dose of E₂ given for two weeks causes a rapid proliferation of the endometrium akin to hyperplasia while benzopyran compound K-1 antagonized the proliferative and hyperplastic effects of estradiol. In estradiol-treated groups, uterine hematoxyline/eosin staining clearly showed the histological changes e.g. increased EA, LA, LEH, GA, and G/S ratio in comparison to control group. Interestingly, all these changes were reversed when K-1 was administered along with E₂. These E₂-antagonizing effects appear to be mediated via inhibition of ERα-ERE/AP-1 mediated transcriptional activation (Kharkwal et al., 2011) thereby leading to suppression of E₂-induced proliferation marker genes e.g. PR, PCNA and cyclinD1. In addition, the E-induced expression of proto-oncogenes (Weisz and Bresciani, 1988; Gunin et al., 2004; Maia et al., 2002) was suppressed under the influence of K-1 thereby suppressing the hyperplastic growth of uterus.

In classical pathway, ER binds to their specific ligands, dimerizes and then interacts with ERE located in the promoter region of estrogen responsive gene (Nilsson et al., 2001; Björnstrom and Sjoberg 2005). The present study as well as our earlier study have shown that benzopyran compound K-1 efficiently antagonizes ERE-mediated estrogen action (Kharkwal et al., 2011). Apart from this, ERs also act through alternative pathway where receptors interact with DNA indirectly through protein-protein contact via AP-1 transcription factors (Kushner et al., 2003). AP-1 is an important transcription factor that governs the expression of genes involved in intercellular communication, amplification and primary pathogenic signals spreading as well as initiation and acceleration of tumorigenesis (Shaulian and Karin, 2002). We found that treatment with K-1 decreased the expression of AP-1 transcription factors viz. c-fos, c-jun in estradiol-induced conditions. The compound decreased the expression of AP-1 target genes, such as cyclinD1, PCNA and c-myc which are associated with cell cycle and tumor induction respectively.

Estrogen also has rapid, nongenomic effects in endometrium. It activates several membranous or cytoplasmic kinase cascades, including the phosphatidylinositol 3-phosphate (PI3K/Akt) cascade, a signaling pathway that plays a key role in cell survival and apoptosis (Kayisli et al., 2004; Gielen et al., 2007; Zhang et al., 2009).
We demonstrated that benzopyran compound K-1 reduced the expression of phosphorylated Akt which may be the upstream event responsible for subsequent reduction of AP-1 mediated transactivation.

Akt phosphorylates XIAP (X-linked inhibitor of apoptosis protein), leading to the inhibition of ubiquitination/degradation of XIAP which is considered a potent inhibitor of caspases and apoptosis (Dan et al., 2004). XIAP suppress cell death by inhibiting caspase-3, -7 (Takahashi et al., 1998; Scott et al., 2005) and modulates the Bax/cytochrome c pathway by inhibiting caspase-9 (Nicholson, 1996; Shiozaki et al., 2003). In this regard, a decrease of XIAP levels as shown by our compounds could induce the apoptotic process, at least in part through activation of procaspases (Sasaki et al., 2000). The activated caspase signaling and the presence of cleaved fragments of caspase-9,-3 and cleaved PARP in treated cells demonstrated the involvement of mitochondrial (intrinsic) pathway of apoptosis triggered by compound K-1.

Akt has been shown to phosphorylate and activate the cyclic AMP-response element-binding protein, which increases the transcription of anti-apoptotic genes, such as Bcl-2 (Pugazhenthi et al., 2000). It also inhibits the activation of Bax during apoptosis (Yamaguchi and Wang, 2001; Yuan et al., 2003). Translocation of Bax from the cytosol to the mitochondria results in the release of cytochrome c. Our results demonstrated that the reduced Akt activity may be responsible for significant down-regulation of the expression of anti-apoptotic Bcl-2 and the up-regulation of pro-apoptotic Bax which indicates that the Bax, Bcl-2 and caspase proteases are involved in regulating K-1-induced apoptosis. Collectively, our results suggest that benzopyran compound is a potent apoptosis inducer in uterus and can be used as a therapeutic agent for endometrial hyperplasia.

In conclusion, we have characterized the inhibitory effects of benzopyran compound K-1 on E₂ - induced uterine growth in hyperplastic condition in rat uterus. The compound interfered with Akt activity, reduced ERE- and AP-1-regulated gene expression, and induced apoptosis through caspase-9 mediated intrinsic pathway. Interestingly, compound also lacked inherent estrogenicity as was evident from the uterine weight and the proliferation markers data. Results indicate that benzopyran
derivative K-1 can serve as candidate molecule for use as preventive as well as therapeutic agent for endometrial hyperplasia. However, further studies in primary cells from human endometrial hyperplasia would be required with a view to explore the molecular and cellular mechanism of action of this benzopyran derivative in humans.

**********