Inhibitory effect of 2, 3-diaryl-2H-1-benzopyran derivative on estrogen-induced uterine hyperplasia and endometrial cancer

**STUDY-3.3**

*Anti-tumorigenic action of 2-[piperidinoethoxyphenyl]-3-[4-hydroxyphenyl]-2H-benzo(6) pyran in ishikawa xenograft*
Introduction

Endometrial cancer is the most common neo-plastic disease among women. The majority of the cases are treated successfully with surgery. Despite high cure rates, a significant number of patients have advanced disease or developed metastasis, requiring systematic therapy (Fleming, 2007). Chemotherapy and radiation are options for advanced disease, and hormonal therapy with progestin is another possibility. Because of its minimal side effects, progestin is often used to treat patients with metastatic or recurrent endometrial cancer (Dai et al., 2005). Unfortunately, progestin treatment leads to depletion of progesterone receptor within the target tissue and thus causing response failure in adjuvant settings (Markman et al., 1992; Satyaswaroop et al., 1992; Neijt, 1994). As currently there is no effective therapy available for advanced or recurrent endometrial cancer, continuous efforts are being made towards the development of efficacious therapy for endometrial cancer.

Unopposed estrogen stimulation of the endometrium is the classic etiologic factor associated with the development and growth of endometrial cancer (Modugno et al., 2005). Estrogen exerts most of its transcriptional effects through the “genomic” or “classical” pathway. However, in addition to this well-established genomic pathway, estrogen can exert early physiological effects that are extremely rapid and are mediated via membrane estrogen receptor which has been characterized as G protein-coupled seven trans-membrane receptor, GPR30. Interestingly, cellular activation by GPR30 occurs through a mechanism involving transactivation of epidermal growth factor receptors (EGFRs), activation of mitogen activated protein kinase (MAPK), adenyl cyclase, and phosphoinositide 3-kinase (PI3K) (Filardo et al., 2000; Filardo et al., 2002; Thomas et al., 2005; Du et al., 2012). MAPKs phosphorylate specific serines and threonines of target protein substrates and regulate cellular activities ranging from gene expression, mitosis, movement, metabolism, survival, and programmed cell death. The key MAPK cascade is ERK, also known as classical Ras/Raf/MAPK kinase/MAPK pathway (Roberts & Der, 2007). In the nucleus, ERK1/2 is supposed to phosphorylate and activate some transcription factors, e.g. c-
myc, c-jun, c-fos and cyclinD1. Recently, several reports, have described estrogen/antiestrogen binding and activation properties of the G protein-coupled receptor (GPR)30, which has been proposed as a candidate for triggering a broad range of biological activities initiated at the level of the plasma membrane by the several agents (Maggioni et al., 2004; Revankar et al., 2005; Thomas et al., 2005; Vivacqua et al., 2006(a); Vivacqua et al., 2006 (b); Albanito et al., 2007; Albanito et al., 2008; Vivacqua et al., 2009).

Different classes of synthetic compounds have been developed so far which are capable of antagonizing ER action in reproductive tissues and, in particular, of blocking estradiol-stimulation of cellular growth in uterine tissue (Katzenellenbogen et al., 1995). In a quest to design nonsteroidal pure antiestrogens, benzopyran derivatives synthesized at Central Drug Research Institute, displayed significant anti-estrogenic activity and inhibit uterine growth (Sharma et al., 1990; Kharkwal et al., 2011; Fatima et al., 2012; Chandra et al., 2011). Since these compounds possess anti-estrogenic activity with high oral bio-availability, it was considered of interest to explore their therapeutic potential in endometrial cancer.

Recently, we have reported the apoptosis-inducing activity of three benzopyran derivatives in endometrial carcinoma cells (Kapil et al., 1990). The current study was undertaken to demonstrate and explore the anti-tumorigenic action of one of the above identified compounds from our laboratory viz., 2-[piperidinoethoxyphenyl]-3-[4-hydroxyphenyl]-2H-benzo(b) pyran (K-1), in endometrial cancer cells and in xenograft mouse model. Herein, we also attempted to define its mechanism of action by studying the modulation of non-genomic GPR30/ EGFR/MAPK signaling pathway which regulates the cellular growth and apoptosis in endometrial adenocarcinoma.
Material and methods

Compound

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

Cell culture and reagents

Human endometrial cancer cell line, Ishikawa was purchased from European Collection of Cell Cultures. They were maintained in MEM supplemented with 15% fetal bovine serum (FBS). Cells were cultured at 37°C and 5% CO₂. Prior to experiments, cells were cultured in phenol red-free MEM supplemented with 10% charcoal stripped fetal bovine serum.

Anti-PCNA, -ERα, -PR, cytokeratin-7, c-myc, β-catenin, p-c-jun, c-jun, c-fos, p21, p27, cyclin D1, cdk4, p-CREB, CREB, PUMAα, NOXA, Bax, Bcl-2, and -β-actin antibodies were purchased from SantaCruz, CA, USA and antibodies for pMEK, MEK, pERK, ERK, cleaved caspase-3, PARP, Bcl-xl were purchased from Cell Signalling Technology. BrdU cell proliferation assay kit was purchased from Calbiochem, In Situ Cell Death Detection Kit was procured from Roche, Mannheim, Germany. p-EGFR ELISA kit was purchased from Invitrogen, Carlsbad, CA. Immuno-Blot™ PVDF membrane was purchased from Millipore, MA, USA. ECL reagent and ECL Hyperfilm was purchased from GE Healthcare, USA.

GPR30 agonist (GPR109b) and antagonist (G15) were purchased from Calbiochem, San Diego, CA).

All culture media and other reagents were purchased from Sigma-Aldrich, USA.

Animal preparation, xenograft tumors and compound treatment
All experimental procedures were done according to the standards specified by Institutional Animal Ethics Committee of Central Drug Research Institute, Lucknow. Ishikawa cells in normal saline were implanted into 6-week-old athymic nude mice bearing the nu/nu gene [NIH(s) (nu/nu)] and housed under pathogen-free conditions. The tumor (300–350 mm³) bearing mice were treated with K-1 (200 µg/kg body weight doses, per day for 14 days. Animals of control group were treated with vehicle only. Xenografted tumor volume was measured using Vernier callipers (major and minor axis) and tumor volume was calculated by the equation: \( V = \frac{L \times W^2}{2} \) (mm³), where \( L = \) length and \( W = \) width.

After euthanasia, animals were dissected for removal of tumors and various other organs for fixation in 4% formaldehyde for TUNEL staining and routine histology. Tissues were processed as per standard protocol (Nair et al., 2003).

**Cell proliferation assay**

Cell viability was determined by MTT assay. Ishikawa cells were seeded (2.5 × 10³ cells/ well) into 96-well plate and treated with K-1 (100nM, 1µM, 5µM, 7.5µM, 10 µM, 12.5µM and 15µM) for 48h. At the end of incubation, MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (0.5 mg/ml) (Sigma) was added and incubated for 2h at 37°C. After 2h of incubation, supernatants were removed and 100µl of DMSO was added. The formazan crystals formed inside the viable cells were solubilized in DMSO and the OD was read with Microquant (Biotech, USA) at 540 nm. The IC₅₀ values for each compound were determined by Compusyn software. The experiments were performed three times with five replicates in each.

**Enzyme linked immunosorbant assay (ELISA)**

Phosphorylation level of EGFR was quantified by using ELISA kit (Invitrogen). In brief, Ishikawa cells were incubated with GPR109b (GPR30agonist), or G15 (GPR30 antagonist) (100nM) or K-1 at various concentrations (7.5µM and 10µM) in the
presence of GPR109b agonist, for 6h. At the end of incubation, cell lysate was prepared by lysing the cells in buffer containing 10 mM Tris pH7.4, 100mM NaCl, 1mM EDTA, 1mM EGTA, 1mMNaF, 20mM Na4P2O7, 2mMNa3VO4, 1% TritonX-100, 10% glycerol, 0.1% SDS and 0.5% deoxycholate supplemented with protease and phosphatase inhibitors. EGFR activation was measured by following the manufacturer’s instructions. OD was taken with Microquant ELISA reader (Biotech, USA) at 450 nm. The experiments were performed three times with three replicates in each.

In tumor xenograft, the ratio of p-EGFR and EGFR were determined using above mentioned ELISA kit.

**Western blot analysis**

K-1 treated cells and K-1 treated tumor from xenograft model, were lysed in lysis buffer (50 mM Tris pH-7.4, 150 mM NaCl, 1% nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS, 1 µM sodium orthovanadate) emented with protease inhibitor cocktail (Sigma). Equal amounts of protein were separated by gel electrophoresis and then transferred to Immuno-Blot™ PVDF membrane (Millipore). The membrane was blocked with 5% skimmed milk and then incubated with a 1:5000 dilution of primary antibody overnight at 4°C. The membrane was then washed and incubated with a secondary peroxidase- conjugated antibody for 1h. Antibody binding was detected using enhanced chemiluminescence detection system (GE Healthcare). After developing, the membrane was stripped and re-probed using antibody against β-actin to confirm equal loading. Each experiment was repeated three times to assess for consistency of results. Quantitation of band intensity was performed by densitometry using Quantity One_ software (v.4.6) and a Gel Doc imaging system (Bio-Rad).

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

TUNEL staining was performed by using an in situ cell death detection kit (Roche, Stockholm, Sweden) to demonstrate the apoptosis in uterine sections. The
manufacturer’s protocol was followed for paraffin-embedded sections. The slides were mounted in 3:1 Vectashield (Vector Laboratories, Burlingame, CA): 4’, 6-diamino-2-phenylindole (Invitrogen, Carlsbad, CA) and sealed. The sections were examined under light microscope (Nikon 80i), and images were captured using an NIS-Elements F 3.0 camera (Nikon).

**Statistical Analysis**

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

In vitro studies

Effect of compound K-1 on cell proliferation

To determine whether benzopyran compound K-1 has antiproliferative activity on Ishikawa cells, we examined the effect of K-1 on cell viability by the MTT assay. Fig 3.3.1 illustrates that MTT staining was reduced in K-1 treated Ishikawa cells in a dose dependent manner which indicates that compound K-1 reduced viability of Ishikawa cells (p<0.05 to p<0.001).

![Graph showing the effect of compound K-1 on cell proliferation](image)

**Fig 3.3.1** Benzopyran compound K-1-mediated growth suppression in endometrial adenocarcinoma Ishikawa cells. Cells were treated with various concentrations (100nM, 1μM, 5μM, 7.5μM, 10μM, 12.5μM and 15μM) of compound K-1 for 48h. The level of cell proliferation was measured using MTT assay. The percentage of viable cells was calculated as the ratio of treated cells to the control cells. Results are expressed as mean ± SEM, n = 5. p values are a-p<0.001, b-p<0.01, c-p<0.05 and d-p>.05 vs. control.

Effect of compound K-1 on the activation of EGFR in vitro and EGFR phosphorylation in xenograft tumor in vivo

As determined by ELISA, GPR109b which is agonist of GPR30 enhanced the activation of EGFR whereas G15 (GPR30 antagonist) suppressed the EGFR activation significantly (p<0.001) after 6h. When cells were treated with benzopyran
compound K-1 in presence of GPR109b, there was a significant decrease observed in the activation of EGFR even at 7.5µM concentration (p<0.001) (Fig 3.3.2 A). Where as in case of ishikawa xenograft, the ratio of p-EGFR/EGFR, determined by ELISA, was found to decrease in K-1 treated groups (Fig 3.3.2 B).

![In-vitro](image1)

**Fig.3.3.2 (A&B) Effect of compound K-1 on activation of EGFR.** Quantified degree of p-EGFR relative to total EGFR expression as determined by ELISA in vitro (A) and in vivo (B). 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 or VT and e-p<0.001, f-p<0.01, g-p<0.05 and h-p>.05 vs. GPR109b agonist- treated group.

In vivo studies

Effect of compound K-1 treatment on growth of xenograft tumor in mice

To examine the effect of compound K1 in xenograft, tumor was induced in athymic mice. It was found that compound K-1 at dose 200 µg/kg body weight/day, reduced the tumor size significantly as observed after 14 days (Fig.3.3.3 A & D) whereas no significant change was observed in body weight in the vehicle treated group (Fig.3.3.3.C). As illustrated in Fig.3.3.3 B, it was observed that in K-1 treated mice, tumor size was reduced significantly as compared to that of vehicle treated group.
From the haematoxylin-eosin–stained sections of dissected tumor, the cellular apoptosis was evident in compound treated group (Fig.3.3.3 D) in comparison to the tumor from the control group.

**Treatment of K-1 starts**

**Fig 3.3.3.** (A) Effect of compound K-1 on tumor progression in a mouse xenograft model. (A) Fig. shows reduction in tumor size after treatment with compound K-1 after 14 days of continuous treatment. (Number of animals per group =6 to 8. Results are expressed as mean ± SE, n = 5.)
**Fig 3.3.3 B & C.** Graph showing tumor volume changes and average body weights of the mice within 14 days after initiation of treatment. (Number of animals per group = 6 to 8. Results are expressed as mean ± SE, n = 5.

**Figure 3.3.3.D.** The upper panel of fig showed the size of the dissected out tumors whereas the lower panel showed the simples histology of that dissected out tumours from vehicle treated group and the compound K1 treated group (Number of animals per group = 6 to 8. Results are expressed as mean ± SE, n = 5.

**Effect of compound K-1 treatment on Histomorphology of liver, lungs, spleen, uterus and kidney**

To see the effect of compound K1 on normal body part of nude mice simple histology was perfomed in various body part of nude mice such as liver, lungs, spleen, uterus and kidney. Histomorphology results revealed that compound K1 did not show any marked change in liver, lungs, spleen, uterus and kidney in the compound treated mice as compared to vehicle treated control group (Fig 3.3.4).
Fig. 3.3.4. Representative sections of kidney, liver, lungs, spleen and uterus of control, K-1 treated and vehicle treated mice at indicated doses, photomicrographs of histological sections was captured. Number of animals per group = 6 to 8.

Effect of compound K-1 treatment on the expression of proliferation markers in xenograft tissue

To analyze the effect of compound K-1 on proliferation markers, immunoblotting of proliferation markers such as PCNA, β-catenin, c-myc, ERα and PR was performed in xenograft tissue. A significant down-regulation of proliferation markers were observed in the compound treated group in comparison to vehicle treated group (Fig. 3.3.5 A).

Densitometric analysis showed that compound K-1 caused ~65% reduction in PCNA (p<0.01), ~40% in β-catenin (p<0.05), ~60% reduction in c-myc (p<0.001), 30%
reduction in ERα (p<0.05) and ~55% reduction in PR (p<0.001) as compared to vehicle treated group (Fig 3.3.5 B).

**Fig 3.3.5 A&B.** Western blot analysis of proliferation markers such as PCNA, β-catenin, c-myc, ERα and PR in tissue lysate. Samples were subjected to Western Blotting with antibodies against markers as described in ‘Materials and Methods’. Equal amounts of cell lysate protein (30μg) were analyzed in each lane. β-actin was used as a control to correct for loading. (F) Densitometric analysis of western blot. Quantification of protein expression levels are shown as fold changes. Number of animals per group = 6 to 8. Results are expressed as mean ± SE, n = 5.

**Effect of compound K-1 on MAPK signaling in xenograft tissue**

The ratio of p-EGFR/EGFR, as determined by ELISA, was found to decrease in xenograft tissue of K-1 treated mice (Fig 3.3.6 A&B). Further, to see the effect of compound K1 on MAPK signaling, immunobloting experiments for p-MEK, MEK, p-ERK, ERK and for downstream effectors such as p-cJun, c-jun and fos were performed. It was observed that the expression of p-MEK, p-ERK, p-cjun and c-fos were significantly reduced as compared to vehicle treated group (Fig. 3.3.6 A). The densitometric analysis showed that compound K-1 caused ~36% reduction in pMEK (p<0.05), ~45% reduction in pERK (p<0.05), ~50% reduction in p-cjun (p<0.001) and ~50% reduction in c-fos (p<0.01) (Fig.3.3.6 B).
**Fig. 3.3.6 (A&B).** Western blot analysis of MAPK markers and cell cycle regulatory genes in tissue lysates of xenograft mice. Samples were subjected to Western Blotting with antibodies against markers as described in ‘Materials and Methods’. Equal amounts of cell lysate protein (30μg) were analyzed in each lane. β-actin was used as a control to correct for loading. Densitometric quantification 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.

**Effect of compound K-1 on expression of cell cycle regulatory genes in xenograft tissue**

To see the effect of compound on cell cycle regulatory genes such as p21, p27, cyclinD1 and cdk4, immunoblotting was performed. Fig. 3.3.7.A clearly shows a significant induction of p21 and p27 gene in K-1 treated group which led a significant reduction in the expression of cyclinD1 and cdk4 as compare to vehicle treated group.

The densitometric analysis showed that compound K-1 caused ~ 2 times induction in p21 (p<0.001), ~ 1.6 times induction in p27 (p<0.01), ~36% reduction in cdk4 (p<0.05) and ~55% reduction in expression at 12.5μM concentration (p<0.001) (Fig. 3.3.7 B).
Fig. 3.3.7 A&B. Western blot analysis of MAPK markers and cell cycle regulatory genes in tissue lysates of xenograft mice. Samples were subjected to Western Blotting with antibodies against markers as described in ‘Materials and Methods’. Equal amounts of cell lysate protein (30μg) were analyzed in each lane. β-actin was used as a control to correct for loading. Densitometric quantification 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.

Effect of compound K-1 on apoptosis in xenograft tissue

In order to assess the effect of benzopyran compound K-1 on the induction of apoptosis in xenograft tissue, TUNEL assay was performed in vehicle treated and K1 treated groups. As illustrated in Fig. 3.3.8, compound K-1 caused significant apoptosis as compared to vehicle treated group. Positive controle and negative controle groups were also be analysed for the confirmation of results.
Fig 3.3.8 Representative sections from the tumor xenografts dissected out from treated mice stained for TUNEL showing cells with fragmented DNA in treatments with K-1 and vehicle treated control tumor. Note the large number of TUNEL positive cells with Greenish yellow deposit in K-1 treated group. Results are expressed as mean ± SEM, n = 3.

Analysis of apoptotic markers

Further to analyse the apoptotic markers, immunobloting was performed. Compound K-1 reduced the expression of p-CREB in a dose-dependent manner which enhanced the expression of PUMAα, Noxa and Bax. The expression of anti-apoptotic gene such as Bcl-2 and Bcl-xl were found to be reduced in K-1 treated group. It was also observed that K1 enhanced the expression of cleaved caspase-3 which led to the increased cleavage of PARP (Fig 3.3.9 A).

Densitometric analysis showed that compound K-1 caused ~49 % reduction in p-CREB, (p<0.01), ~1.5 times induction in PUMAα (p<0.01), ~1.4 times induction in
Noxa (p<0.01), ~1.8 times induction in Bax (p<0.001), ~1.6 times induction in cleaved caspase-3 (p<0.01) and ~1.8 times induction in cleaved PARP (p<0.001), ~80% reduction in Bcl-2 and ~60% reduction in Bcl-xl (p<0.001) (Fig 3.3.9B).

**Fig 3.3.9 (A&B)** Western blot analysis of apoptotic markers such as p-CREB, CREB, PUMAα, NOXA, Bax, Bcl-2, Bcl-xl, cleaved caspase-3 and cleaved PARP in xenograft tissue lysate. Samples were subjected to Western Blotting with antibodies against markers as described in ‘Materials and Methods’. Equal amounts of cell lysate protein (30μg) were analyzed in each lane. β-actin was used as a control to correct for loading. Densitometric quantification 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.

**Cytotoxicity effect of compound K-1**

To see the cytotoxic potential of compound K-1, histological sections from the kidney from the animals of all three groups (compound treated group, vehicle treated group and control group), were stained for TUNEL assay. No TUNEL- positive cells were
visible in the kidney of mice receiving treatments with K-1 (data not shown) which confirmed that compound was not toxic to kidney (Fig 3.3.10).

**Fig.3.3.10.** Representative sections of the kidney from Control, K-1 treated and vehicle treated animals stained for TUNEL positive cells showing reactivity with treatments of compound K-1. Number of animals per group =6 to 8.
Discussion

Although antiestrogens are widely used in the prevention and treatment of estrogen-responsive cancers such as breast cancer, the effects of pure non-steroidal antiestrogens on endometrial cancer are poorly understood. Recently, benzopyran’s role as a potentiating factor in killing cancer cells has been shown by its antiproliferative actions in inducting the cell death in endometrial cancer cells (Fatima et al., 2012). However, the mechanism by which benzopyran compounds exert cytotoxic activity is not completely understood. In the present study, we have examined the effects of 2-[piperidinoethoxyphenyl] -3- [4-hydroxyphenyl]- 2H-benzo (b) pyran (K-1) on GPR30/ EGFR/ MAPK -mediated signaling and cell survival/apoptosis in human endometrial cancer cells and in xenograft mouse model. Compound K-1 showed cytotoxic effects in human Ishikawa and in human primary endometrial cancer cells and caused inhibition of growth of xenograft tumor. Findings from our earlier in-vitro studies and the current in-vivo studies support the hypothesis that benzopyrans possess ability to induce apoptosis and evoke Ishikawa xenograft tumor regression.

Our earlier studies have clearly shown that benzopyrans efficiently interfered with genomic action by antagonizing the ERE-mediated classical estrogen action (Kharkwal et al., 2011; Fatima et al., 2012). We also found that benzopyran compounds exposure decreased non-classical AP-1 mediated transcriptional activation in endometrial cancer cells (Fatima et al., 2012). Although intracellular estrogen receptors have been demonstrated to be capable of mediating many responses, the signaling capabilities of 7-transmembrane receptor i.e. GPR30 (also referred to as ‘mER’) in response to estrogen have just begun to be described (Filardo et al., 2000, Prossnitz et al., 2007). Estrogen-mediated GPR30-dependent activation of the MAP kinase ERK1/2 via EGFR transactivation was first described by Filardo et al (Filardo et al., 2000, Filardo et al., 2002a; Filardo et al., 2002b).
In the present study, it was evaluated and demonstrated that in endometrial cancer cells, compound K1 interferes with GPR30/EGFR-mediated signaling and manifests the ability to reduce cellular proliferation. Ishikawa cells treated with GPR109b which is a agonist of GPR30, significantly enhanced the activation of EGFR whereas antagonist of GPR30 i.e. G15 significantly reduced the activation of EGFR. Interestingly, when cells were treated with compound K-1 in the presence of GPR109b (GPR30 antagonist), a significant decrease was observed in the activation of EGFR at 7.5µM concentration which further suppressed the activation of ERK. Although no direct evidence is available, the possible mechanism of EGFR inhibition by K-1 may involve estrogen antagonism at GPR30 by interfering with either ligand binding to GPR30 or with its activation. However, the direct effects of compound K-1 on inhibition of EGFR activation also cannot be ruled out. These phenomenon may cause the ultimate suppression of downstream signaling responsible for preventing the growth and cell cycle progression in endometrial cancer cells.

It has been demonstrated by several investigators that transient activation of ERK1/2, a downstream effector of EGFR, plays a pivotal role in cellular proliferation and reduces cell cycle arrest and causes apoptosis (Roux et al., 2004; Meloche et al., 2007; Cagnello et al., 2011; Mebratu, et al., 2009). In our study, it was interesting to note that compound K-1 suppressed the phosphorylation of ERK1/2, but not of JNK and p38 in endometrial cancer cells and similar results were observed in tumor xenograft tissue also. Suppressed phosphorylation of ERK led to inhibition of cellular proliferation and induction of apoptosis as observed in both in-vitro and in in-vivo experiments. The reduced expression of p-ERK led to decrease in proliferation by reducing the expression of p-cjun, c-fos, c-myc, cyclinD1. K1 reduced the expression of other key proliferation markers such as PCNA, β-catenin, ERα, PR during the inhibition of xenograft tumor growth. In addition, the phosphorylation of CREB was decreased which results into enhanced expression of PUMAα, Noxa, cleaved caspase-3, cleaved PARP, the characteristic markers of apoptosis. The data on analysis of cell cycle and the major G1 phase regulators (cyclin D1, cdk4, p21, p27) indicated that K-1 was capable of inducing cell cycle arrest and subsequently regulating cell cycle -related effectors which led to regression of Ishikawa xenograft tumor.
The present study provides the evidence that K-1 inhibited endometrial cancer cellular growth and tumor xenograft growth via interfering with GPR30/EGFR/ERK1/2 activation. Our compound showed dual targeting i.e. non-genomic GPR30 signaling and genomic ER signaling in endometrial cancer cells. To conclude, our results have provided a rational that benzopyran derivative K-1 could be developed as a potential chemotherapeutic agent against human endometrial cancer. Such therapeutic agents may also be able to prevent the possibility of cross reactivity between two pathways in case of resistance caused by existing therapies.