Am J Cancer Res 2021;11(6):3335-3353
www.ajcr.us /ISSN:2156-6976/ajcr0133920

Original Article

SERMs suppresses the growth of ERα positive cervical cancer xenografts through predominant inhibition of extra-nuclear ERα expression

Balaji Ramachandran[1], Kanchan Murhekar[2], Shirley Sundersingh[2]

[1]Department of Molecular Oncology, Cancer Institute (W.I.A), No. 38, Sardar Patel Road, Adyar, Chennai 600 036, India; [2]Department of Oncopathology, Cancer Institute (W.I.A), No. 38, Sardar Patel Road, Adyar, Chennai 600 036, India

Received April 5, 2021; Accepted May 5, 2021; Epub June 15, 2021; Published June 30, 2021

Abstract: The role of estrogens and estrogen receptors (ER) in cervical cancer (CC) is not well established. However, epidemiological studies and abundant evidence from genetically engineered mouse models support such hypothesis. In this study, we have addressed estrogen responsiveness in a human CC cell line xenograft mouse model. We assessed the sensitivity of Ethynyl Estradiol (EE), SERMs (fulvestrant, MPP) and a non-SERM (EGCG) to competitively modulate the growth of ERα+^{ve} MS751 CC xenografts. We also checked the agonistic-antagonistic propensity of the above treatments to alter the histology of ovariectomised mouse uterine cervix. Chronic EE treatment encouraged the growth of ERα+^{ve} MS751 CC xenografts, while SERMs and EGCG significantly decreased tumor formation. SERMs were found to inhibit ERα expression, localized within cytoplasmic and membrane compartments. Conversely, ERα was not inducible and EE administration suppressed the growth of ERα-^{ve} HeLa CC xenografts. SERMs competitively induced atrophic features to uterine cervix, with MPP giving rise to mucinous metaplasia in the ectocervix. We have demonstrated that, estrogen sensitivity mediated through ERα has promoted CC tumorigenesis. This in turn was modulated by SERMs, predominantly through inhibition of extra-nuclear ERα expression. Though, induction of hyperestrogenic status in the ectocervix, might underrate the utility of SERMs in ERα+^{ve} CC.

Keywords: SERMs, MPP, fulvestrant, anti-estrogen, estrogen, cervical cancer, xenografts, Cytoplasmic ERα, membrane ERα

Introduction

In terms of incidence and mortality, cervical cancer (CC) is ranked as the fourth most cancer in women worldwide and second in India [1, 2]. It is known that, persistent Human papillomavirus (HPV) infection is crucial for the development of CC [2-7]. With the stringent presence of HPV infection as a strongest factor, several other important risk factors such as immunosuppression, smoking, parity and chronic oral contraceptive usage were known to augment the likelihood of developing CC [2]. Most cases of CC rise in the transformation zone of cervix that is very sensitive to estrogens [8, 9].

With reference to life style observations made in HPV infected women, repeated parity and usage of oral contraceptives have shown to increase the risk of CC [5-7, 10-12]. These epidemiological observations raise the likelihood that hormones such as estrogen, in the presence of HPV infection might affect the cancers of the cervix [13]. Indeed, it has been shown by several investigators that mere presence of HPV infection is not a mandate to develop CC, but may require additional synergistic factors such as estrogens to induce the expression of HPV oncogenes and subsequent loss of p53 [4, 13-16].

In *vivo* studies with transgenic mouse models have consistently demonstrated the role of estrogens in driving HPV+^{ve} CC. K14E6/E7 transgenic mouse, implanted with estrogen pellets developed aggressive estrogen receptor α (ERα) dependent CC [17]. Upon estrogen withdrawal, this mouse model responded by

generating less aggressive tumors [3]. CC development in this mouse model was inhibited by selective estrogen receptor modulators (SERMs) such as fulvestrant and raloxifene [18]. K14E6/E7 (ERα-/-) knock out mouse model failed to induce CC with estrogen treatment [4]. K14E6/E7 mice with a knocked-in mutant DNA-binding domain failed to bind to estrogen responsive elements (EREs) and did not respond to estrogens [19]. The data is consistent with the findings from other CC mice models [9, 20, 21]. Observations made from in *vivo* models, allowed us to conclude that, ERα is required for the development of atypical squamous metaplasia (ASM). ASM is considered as the initial phase of cervical carcinogenesis, prior to the development of cervical intraepithelial neoplasias (CINs). ERα in the presence of active HPV oncogenes, leads the process of carcinogenesis from ASM, progress through CINs to CC [4, 13, 18].

However, studies with clinical CC specimens have reported contradictory findings of claiming both continuance [22-24] as well as loss of ERα expression during CC progression [25-30]. In view of the inconsistencies between the published reports, the continuity or loss of ERα expression during the progression of CC is indecisive [31].

While the role of estrogens and ERα in the development of CC is not well established, accumulating information in support of such an association is clearly documented and the significance of ERα in CC is gaining momentum. It is important to understand whether, ERα is a druggable driver of CC, before it can even be considered as a therapeutic target. Although it is uncertain as of now, if ERα could be regarded as a therapeutic target for CC, the suitability and utility of SERMs has to be considered as a natural choice of ERα antagonists. In this study, we explored the sensitivity of two SERMs [Methylpiperidino pyrazole (MPP), fulvestrant] and a non-SERM [Epigallocatechin Gallate (EGCG)] to alter the histology of normal mouse cervix and to bring down tumor burden in ERα+ve and -ve human CC cell line xenograft mouse model in a longitudinal treatment period. We also tested if the estrogen; Ethynyl Estradiol (EE), could selectively encourage the growth of ERα+ve CC xenografts.

Materials and methods

Chemicals

MPP, EGCG and EE were purchased from Cayman chemical company, USA. Fulvestrant and APTES were purchased from Sigma, DMSO from MP Biomedicals, California, USA, ketamine from Aneket, Neon Laboratories Limited, Palghar (Thane), India and Xylazine from Indian Immunologicals Limited, Hyderabad, India.

Cell lines

MS751 (ATCC, USA), HeLa and MCF-7 cell lines (NCCS, India) were cultured in DMEM (Himedia, India) supplemented with 10% FBS (Invitrogen, USA), 100 µg/L penicillin and 100 µg/L streptomycin. Cells were maintained at 37°C in a 5% Co_2 incubator.

Cell line block preparation

Cell lines were treated with or without 10 nM EE for 48 Hours or until 175 cm^2 culture flasks reach 80% confluence. Cells were harvested and cell viability was verified with 0.4% trypan blue (sigma) exclusion, which showed 95% live cells. Cells were pelleted by centrifugation at 2000 rpm/10 minutes with 10 ml of 100% isopropanol. The resulting cell pellet was stained with a drop of Eosin and fixed in 10% neutral buffer formalin (NBF) overnight and further processed into paraffin embedded cell blocks.

Experimental animals

Healthy adult female (nulliparous) swiss albino mice of 4-6 weeks of age, were purchased from King Institute of Preventive Medicine, Chennai, India and housed at the departmental animal facility in clean polypropylene cages.

Specific Pathogen Free female Ncr Nude (NCRNU) mice (4-6 weeks age) were procured from Vivo BioTech Ltd., Hyderabad, India, an authorized Breeder & Distributor of Taconic Biosciences, Inc. These mice were maintained in Individually Ventilated Caging (IVC) system, housed in clean room facility (Class 10,000).

Mice were fed *ad-libitum* with irradiated Altromin 1324P (Phytoestrogen poor) diet and maintained in autoclaved corn cob bedding. Animal experiments were carried out with prior

approval from the Institutional Animal Ethics Committee of Cancer Institute (W.I.A), Adyar, Chennai, India with project approval number (# CIWIA122012, # CIWIA012015, # CIWIA 072016). Animal housing conditions were maintained as reported [32].

Study design

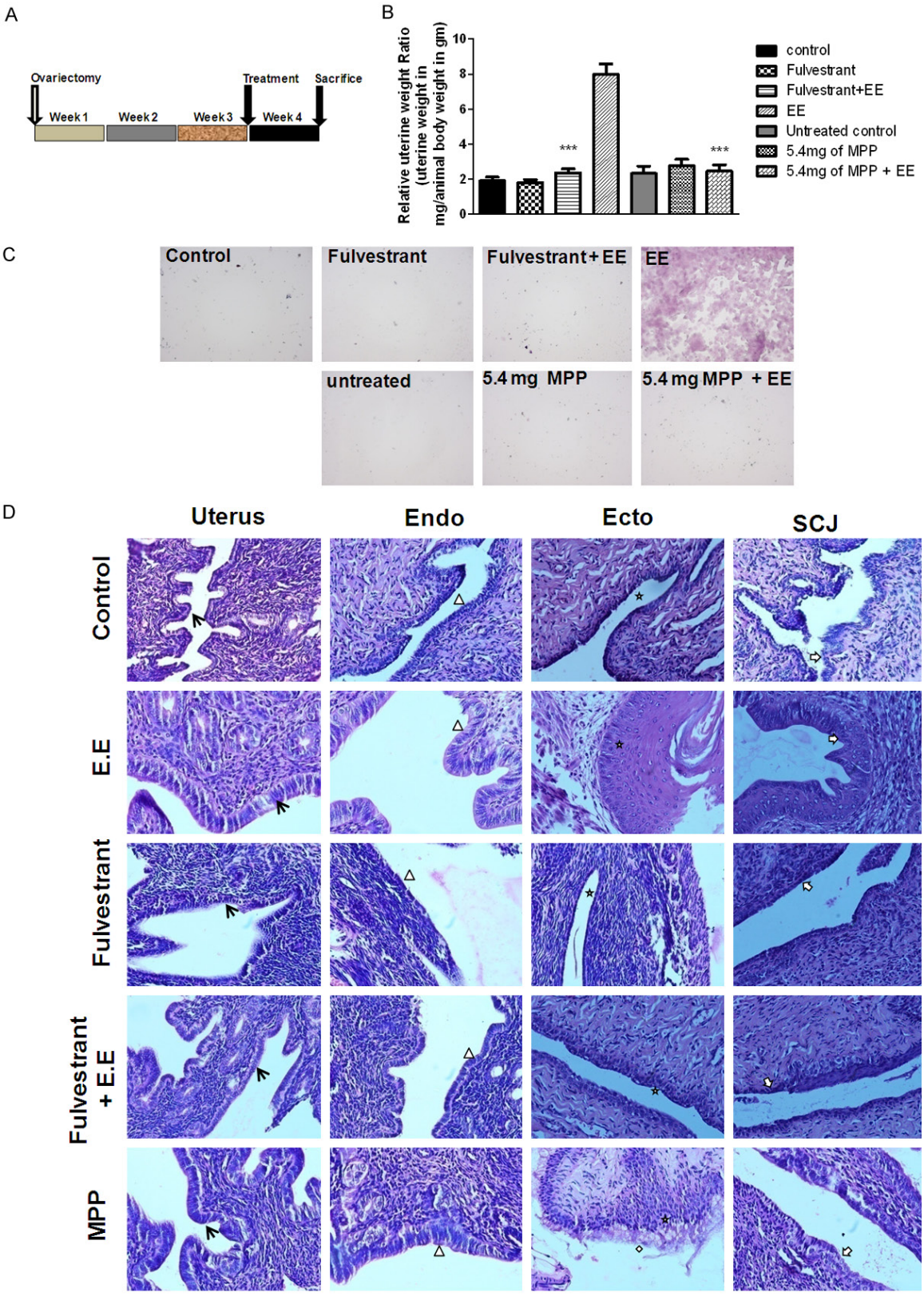
Acute (LD_{50}) and repeated dose toxicity: Swiss albino mice were used in this study. Acute dose toxicity was evaluated at a range of doses, as per OECD No. 425 guidelines. Dosing recommendations were calculated based on probit analysis by OECD software program no. 425. This method was adopted to minimize animal usage and the requirement of the compound under investigation. Initial dosing (i.p route - 100 ul volume) was done with a limit test of 2000 mg/kg (1 mouse) and mortality was observed immediately at this dose range. Main test was carried out as per the directions of dose limits specified by the software as; with doses of (1 mouse) 550, (3 mice) 175, (2 mice) 55 mg/kg body weight. Short and long-term outcomes (in terms of animal death or survival due to treatment, as end point) were plotted based on observations within the initial 48 hours and after several days (14 days) after treatment, respectively. The OECD software calculated the i.p LD_{50} of MPP as 175 mg/kg.

Based on LD_{50} values, dose range for maximum tolerated dose (MTD) experiment was calculated, taking 1/10 of LD_{50}, as a starting higher dose with a default dose progression factor of 3.2 (which corresponds to a dose progression of one half log unit). A range of doses were chosen as; control (0), 17.5, 5.46, 1.70, 0.53 mg/kg body weight, dosed through i.p route (100 ul volume). Mice were randomized into various experimental groups (n = 5 per group) based on body weight. MPP was dissolved in 4% DMSO diluted in sterile saline as vehicle, irrespective of treatment routes. Various concentrations of MPP were freshly prepared, immediately before the treatment. Control group received mock treatment with vehicle alone. The animals were consecutively treated for a period of 14 days and maintained for an additional 14 days of treatment free observation period. Whole animal body weight was measured twice a week throughout the experiment and the data is expressed as % body weight change during treatment, in comparison to day 1 body weight of the same animal, just before the initiation of treatment.

At the end of the study, the animals were fasted overnight and sacrificed by ketamine overdose. Whole blood and serum were collected by cardiac puncture for hematological and biochemical analysis, respectively. Immediately after sacrifice, the animals underwent necropsy. A gross anatomo-pathological investigation was carried out before organ excision. The following organs were sampled for histopathological examination and fixed in 10% neutral buffered formalin (NBF); liver, kidney, stomach, small and large intestines, brain, spinal cord, spleen, heart, thymus, lungs, trachea, uterus with ovary and bone marrow. Fixed organs were paraffin embedded, sectioned and stained with haematoxylin and eosin.

Uterotrophic assay

Swiss albino mice were ovariectomised at an age of around 6 to 8 weeks, by the procedure described in the OECD guidelines no. 440. Animals were anaesthetized with Ketamine (100 mg/kg/i.p) and Xylazine (10 mg/kg/i.p) prior to surgery. Ovariectomised mice were maintained for at least 21 days before the start of the main experiments, to wash of endogenous estrogens (**Figure 1A**) and randomized based on body weight into the following groups: (1). untreated control; (2). vehicle control; (3). fulvestrant (400 µg/kg/day/s.c); (4). EE (0.6 µg/kg/day/s.c); (5). EE (0.6 µg/kg/day/s.c) + fulvestrant (400 µg/kg/day/s.c); (6). MPP (5.4 mg/kg/i.p); (7). MPP (5.4 mg/kg/i.p) + EE (0.6 µg/kg/day/s.c); (8). EGCG (67.8 mg/kg/i.p); (9). EGCG (67.8 mg/kg/i.p) + EE (0.6 µg/kg/day/s.c); (10). EGCG (21.1 mg/kg/i.p); (11). EGCG (21.1 mg/kg/i.p) + EE (0.6 µg/kg/day/s.c). We have chosen MPP dosage (MTD as 5.4 mg/kg/day) from the present study and EGCG as 67.8 and 21.1 mg/kg, based on previous report [32]. MPP and EGCG were dissolved and administered in 4% DMSO. Stock solution of fulvestrant was prepared in 100% DMSO and diluted to the required dose with 0.9% sterile saline *per se* to a final concentration of 2% DMSO. The treatments were carried out for a period of 7 days and the animals were immediately sacrificed (by overdose of ketamine), 24 hours after the last dose. In combination groups, fulves-


A
Ovariectomy
Treatment
Sacrifice
Week 1
Week 2
Week 3
Week 4
B
Relative uterine weight Ratio (uterine weight in mg/animal body weight in gm)
10
8
6
4
2
0
***
***
control
Fulvestrant
Fulvestrant+EE
EE
Untreated control
5.4mg of MPP
5.4mg of MPP + EE
C
Control
Fulvestrant
Fulvestrant+EE
EE
untreated
5.4 mg MPP
5.4 mg MPP + EE
D
Uterus
Endo
Ecto
SCJ
Control
E.E
Fulvestrant
Fulvestrant + E.E
MPP

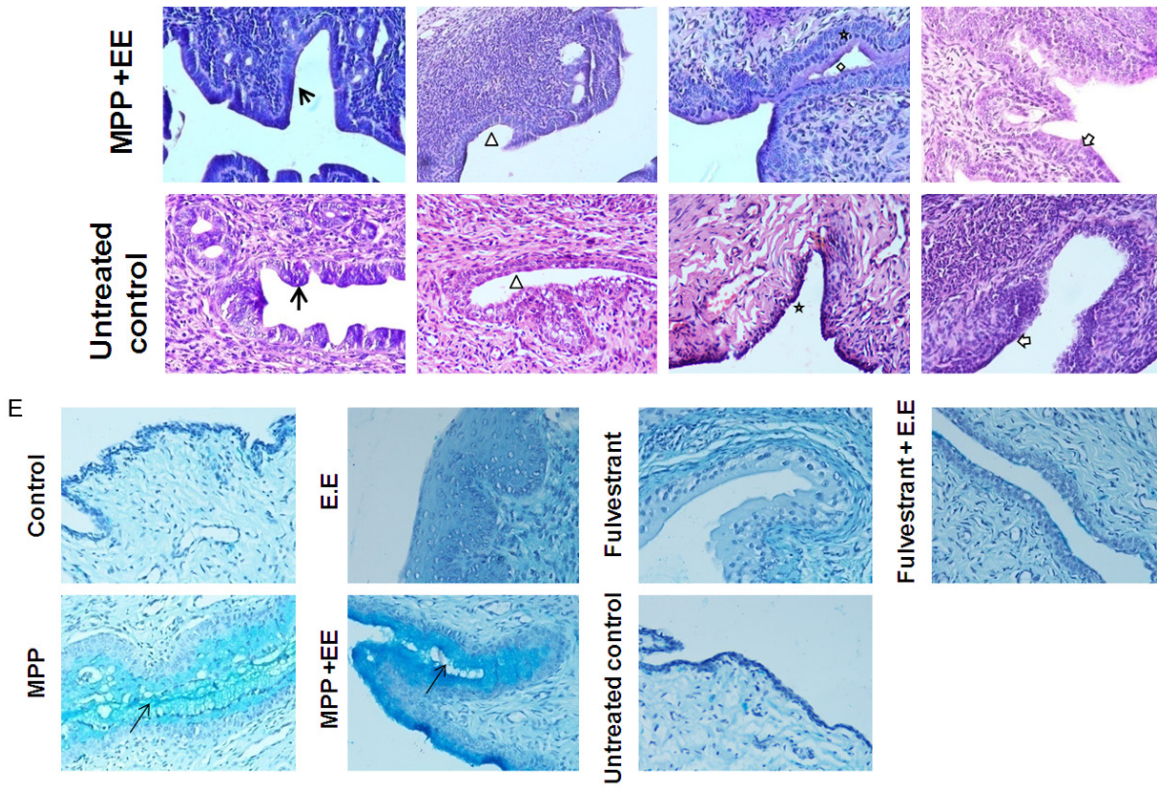


Figure 1. A. Timeline of uterotrophic assay experimental design. B. Uterine wet weights during sacrifice, 24 hours post 7 days consecutive administration of various treatments. Data represents Mean ± SEM (n = 5) and * indicates a significant difference between EE *versus*, fulvestrant + EE, MPP 5.4 mg/kg/day + EE as assessed by one-way ANOVA plus Bonferroni post hoc test (***P < 0.001). C. Photomicrographs of haematoxilyn and eosin stained vaginal smears obtained just prior to sacrifice, post 24 hours after the last dose of 7 days consecutive treatments (Magnification 10×). D. Photomicrographs of uterus, endocervix, ectocervix and Squamocolumnar junction (SCJ) of ovariectomised swiss albino mice, demonstrated competitive anti-estrogenic effects of SERMs (fulvestrant and MPP), against EE induced uterotrophy, during a 7 day treatment period. Uterus; black arrows indicate endometrial lining, endocervix; triangle indicate columnar cells, ectocervix; star indicate squamous cells, diamond indicate mucinous metaplasia, SCJ; Junction between columnar and squamous cells indicated by white arrow marks (Magnification 40×). E. Representative images of ectocervix of ovariectomised swiss albino mice, obtained after treatment with EE and SERMs (fulvestrant and MPP) during a 7 day treatment period. Arrows indicate mucin secreting squamous cells (Magnification 40×).

trant, MPP and EGCG were administered 30 minutes prior to EE treatment. During sacrifice, the procedure described in the OECD guidelines 440 was followed, to retain both uterine horns and cervix. Vagina was removed from the uterus just below the cervix, so that the cervix remains with the uterine body. Blotted uterine weight was measured immediately after excision and rapidly fixed in 10% NBF. Fixed tissues were paraffin embedded, sectioned and stained with haematoxylin and eosin. Whole animal body weight was recorded at the time of sacrifice and normalized with uterine wet weights and the data is expressed as relative uterine weight ratio as Mean ± SEM (n = 6). For mucin staining, deparaffinized uterus tissue slides were stained with 1% alcian blue solution in 3% acetic acid and counterstained with haematoxylin.

Vaginal smears were taken before the start of the study (to ascertain the success of ovariectomy) and also before sacrifice. Vaginal cytology was determined by flushing the vagina with 70 ul of sterile saline and smeared into a microscopic slide. The smear was air dried, fixed with isopropanol and stained with haematoxylin and eosin solution (each 30 seconds) and finally rinsed gently in running tap water and air dried. Vaginal cytology (estrous phase) was observed under oil immersion with Leica DMRXA upright microscope with 10× magnification.

In vivo efficacy studies

In vivo EE treatment: We measured the average water consumption of Ncr Nude (NCRNU) mice for one week. Fresh water bottle changed and consumption measured at 3.00 pm, daily. We ensured no leakage of sipper bottle during the measurement and also throughout the study. In an average, a single mouse was found to consume 3.6 mL of water during 24 hours period. Accordingly, we calculated and administered pharmacological EE exposure in an average animal as 1 μg/kg/per day in drinking water. EE stock solution was prepared in 100% ethanol and diluted with drinking water to final dose of exposure.

Generation of donar xenografts: 10 million cells each of MS751 and HeLa cells in FBS free DMEM were re-suspended in 50% Matrigel (Corning, India) and injected subcutaneously into the flank of each animal (10 mice per cell line). The donar animals were switched to EE treatment in drinking water as described above or with *ad-libitum* drinking water (5 mice per treatment), one week before cell line implantation until sacrifice on day 91. The injected area was monitored for tumor formation by physical palpation. Throughout the study, changes in tumor volume were measured with the aid of vernier calipers and the tumor volume was calculated using the formula: $V\ (mm^3) = a \times b^2/2$ (a = largest diameter and b = smallest diameter). Xenograft bearing mice (under EE treatment in drinking water) were sacrificed as soon as the tumor reached sufficient sizeable material for implantation into the experimental set. On the day of sacrifice, animals were euthanized; donor tumors were aseptically retrieved, sliced into 30 mm^3 pieces and re-implanted into the experimental animals with the aid of a sterile trochar (Innovative Research of America, USA).

Experimental group and drug treatments: We employed only first passage xenografts, with a notion that, tumors with a low passage will be more identical to the original cancer cell line. First passage tumors are typically believed to have cells of human cellular origin, with less murine components. Animals were monitored for post-implantation illness and wound closure. Based on tumor volume and body weight (BW), animals were randomized into the following treatment groups with 5 animals per group: (1). Control; (2). Fulvestrant (1 mg/kg/day s.c. route); (3). Cisplatin (2 mg/kg/i.p treated once every three days); (4). MPP (5.4 mg/kg/i.p daily); (5). MPP (5.4 mg/kg/i.p daily) + Cisplatin (2 mg/kg/i.p treated once every three days); (6). EGCG (21.1 mg/kg/i.p daily); (7). EGCG (21.1 mg/kg/i.p daily) + Cisplatin (2 mg/kg/i.p treated once every three days). All the animals were switched to EE treatment in drinking water 1 week prior to donar xenograft implantation and continued throughout the entire duration of the study, irrespective of treatment groups. MPP and EGCG were dissolved in 4% DMSO diluted in sterile saline as vehicle. Cisplatin was dissolved in sterile water for injection.

Drug treatments were initiated as soon as the xenografts reach a tumor volume of 120 to 150 mm^3. During the study period, tumor volume growth dynamics was measured twice a week. Animal body weight loss/gain was also monitored twice weekly. Mice were sacrificed on day 16 and the tumours were collected and immediately fixed in 10% NBF. Further, whole animal was subjected to necropsy and selected organs such as liver, lung and uterus were excised for 10% NBF fixation.

Immunohistochemistry: For antigen retrieval, 10% NBF fixed paraffin embedded tissues were sectioned in APTES coated slides and incubated in TE buffer pH 9 in a pressure cooker for 8 minutes. Sections were immune stained with 1:50 dilution of anti-ERα (sc-8002 or sc-8005) from Santa Cruz Biotechnology, Inc and visualized with Dako REAL™ EnVision™/HRP, Rabbit/Mouse (ENV) reagent.

Statistics

Statistical analysis (explained in figure legends) was performed using GraphPad Prism version 5.01 for Windows, GraphPad Software (San Diego, California, USA). The same was used to generate figures and graphics. Data expressed as mean ± standard error mean (SEM).

Results

Estimation of i.p LD_{50} and MTD for MPP

To our knowledge, there is no data regarding acute toxicity and MTD of MPP through i.p route. We wanted to determine the maximum dose tolerable to the animals, in order to reduce

Table 1. Percentage change of body weight in comparison with day 1, observed during 14 days consecutive treatment of MPP, followed by 14 days observation period. Doses used are control (0), 17.5, 5.4, 1.7 and 0.5 mg/kg/i.p. Data expressed as Mean ± SEM (n = 5) and * indicates a significant difference from control, as assessed by two-way ANOVA plus Bonferroni post hoc test Control Vs treatment/time (*P < 0.05; **P < 0.01)

Treatment	Control	17.5 mg/kg/i.p	5.4 mg/kg/i.p	1.7 mg/kg/i.p	0.5 mg/kg/i.p
day 1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
day 4	0.5±0.5	0.0±0.0	-0.4±1.6	0.7±0.7	0.0±0.0
day 8	1.9±0.8	0.6±1.3	-0.6±0.6	1.5±1.8	2.4±1.2
day 11	3.7±2.5	-5.2±2.6*	-0.7±0.7	-1.5±0.9	1.8±2.8
day 15	4.7±3.4	-7.4±2.4**	-1.2±2.1	-0.7±0.7	-0.0±2.1
day 18	5.3±2.9	-5.0±2.6**	0.7±1.7	-0.7±0.7	0.6±2.6
day 22	9.2±5.2	-1.1±2.8**	4.9±1.7	2.1±1.4	4.3±6.2
day 25	6.8±3.9	1.3±3.4	5.6±1.7	2.1±1.4	4.8±3.2
day 29	9.6±3.4	2.7±2.9	4.1±1.9	2.1±1.4	6.7±5.0

animal mortality during the course of longitudinal studies. In the acute study, we considered death as end point, after single dose administration of MPP. We observed immediate animal mortality within 48 hours at (1 mouse) 2000, (1 mouse) 550, (2 mice) 175 mg/kg. There were no signs of toxicity or any other abnormalities with (1 mouse) 175 and (2 mice) 55 mg/kg, even after 14 days of observation period. The OECD no. 425 software calculated i.p LD_{50} as 175 mg/kg.

Next, we estimated the i.p MTD with a range of MPP doses as: control (0), 17.5, 5.46, 1.70, 0.53 mg/kg body weight. The animals were consecutively treated for 14 days, followed by 14 days observation period without treatment, to check for long-term adverse effects. MPP was well tolerated and no abnormalities or mortality were observed during the treatment period. There was a trend of decrease in body weight from day 11 to day 22, with a maximum decrease in body weight (around 7%) observed with the highest dose (17.5 mg/kg) around day 15 post treatment *versus* 10% limit (IUPAC [International Union of Pure and Applied Chemistry] definition) (**Table 1**). However, we did not observe any significant decrease in body weight in 5.4, 1.7 and 0.5 mg/kg/day treatment groups. None of the treatments exhibited any signs of toxicity and there is no significant change in biochemical, hematological parameters and histo-pathological scores (data not shown). Due to significant decrease in whole animal body weight, 17.5 mg/kg dose was discarded and a MTD dose of 5.4 mg/kg/bw was carried forward for future studies.

Uterus is responsive to EE and SERMs

Uterotrophic assay is a classical, gold standard method to assess ERα activity. We carried out uterotrophic assay to determine the extent to which, the chosen compounds could inhibit ERα before treating them in CC xenografts. Animals were ovariectomised to prevent the interference of endogenous estrogens and fed with phytoestrogen-poor feed, to get rid of dietary estrogens. Animals were randomized into various treatment groups, as described in the materials and methods section. Measurement of uterine wet weights showed that, 7 days consecutive treatment with Fulvestrant and MPP treatments did not induce any signs of uterotrophy. There is also no difference in the uterine wet weights, between vehicle treated and untreated controls. As expected, EE significantly increased the uterine wet weight. Treatment with fulvestrant and MPP significantly reduced EE induced uterotrophy to 70.63% (*p* value < 0.001) and 69.51% (*p* value < 0.001), respectively (**Figure 1B**). The success of uterine inhibition was further verified by vaginal cytology. Exfoliated cells from the vaginal cavity confirmed the success of ovariectomy, with literally no cells in field and arrested at the diestrous phase. All the treatments exhibited good inhibition and competitively neutralized (Fulvestrant or MPP + EE) the cellular morphology observed in the EE treatment group. Vehicle treated and untreated controls, fulvestrant and MPP treatments did not exhibit any changes in vaginal cytology (**Figure 1C**).

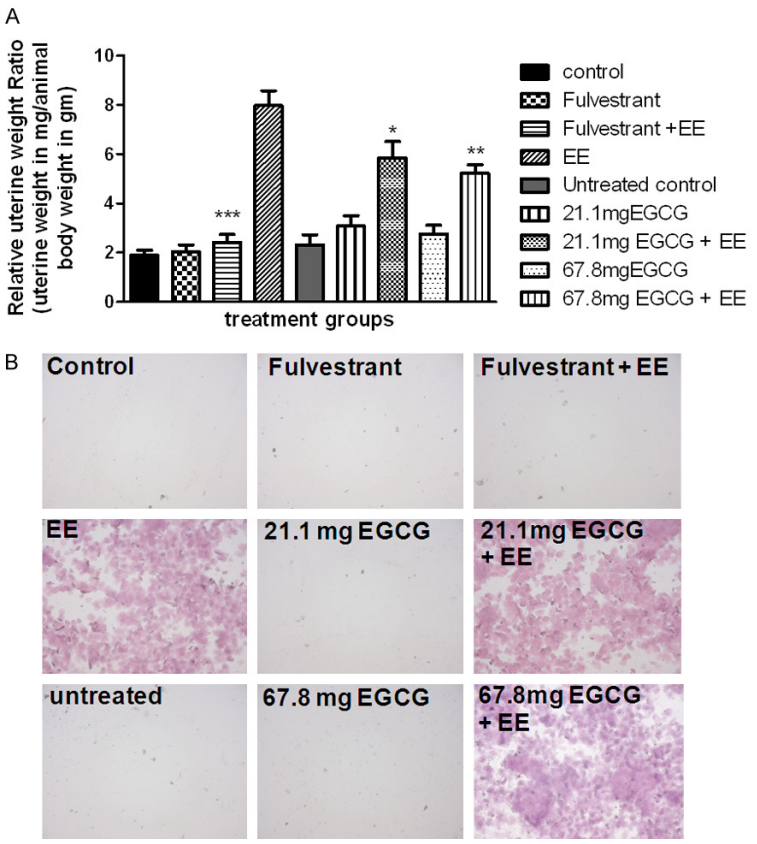


Figure 2. A. Uterine wet weights during sacrifice, 24 hours post 7 days consecutive administration of various treatments. Data represents Mean ± SEM (n = 5) and * indicates a significant difference between EE *versus*, fulvestrant + EE, EGCG 21.1 mg/kg/day + EE, EGCG 67.8 mg/kg/day + EE as assessed by one-way ANOVA plus Bonferroni post hoc test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). B. Photomicrographs of haematoxilyn and eosin stained vaginal smears obtained just prior to sacrifice, post 24 hours after the last dose of 7 days consecutive treatments (Magnification 10×).

MPP induced hyper-estrogenic status in the ectocervix

Next, we set to compare the histological changes observed in uterine cervix in various treatment groups. Control (vehicle treated and untreated) groups showed normal endometrium, myometrium, endometrial glands, epithelial lining of uterus, single columnar and cuboidal lining of the endo and ectocervix, respectively. The positive control, EE exhibited endometrial glandular hyperplasia, increase in the secretory endometrium, uterine cavity, number of glands to stroma ratio, glandular epithelial lining and papillary formation of epithelium. EE treatment also showed multilayering of endo cervix and thickened squamous epithelium with maturation effects in the ectocervix. Fulvestrant group with or without EE treatment, showed atrophic changes, both in the uterus and cervix and completely neutralized the effects of EE. MPP also completely neutralized the uterotrophic effects of EE, with minimal hyperplasia of the endometrium and features of enlarged glands. It is important to note that, minimal uterotrophic features exhibited by MPP + EE group was completely absent in fulvestrant + EE treatment group. Indeed, MPP treatment alone exhibited some mild agonistic features, such as enlargement of uterine glands. On the other hand, MPP and MPP + EE treated groups exhibited multilayering columnar cells of endocervix and mucinous metaplasia of ectocervix (**Figure 1D**). Alcian blue staining confirmed the presence of mucinous metaplasia, particularly in the ectocervix of MPP treatment, irrespective of EE co-treatment (**Figure 1E**).

EGCG is non-responsive to uterotrophic assay

We assessed the ability of EGCG, a non-SERM to competitively modulate uterine weights. EGCG at 21.1 (26.59% with *p* value < 0.05) and 67.8 (34.50% with *p* value < 0.001) mg/kg/bw inhibited EE induced uterotrophy *versus* EE treatment. The percentage inhibition is less compared to fulvestrant (69.76%) (**Figure 2A**). This indicates that EGCG is not as potent as fulvestrant. EGCG at two different doses (21.1 and 67.8 mg/kg/bw) did not exhibited any agonistic or antagonistic activity. Moreover, vaginal smear analysis indicated that, EGCG at two different doses were unable to completely neutralize EE induced uterotrophic effects (**Figure 2B**). In addition, observation of the histological architecture led us to conclude that, there are no differences between EGCG + EE and EE treatment groups. No atrophic changes were noted in any of the EGCG with or without EE, treatment groups. In uterus and cervix,

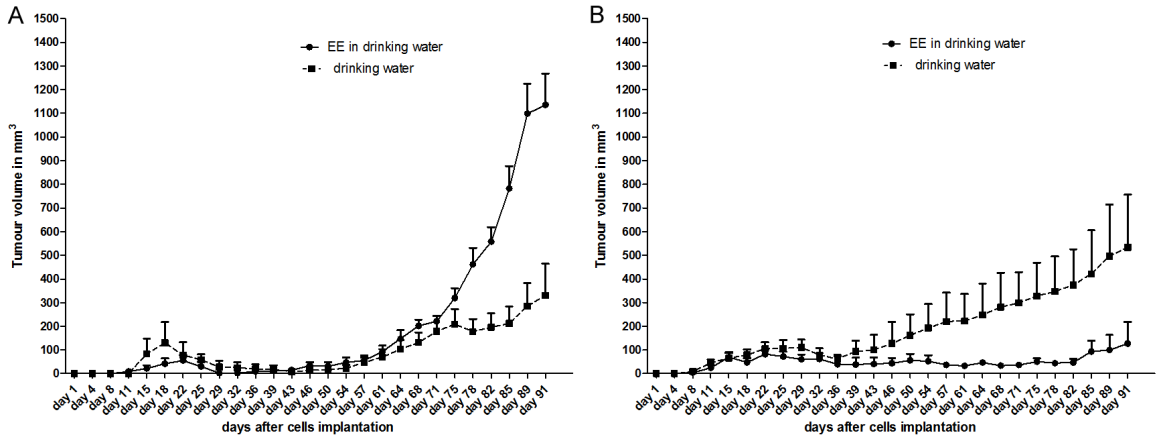


Figure 3. Effect of EE (1 µg/kg body weight/day) on the growth dynamics of donor MS751 ERα+ve and HeLa ERα-ve CC xenografts during 91 days exposure period. Data expressed as Mean ± SEM (n = 5). The data was analyzed by two-way ANOVA and Bonferroni test. [a]P < 0.05, [b]P < 0.01, [c]P < 0.001 control *versus* treatment and time. A. MS751 EE in drinking water *versus* plain drinking water, showed significance on day 78 ([b]), day 82, 85, 89 and 91 ([c]). B. HeLa EE in drinking water *versus* plain drinking water, showed significance on day 89 and 91 ([a]).

EGCG at an increased dose (67.8 mg/kg/bw), was not capable of reversing EE induced histological changes (data not shown).

EE treatment increases the growth of ERα+ve and suppresses the growth of ERα-ve CC xenografts

Two HPV-18+ve cell lines were chosen; MS751 is ERα+ve [28, 33] and HeLa is ERα-ve [34-36]. We have chosen EE for our study due to the reason that, EE is the predominant estrogenic component of combined oral contraceptives. Both MS751 and HeLa cell line xenografts under EE treatment appeared to grow around starting from the second week of post-cell line injection. We found that chronic EE treatment in drinking water significantly (from day 78 till day 91) enhanced the growth pattern of MS751 xenografts (with a fold change of 2.43 on day 91), compared to MS751 xenografts consuming plain drinking water (**Figure 3A**). Surprisingly, we observed significant inhibition (from day 89 till day 91), in the growth pattern of HeLa xenografts consuming EE in drinking water (with a fold change of 3.20 on day 91) (**Figure 3B**). This contradictory pattern allowed us to conclude that, estrogen sensitivity mediated through ERα positivity, could increase the growth of CC xenografts.

SERMs and EGCG inhibited the growth of ERα+ve CC xenografts

All the drug treatments showed significant inhibition of MS751 xenografts, starting from day 7 of treatments *versus* vehicle treated controls. Fulvestrant treatment showed significant tumor inhibition on day 13 ($P < 0.05$), day 16 ($P < 0.001$) *versus* vehicle treated controls with a fold change of 0.80 on day 16. The positive control, cisplatin showed significance on day 7 ($P < 0.05$), day 10 ($P < 0.05$), day 13 and 16 ($P < 0.001$) with a fold change of 1.72 on day 16 *versus* vehicle treated controls. MPP, showed significance on day 7 ($P < 0.05$), day 10 ($P < 0.01$), day 13 and 16 ($P < 0.001$) (1.30 fold change on day 16) and MPP + Cisplatin, showed significance on day 7 ($P < 0.05$), day 10, 13 and 16 ($P < 0.001$) (2.71 fold change on day 16) *versus* vehicle treated controls. EGCG treated groups showed significance on day 13 ($P < 0.05$), day 16 ($P < 0.001$) and EGCG + Cisplatin, showed significance on day 10 ($P < 0.05$), day 13 and 16 ($P < 0.001$), with a fold change of 1.16 and 1.41, respectively *versus* vehicle treated controls (**Figure 4A** and **4B**). The study was terminated on day 16, due to around 10% loss of whole animal body weight in MPP + Cisplatin and EGCG + Cisplatin groups (**Table 2**). Animals were sacrificed (by over dose of Ketamine) as per CPCSEA guidelines. MS751 xenografts, liver, lung and uterus were immediately removed for 10% NBF fixation. Histopathological analysis of MS751 xenografts indicated the presence of poorly differentiated epidermoid carcinoma. With fulvestrant and MPP treatment, a significant inhibition of tumour growth was observed in combination with cisplatin *versus* cisplatin treatment group alone. Both fulvestrant and MPP treated tumors, showed the presence of viable tumour

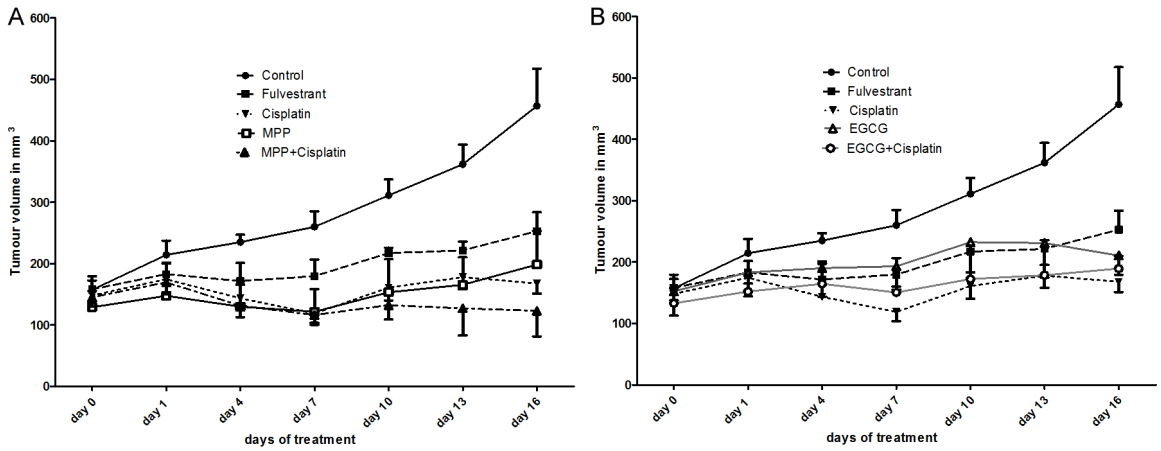


Figure 4. Effect of SERMs (Fulvestrant and MPP) and non-SERM (EGCG) to inhibit EE induced growth of MS751 ERα+ve CC xenografts during 16 days treatment period. Values represent, Mean ± SEM (n = 5). The data was analyzed by two-way ANOVA and Bonferroni test. [a]P < 0.05, [b]P < 0.01, [c]P < 0.001 control *versus* treatment and time. Control *versus* fulvestrant, showed significance on day 13 ([a]), day 16 ([c]). Control *versus* Cisplatin, showed significance on day 7 ([a]), day 10 ([a]), day 13 and 16 ([c]). Control *versus* MPP, showed significance on day 7 ([a]), day 10 ([b]), day 13 and 16 ([c]). Control *versus* MPP + Cisplatin, showed significance on day 7 ([a]), day 10, 13 and 16 ([c]). Control *versus* EGCG, showed significance on day 13 ([a]), day 16 ([c]). Control *versus* EGCG + Cisplatin, showed significance on day 10 ([a]), day 13 and 16 ([c]). Control, fulvestrant, and cisplatin groups are the same in left (A) and right (B) panel.

Table 2. Percentage change in body weight in comparison with day 0, observed during 16 days of longitudinal treatment period. Data represents Mean ± SEM (n = 5) and * indicates a significant difference from control, as assessed by two-way ANOVA plus Bonferroni post hoc test Control *versus* treatment/time (*P < 0.05; **P < 0.01; ***P < 0.001). Doses were the following: Fulvestrant; 1 mg/kg/day/s.c, Cisplatin; 2 mg/kg/i.p once every 3 days, MPP; 5.4 mg/kg/i.p/day, EGCG; 21.1 mg/kg/i.p/day

Treatment[a]	Control[a]	Fulvestrant[a]	Cisplatin[b]	MPP[a]	MPP[a] + Cisplatin[b]	EGCG[a]	EGCG[a] + Cisplatin[b]
day 0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
day 1	1.5±1.6	0.8±0.8	2.2±0.9	1.2±1.2	2.0±1.1	1.5±0.9	1.9±1.1
day 4	3.1±1.4	1.6±0.9	0.7±0.7	2.4±1.2	5.0±3.5	0.1±1.3	2.8±1.8
day 7	1.5±2.0	1.6±0.9	0.7±1.4	0.0±0.0	3.0±1.9	1.1±2.2	1.8±1.0
day 10	0.0±1.2	0.1±1.9	1.5±0.9	0.0±2.0	-3.8±4.3	3.4±2.4	-2.1±2.4
day 13	0.7±1.9	-0.6±2.1	-2.3±2.0	-1.1±1.1	-8.7±4.3**	-2.1±2.1	-7.5±1.7**
day 16	0.7±3.2	-2.2±1.4	-3.1±1.5	-2.4±1.2	-10.7±3.3***	-2.3±1.5	-7.5±1.7*

[a]Treatments were administered on a daily basis. [b]Cisplatin-administered once every 3 days.

cells with less necrosis (10 to 30%). Cisplatin, EGCG and EGCG + Cisplatin groups exhibited extensive areas of necrosis exhibiting features of cytotoxicity (**Figure 5**). Liver tissue did not indicate any signs of drug induced toxicity or metastasis. We observed mucinous metaplasia in the ectocervix of MPP alone and MPP + EE treatment groups.

Next, donar HeLa xenografts consuming plain drinking water, with features of poorly differentiated carcinoma, were subsequently reimplanted into experimental animals (under EE treatment in drinking water primed one week before implantation). These xenograft implants failed to grow, reconfirming the earlier trend with donar HeLa xenografts (data not shown). We did not obtained sufficient tumor material (for subsequent implantation into the experimental groups), from donar HeLa xenografts that consumed EE in drinking water. Hence, we are unable to perform MPP, fulvestrant, Cisplatin and EGCG treatments with HeLa xenografts.

SERMs inhibits ERα expression predominantly localized in plasma membrane and cytoplasm

Control MS751 tumors showed strong to moderate cytoplasmic and membrane ERα staining,

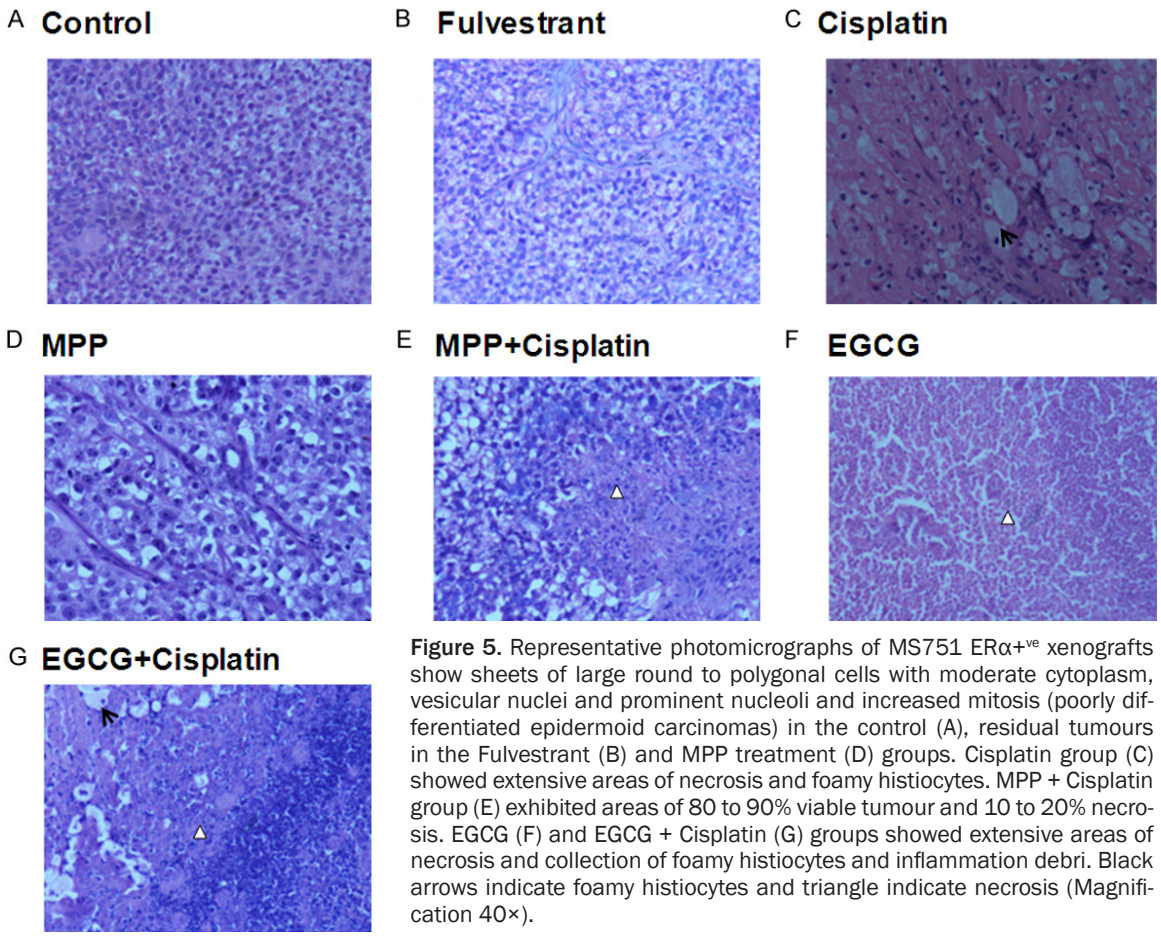


Figure 5. Representative photomicrographs of MS751 ERα+ve xenografts show sheets of large round to polygonal cells with moderate cytoplasm, vesicular nuclei and prominent nucleoli and increased mitosis (poorly differentiated epidermoid carcinomas) in the control (A), residual tumours in the Fulvestrant (B) and MPP treatment (D) groups. Cisplatin group (C) showed extensive areas of necrosis and foamy histiocytes. MPP + Cisplatin group (E) exhibited areas of 80 to 90% viable tumour and 10 to 20% necrosis. EGCG (F) and EGCG + Cisplatin (G) groups showed extensive areas of necrosis and collection of foamy histiocytes and inflammation debri. Black arrows indicate foamy histiocytes and triangle indicate necrosis (Magnification 40×).

with mild to moderate nuclear staining in confined areas and large regions of nuclear negative areas. Extensive necrosis was observed in the center of tumour sections, followed by viable tumour cells in the periphery, *per se* indicated the aggressiveness of developed tumours. MS751 tumours treated with fulvestrant and MPP showed less mitosis and the tumour cells were found to be negative for ERα. MPP + Cisplatin group was also found to be negative for ERα with extensive necrosis. As expected, Cisplatin, EGCG and EGCG + Cisplatin treatments exhibited large areas of necrosis, with non-specific background staining. The positive control, MCF-7 showed strong expression of nuclear ERα. ERα expression was undetectable in EE treated HeLa. Conversely, EE treated MS751 cells showed ERα expression in the cell membrane and cytoplasm. Nuclear ERα was completely absent in MS751 (Magnification 40×) (**Figure 6**).

Discussion

In this study, we employed a simple human CC cell line xenograft mouse model to investigate the association between estrogens and ERα in HPV+ve CC. Our findings have reconfirmed such association that has been extensively demonstrated with epidemiological studies [5-7, 10-12] and genetically engineered mouse models [3, 4, 9, 17-21, 37, 38].

Uterine cervix is sensitive to anti-estrogens

Estrogens are necessary for the normal physiological differentiation and maturation of uterine cervix. In fertile women, ER and progesterone receptor (PR) are highly expressed in the normal epithelium of the transformation zone, compared to the ectocervical epithelium [8]. ERα expression in the squamous epithelium of uterine ectocervix changed dynamically with respect to different phases of menstrual cycle [39]. In this study, we wanted to understand the responsiveness of normal uterine cervix to selected SERMs (Fulvestrant and MPP) and non-SERMs (EGCG) that were otherwise, efficacious in suppressing CC tumour growth. We found that fulvestrant can exert direct inhibitory effects on the cervix. On the other hand, MPP

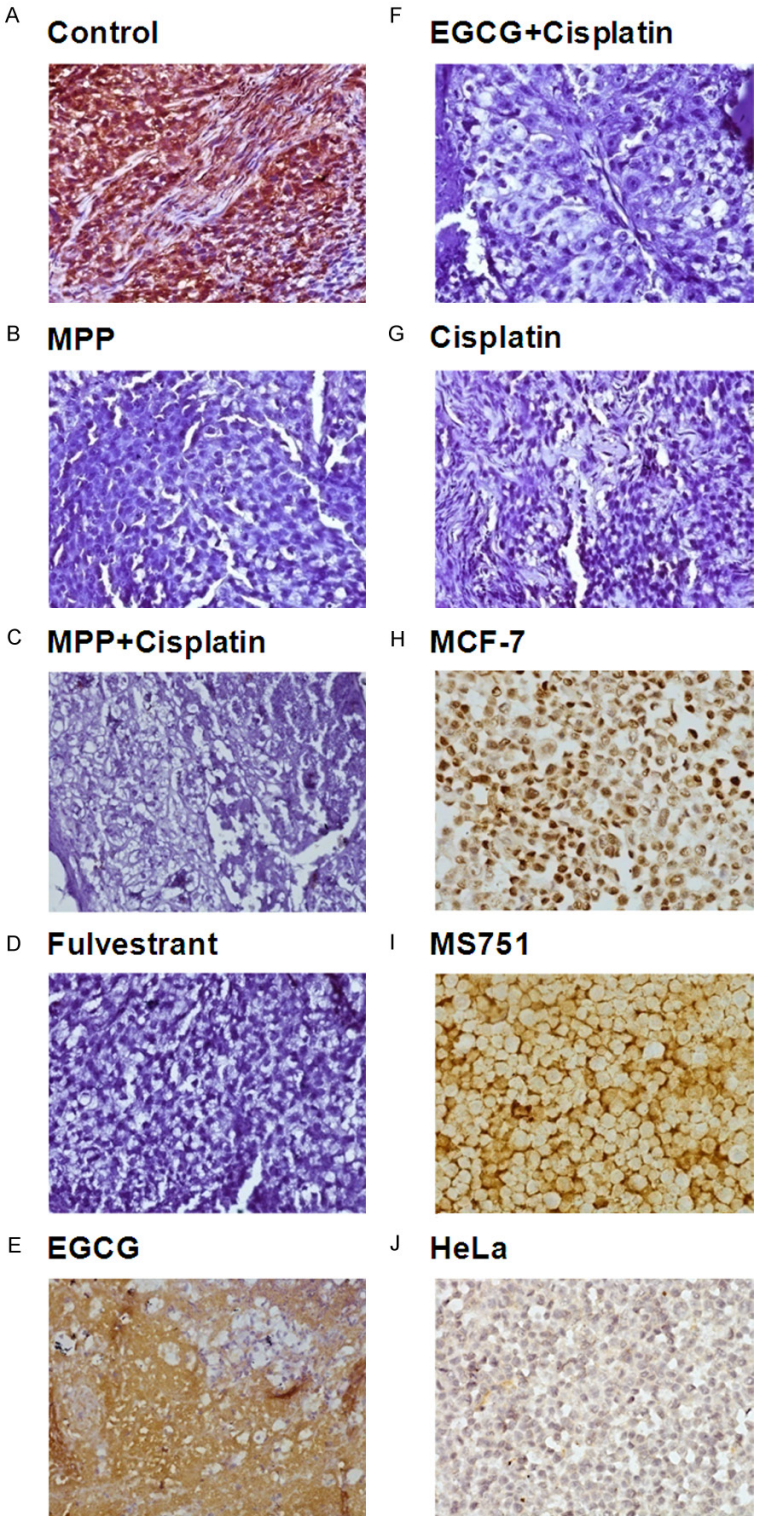


Figure 6. Representative photomicrographs from immunohistochemical staining of ERα expression in MS751 ERα+ve tumor xenografts shows predominant cytoplasmic and membrane ERα expression with limited nuclear ERα staining in confined areas of control tumours (A). Treatment with MPP, MPP + Cisplatin and fulvestrant showed loss of ERα in all the extra-nuclear compartments (B-D). Extensive necrosis of EGCG, EGCG + Cisplatin, Cisplatin showed widespread background staining with few viable areas of tumor cells (E-G). ERα was readily detectable in the nucleus of MCF-7 (H), cell membrane and cytoplasm of EE treated MS751 cells (I) and undetectable in EE treated HeLa cells (J) (Magnification 40×).

induced mucinous metaplasia only in the ectocervix, the most prominent site of initiation of cervical carcinogenesis. On the other hand, we did not observe the same with the other SERM, fulvestrant. Mucinous metaplasia of ectocervix is an uncommon finding due to estrogen sensitiveness of the squamous cells, owing to hyperestrogenic states and otherwise, physiologically found in the endocervix [40]. It's worth to note that 75 to 90% of CC are squamous cell carcinomas [41]. This shows the difference that exists in the tissue selective actions between SERMs and questions the ability of SERMs to qualify as a therapeutic component for CC. The fact that EGCG was unable to induce any signs of agonism or antagonism in cervix, allowed us to conclude that, EGCG could be a non-specific inhibitor, despite EGCG is known to inhibit ERα expression [42].

Endogenous ERα positivity determines the responsiveness of CC tumours to estrogens

In the present study, we have generated a xenograft mouse model with two human CC cell lines, MS751 and HeLa, known to harbor HPV18 integrated DNA. Both the xenografts formed tumors with features of poorly differentiated carcinoma. MS751 is known to express basal ERα at the protein level [28, 33] and form xenografts without any hormone stimulation [43]. In the present study, mere EE treatment has accelerated the growth of MS751 xenografts in Ncr Nude mice, much earlier compared to small size of tumours, after two months in SCID mice without any estrogen treatment [43]. The observation that, SERMs were able to antago-

nize the growth of MS751 ERα+ve CC xenografts driven by EE, allowed us to speculate that, mere presence of endogenous ERα is the factor responsible for driving CC carcinogenesis. On the other hand, it was observed that, HeLa ERα -ve CC xenografts treated with EE in drinking water failed to develop tumours. This is in line with the observation from other reports. Li et al demonstrated that HeLa cells upon in *vitro* E2 (17-β Estradiol) treatment in doses ranging from 0 to 10 μM concentrations showed a dose and time dependant increase in cell death [44]. In another study, E2 treatment ranging from 0 to 100 nM concentrations did not show any change in cell survival, neither an increase in ERα mRNA expression. This could be ascribed due to fundamental differences in E2 as a function of dose. High concentrations of E2 may lead to apoptosis in ERα-ve HeLa cells [44, 45]. Bulzomi has shown that E2 at 10 nM concentration reduced cell numbers of mock vector transfected HeLa cells suggesting an ERα-independent cytotoxic effect of E2 [46]. Hence, the pattern of growth inhibition observed with HeLa xenografts might be a result of apoptosis due to consecutive treatment with a strong estrogen, EE. On the other hand, Kozasa and co-workers have shown that administration of E2 or impregnation led to tremendous increase in growth of HeLa tumour xenografts. But, we observed an entirely opposite trend in the present study. We believe that, this difference can be ascribed due to fundamental pharmacological differences between EE and E2. EE is 100 fold much potent compared to E2 [47], which might have contributed to cellular growth suppression. Next, the timing of hormone treatment could influence on the degree of sensitization of cells. In the present study, the animals were primed with EE in drinking water one week before the first implantation of HeLa cells. We can only assume that, HeLa cells might have faced the cellular inhibitory effects of EE, before these cells try to establish themselves to survive the challenging in *vivo* pressure. Nevertheless, our results are supported by a 21 day study, which administered FSH to HeLa xenografts, which resulted in growth suppression [48]. Indeed, it was demonstrated that FSH treatment, before the initiation of HeLa xenografts showed better tumour suppression, rather than post- tumour initiation. E2 treatment was found to inhibit exogenously expressing ERα transfected MDA-MB-231 in *vitro* and also prevented metastasis when implanted in nude mice [49]. Hence, endogenous ERα can be regarded as a determinant of tumorigenesis. In our study, ERα was not exogenously expressed, but EE treatment inhibited HeLa tumour growth. This indicates that estrogen might have inhibited through ERα-independent pathway [50]. Secondly, HeLa cells are found to be sensitive to E2 induced cellular inhibition with or without the presence of exogenous ERα expression. Indeed, it is important to underline the role of cellular factors to choose on estrogen responsiveness and cell fate [36, 46, 51-56].

ERα encourages CC tumorigenesis predominantly from the extra nuclear compartment

Classically, nuclear ERα was considered to be an indicator of ER positivity and the role of extra- nuclear ERα (membrane, cytoplasm and stroma) especially in the context of cancer as a transformed cell, is underemphasized [57, 58]. It is now evident that palmitoylation process allows ERα to be localized to the membrane, in association with caveolin-1. Upon estrogen stimulation, ERα undergoes de-palmitoylation, de-associate with caveolin-1 and therefore allow the activation of rapid signals relevant for cellular proliferation [59, 60]. The activated steroid-receptor responses in turn activate a number of cascading signaling pathways within the cytoplasm/membrane and thus are responsible for the biological responses such as cell growth and survival [61, 62]. In breast cancer, ERα localized to cytoplasm and plasma membrane was shown to up-regulate proliferation and tumorigenic signals [63, 64]. With a similar trend, we could only speculate that, SERMs as a mimic of estrogens, could exert antitumor activity in CC by inhibiting extra-nuclear ERα localization possibly by interfering the palmitoylation and de-palmitoylation signals.

Very limited studies reported the involvement of cytoplasm and membrane ERα in CC. Darne and co-workers observed 54% of 131 squamous cell carcinoma biopsies with cytoplasmic ER positivity [65]. Sun and co-workers have shown that ERα36, a truncated variant isoform of ERα was found to be localized in the plasma membrane and cytoplasm, in both CC tissues and cell lines. ERα36 was shown to regulate migration, invasion and proliferation of CC cells [66].

In CC, estrogens exert their action through receptors such as ERα, ERβ, Prolactin Receptor and G-protein coupled ER which are located both in the nucleus and cytoplasm in contrasting proportions. It has been proposed that the early oncogenes of HPV with respect to HPV subtypes play a role in shifting the location of these receptors during CC progression [67-69]. Indeed, it has been shown that HPV oncogenes independently and synergistically with estrogen, reprogrammed the gene expression profiles of the tumor microenvironment in K14E6/E7 transgenic mouse model [70].

In our study, we found that ERα is predominantly expressed in the cytoplasm and membrane compartments of ERα+ve MS751 CC xenografts. We also found mild to moderate ERα staining localized to the nuclear compartment in confined areas. On the other hand, we observed ERα expression within the cytoplasm and membrane compartments of MS751 cell line block without any traces of nuclear staining. We could only speculate that such a difference in expression pattern might be a result of prolonged stimulation due to incessant EE treatment with an exchange of ERα localization between nuclear, cytoplasmic and membrane compartments. ERα expression was absent in nucleus as well as cytoplasm and membrane of HeLa cells. ERα expression was completely absent in groups treated with fulvestrant and MPP, in addition to tumour growth suppression. This allowed us to conclude that, cytoplasmic and membrane ERα positivity would be the responsible factor in driving the growth of MS751 xenografts. We are unable to recognize the ERα expression status of EGCG and cisplatin treated groups, owing to a heavy non-specific background staining due to necrosis.

Efficacy of SERMs in CC

We observed a trend of significant growth inhibition of MS751 xenografts due to SERMs treatment (fulvestrant and MPP). This is in line with the findings from other CC mouse models [18, 37]. We have demonstrated that MPP has antagonistic effect in uterus and an agonistic effect in the uterine cervix in the form of mucinous metaplasia. MPP is an ERα selective antagonist [71, 72]. MPP belongs to a class of triarylpyrazole with 1000 fold higher selectivity to ERα compared to other non-selective SERMs such as tamoxifen or raloxifene [72]. Fulvestrant is considered to be a pure ER antagonist [73], but do not discriminate between ERα and ERβ, and thus, the individual ER effects cannot be distinguished [71]. EGCG failed to elicit strong inhibition in the uterotrophic assay, which indicated its non-selectiveness to ERα. On the other hand, EGCG has proven anti-tumour activity by interacting with multitude of signaling pathways in a variety of cancers including CC [74-78] and is also capable of down regulating ERα [42, 79]. This explains the narrow differences in the efficacies observed between cisplatin, fulvestrant, MPP and EGCG.

To our knowledge, a xenograft model with human CC cell line was not tested for anti-cancer activity against ERα inhibited by SERMs. The lack of appropriate model to represent a ERα+ve CC model [13] and the hypothetical question of translatability of SERMs towards clinical application in CC was strongly perceived [80]. This model would be very relevant as a direct measure of treatment response, in view of human-like cellular machinery. We thought this would be much more pragmatic due to the fact that SERMs exert tissue specific ERα agonistic/antagonistic actions based on the cellular co-activator/co-repressor complexes [72, 81]. In our study, continuous EE was administered in drinking water that probably triggered the membrane and cytoplasmic ER to encourage the growth of CC xenografts. As expected selective ERα inhibitors (fulvestrant and MPP) superiorly inhibited the growth of ERα+ve MS751 xenografts. Hence, SERMS needs to be tailored to suit the tissue specific machinery present in ERα expressing CC. Alternatively, inhibitors targeted to antagonize specific co activator complex can also be designed. This indirect approach may also help to bring down ERα dependent CC. MPP was found to suppress the growth of CC xenografts. Instead, MPP stimulated mucinous metaplasia of ectocervix, reflecting hyperestrogenic status. Mucinous metaplasia might be an early indication of carcinogenesis event. It is known that SERMS could exert contrasting effects in different tissue targets [72, 81, 82]. Hence, the utility of SERMS in CC is still a benefit of doubt and the present study does not warrant considering the utility of SERMs as a therapeutic option. SERMs demonstrated both promising [83, 84] and inconclusive results [13, 85, 86], that limit

their use, due to lack of evidence from controlled clinical trials. In this study, we explored the capacity of SERMs to attenuate the growth of CC only in the perspective of antagonizing ERα positivity, but we failed to investigate if the chosen SERMS might have inhibited the proliferation of CC through other pathways. SERMs have also been demonstrated to bring down tumour burden independent of ER pathways [87, 88].

In conclusion, we have demonstrated that ERα positivity determines the sensitivity of CC xenografts to both exogenous estrogens and anti-estrogens, with ERα expression predominantly localized to the cell membrane and cytoplasm. SERMs could exert non-specific activities in uterine cervix, which calls for caution to utilize these SERMs in the treatment of CC.

Acknowledgements

This work was supported by Science and Engineering Research Board (SB/YS/LS-49/2013) to RB. The authors wish to thank Kirtana Reddy for proof reading the manuscript.

Disclosure of conflict of interest

None.

Address correspondence to: Balaji Ramachandran, Department of Molecular Oncology, Cancer Institute (W.I.A), No. 38, Sardar Patel Road, Adyar, Chennai 600 036, India. Tel: +914422209150; E-mail: balajiphd@gmail.com

References

[1] Arbyn M, Weiderpass E, Bruni L, de Sanjosé S, Saraiya M, Ferlay J and Bray F. Estimates of incidence and mortality of cervical cancer in 2018: a worldwide analysis. Lancet Glob Heal 2020; 8: e191-e203.

[2] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68: 394-424.

[3] Brake T and Lambert PF. Estrogen contributes to the onset, persistence, and malignant progression of cervical cancer in a human papillomavirus-transgenic mouse model. Proc Natl Acad Sci U S A 2005; 102: 2490-2495.

[4] Chung SH, Wiedmeyer K, Shai A, Korach KS and Lambert PF. Requirement for estrogen receptor alpha in a mouse model for human papillomavirus-associated cervical cancer. Cancer Res 2008; 68: 9928-9934.

[5] Marks M, Gravitt PE, Gupta SB, Liaw KL, Kim E, Tadesse A, Phongnarisorn C, Wootipoom V, Yuenyao P, Vipupinyo C, Rugpao S, Sriplienchan S and Celentano DD. The association of hormonal contraceptive use and HPV prevalence. Int J Cancer 2011; 128: 2962-2970.

[6] Marks MA, Gupta S, Liaw KL, Tadesse A, Kim E, Phongnarisorn C, Wootipoom V, Yuenyao P, Vipupinyo C, Rugpao S, Sriplienchan S, Gravitt PE and Celentano DD. Prevalence and correlates of HPV among women attending family-planning clinics in Thailand. BMC Infect Dis 2015; 15: 159.

[7] Liao SF, Lee WC, Chen HC, Chuang LC, Pan MH and Chen CJ. Baseline human papillomavirus infection, high vaginal parity, and their interaction on cervical cancer risks after a follow-up of more than 10 years. Cancer Causes Control 2012; 23: 703-708.

[8] Remoue F, Jacobs N, Miot V, Boniver J and Delvenne P. High intraepithelial expression of estrogen and progesterone receptors in the transformation zone of the uterine cervix. Am J Obstet Gynecol 2003; 189: 1660-1665.

[9] Elson DA, Riley RR, Lacey A, Thordarson G, Talamantes FJ and Arbeit JM. Sensitivity of the cervical transformation zone to estrogen-induced squamous carcinogenesis. Cancer Res 2000; 60: 1267-1275.

[10] Moreno V, Bosch FX, Muñoz N, Meijer CJ, Shah KV, Walboomers JM, Herrero R and Franceschi S; International Agency for Research on Cancer. Multicentric Cervical Cancer Study Group. Effect of oral contraceptives on risk of cervical cancer in women with human papillomavirus infection: the IARC multicentric case-control study. Lancet (London, England) 2002; 359: 1085-1092.

[11] Muñoz N, Franceschi S, Bosetti C, Moreno V, Herrero R, Smith JS, Shah KV, Meijer CJ and Bosch FX; International Agency for Research on Cancer. Multicentric Cervical Cancer Study Group. Role of parity and human papillomavirus in cervical cancer: the IARC multicentric case-control study. Lancet (London, England) 2002; 359: 1093-1101.

[12] Roura E, Travier N, Waterboer T, de Sanjosé S, Bosch FX, Pawlita M, Pala V, Weiderpass E, Margall N, Dillner J, Gram IT, Tjønneland A, Munk C, Palli D, Khaw KT, Overvad K, Clavel-Chapelon F, Mesrine S, Fournier A, Fortner RT, Ose J, Steffen A, Trichopoulou A, Lagiou P, Orfanos P, Masala G, Tumino R, Sacerdote C, Polidoro S, Mattiello A, Lund E, Peeters PH, Bueno-de-Mesquita HB, Quirós JR, Sánchez MJ, Navarro C, Barricarte A, Larrañaga N,

Ekström J, Lindquist D, Idahl A, Travis RC, Merritt MA, Gunter MJ, Rinaldi S, Tommasino M, Franceschi S, Riboli E and Castellsagué X. The influence of hormonal factors on the risk of developing cervical cancer and pre-cancer: results from the EPIC Cohort. PLoS One 2016; 11: e0147029.

[13] Chung SH, Franceschi S and Lambert PF. Estrogen and ERalpha: culprits in cervical cancer? Trends Endocrinol Metab 2010; 21: 504-511.

[14] Nair HB, Luthra R, Kirma N, Liu YG, Flowers L, Evans D and Tekmal RR. Induction of aromatase expression in cervical carcinomas: effects of endogenous estrogen on cervical cancer cell proliferation. Cancer Res 2005; 65: 11164-11173.

[15] Hellberg D. Sex steroids and cervical cancer. Anticancer Res 2012; 32: 3045-3054.

[16] Shai A, Pitot HC and Lambert PF. p53 Loss synergizes with estrogen and papillomaviral oncogenes to induce cervical and breast cancers. Cancer Res 2008; 68: 2622-2631.

[17] Herber R, Liem A, Pitot H and Lambert PF. Squamous epithelial hyperplasia and carcinoma in mice transgenic for the human papillomavirus type 16 E7 oncogene. J Virol 1996; 70: 1873-1881.

[18] Chung SH and Lambert PF. Prevention and treatment of cervical cancer in mice using estrogen receptor antagonists. Proc Natl Acad Sci U S A 2009; 106: 19467-19472.

[19] Son J, Park JW, Lambert PF and Chung SH. Requirement of estrogen receptor alpha DNA-binding domain for HPV oncogene-induced cervical carcinogenesis in mice. Carcinogenesis 2014; 35: 489-496.

[20] Morales-Peza N, Auewarakul P, Juárez V, García-Carrancá A and Cid-Arregui A. In vivo tissue-specific regulation of the human papillomavirus type 18 early promoter by estrogen, progesterone, and their antagonists. Virology 2002; 294: 135-140.

[21] Park JS, Rhyu JW, Kim CJ, Kim HS, Lee SY, Kwon YI, Namkoong SE, Sin HS and Um SJ. Neoplastic change of squamo-columnar junction in uterine cervix and vaginal epithelium by exogenous estrogen in hpv-18 URR E6/E7 transgenic mice. Gynecol Oncol 2003; 89: 360-368.

[22] Fonseca-Moutinho JA, Cruz E, Carvalho L, Prazeres HJ, de Lacerda MM, da Silva DP, Mota F and de Oliveira CF. Estrogen receptor, progesterone receptor, and bcl-2 are markers with prognostic significance in CIN III. Int J Gynecol Cancer 2004; 14: 911-920.

[23] Shen K, Yueng W and Ngan H. Estrogen and progesterone receptors in normal cervix and primary cervical carcinoma. Zhonghua Fu Chan Ke Za Zhi 1994; 29: 284-288, 317-318.

[24] Bodner K, Laubichler P, Kimberger O, Czerwenka K, Zeillinger R and Bodner-Adler B. Oestrogen and progesterone receptor expression in patients with adenocarcinoma of the uterine cervix and correlation with various clinicopathological parameters. Anticancer Res 2010; 30: 1341-1345.

[25] Nonogaki H, Fujii S, Konishi I, Nanbu Y, Ozaki S, Ishikawa Y and Mori T. Estrogen receptor localization in normal and neoplastic epithelium of the uterine cervix. Cancer 1990; 66: 2620-2627.

[26] Konishi I, Fujii S, Nonogaki H, Nanbu Y, Iwai T and Mori T. Immunohistochemical analysis of estrogen receptors, progesterone receptors, Ki-67 antigen, and human papillomavirus DNA in normal and neoplastic epithelium of the uterine cervix. Cancer 1991; 68: 1340-1350.

[27] Coelho FRG, Prado JCM, Pereira Sobrinho JS, Hamada G, Landman G, Pinto CA, Nonogaki S and Villa LL. Estrogen and progesterone receptors in human papilloma virus-related cervical neoplasia. Brazilian J Med Biol Res 2004; 37: 83-88.

[28] Zhai Y, Bommer GT, Feng Y, Wiese AB, Fearon ER and Cho KR. Loss of estrogen receptor 1 enhances cervical cancer invasion. Am J Pathol 2010; 177: 884-895.

[29] López-Romero R, Garrido-Guerrero E, Rangel-López A, Manuel-Apolinar L, Piña-Sánchez P, Lazos-Ochoa M, Mantilla-Morales A, Bandala C and Salcedo M. The cervical malignant cells display a down regulation of ER-α but retain the ER-β expression. Int J Clin Exp Pathol 2013; 6: 1594-1602.

[30] Nikolaou M, Koumoundourou D, Ravazoula P, Papadopoulou M, Michail G and Decavalas G. An immunohistochemical analysis of sex-steroid receptors, tumor suppressor gene p53 and Ki-67 in the normal and neoplastic uterine cervix squamous epithelium. Med Pregl 2014; 67: 202-207.

[31] Ramachandran B. Functional association of oestrogen receptors with HPV infection in cervical carcinogenesis. Endocr Relat Cancer 2017; 24: R99-R108.

[32] Ramachandran B, Jayavelu S, Murhekar K and Rajkumar T. Repeated dose studies with pure Epigallocatechin-3-gallate demonstrated dose and route dependant hepatotoxicity with associated dyslipidemia. Toxicol Rep 2016; 3: 336-345.

[33] López-Romero R, Rodríguez-Esquivel M, Romero-Morelos P, García-Avilés JE, Serafín-Castillo A, Huerta-Padilla VM, Guerra-Araiza C, Mantilla-Morales A, Monrroy-García A, Aguilar-Urbano MA, Martínez-Castillo MA, Jiménez-Tenorio JA and Salcedo M. The expression of transcription factor BORIS and its association with the estrogen receptor beta (ER-β) in cervical carci-

nogenesis. Int J Clin Exp Pathol 2019; 12: 3208-3221.
[34] Jang ER, Lim SJ, Lee ES, Jeong G, Kim TY, Bang YJ and Lee JS. The histone deacetylase inhibitor trichostatin A sensitizes estrogen receptor alpha-negative breast cancer cells to tamoxifen. Oncogene 2004; 23: 1724-1736.
[35] He Q, Liang CH and Lippard SJ. Steroid hormones induce HMG1 overexpression and sensitize breast cancer cells to cisplatin and carboplatin. Proc Natl Acad Sci U S A 2000; 97: 5768-5772.
[36] Hall JM and McDonnell DP. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 1999; 140: 5566-5578.
[37] Spurgeon ME, Chung SH and Lambert PF. Recurrence of cervical cancer in mice after selective estrogen receptor modulator therapy. Am J Pathol 2014; 184: 530-540.
[38] Jakacka M, Ito M, Martinson F, Ishikawa T, Lee EJ and Jameson JL. An estrogen receptor (ER) alpha deoxyribonucleic acid-binding domain knock-in mutation provides evidence for nonclassical ER pathway signaling in vivo. Mol Endocrinol 2002; 16: 2188-2201.
[39] Mosny DS, Herholz J, Degen W and Bender HG. Immunohistochemical investigations of steroid receptors in normal and neoplastic squamous epithelium of the uterine cervix. Gynecol Oncol 1989; 35: 373-377.
[40] Turashvili G and Childs T. Mucinous metaplasia of the endometrium: current concepts. Gynecol Oncol 2015; 136: 389-393.
[41] Parkin DM, Whelan SL, Ferlay J, Raymond L and Young J. Cancer incidence in five continents. Vol. VIII. Lyon: IARC Press; 2002.
[42] Qiao Y, Cao J, Xie L and Shi X. Cell growth inhibition and gene expression regulation by (-)-epigallocatechin-3-gallate in human cervical cancer cells. Arch Pharm Res 2009; 32: 1309-1315.
[43] Wu TC, Hsieh ST, Purow BW and Kurman RJ. Demonstration of human papillomavirus (HPV) genomic amplification and viral-like particles from CaSki cell line in SCID mice. J Virol Methods 1997; 65: 287-298.
[44] Li D, Chen J, Ai Y, Gu X, Li L, Che D, Jiang Z, Li L, Chen S, Huang H, Wang J, Cai T, Cao Y, Qi X and Wang X. Estrogen-related hormones induce apoptosis by stabilizing schlafen-12 protein turnover. Mol Cell 2019; 75: 1103-1116, e9.
[45] Kozasa K, Mabuchi S, Matsumoto Y, Kuroda H, Yokoi E, Komura N, Kawano M, Takahashi R, Sasano T, Shimura K, Kodama M, Hashimoto K, Sawada K, Nagasaka K and Kimura T. Estrogen stimulates female cancer progression by inducing myeloid-derived suppressive cells: investigations on pregnant and non-pregnant experimental models. Oncotarget 2019; 10: 1887-1902.
[46] Bulzomi P, Bolli A, Galluzzo P, Leone S, Acconcia F and Marino M. Naringenin and 17beta-estradiol coadministration prevents hormone-induced human cancer cell growth. IUBMB Life 2010; 62: 51-60.
[47] Stanczyk FZ, Archer DF and Bhavnani BR. Ethinyl estradiol and 17β-estradiol in combined oral contraceptives: pharmacokinetics, pharmacodynamics and risk assessment. Contraception 2013; 87: 706-727.
[48] Shi X, Qiu S, Zhuang W, Wang C, Zhang S, Yuan N, Yuan F and Qiao Y. Follicle-stimulating hormone inhibits cervical cancer via NF-κB pathway. Onco Targets Ther 2018; 11: 8107-8115.
[49] Garcia M, Derocq D, Freiss G and Rochefort H. Activation of estrogen receptor transfected into a receptor-negative breast cancer cell line decreases the metastatic and invasive potential of the cells. Proc Natl Acad Sci U S A 1992; 89: 11538-11542.
[50] Obrero M, Yu DV and Shapiro DJ. Estrogen receptor-dependent and estrogen receptor-independent pathways for tamoxifen and 4-hydroxytamoxifen-induced programmed cell death. J Biol Chem 2002; 277: 45695-45703.
[51] Maminta ML, Molteni A and Rosen ST. Stable expression of the human estrogen receptor in HeLa cells by infection: effect of estrogen on cell proliferation and c-myc expression. Mol Cell Endocrinol 1991; 78: 61-69.
[52] Treilleux, Peloux N, Brown M and Sergeant A. Human estrogen receptor (ER) gene promoter-P1: estradiol-independent activity and estradiol inducibility in ER+ and ER- cells. Mol Endocrinol 1997; 11: 1319-1331.
[53] Zajchowski DA, Sager R and Webster L. Estrogen inhibits the growth of estrogen receptor-negative, but not estrogen receptor-positive, human mammary epithelial cells expressing a recombinant estrogen receptor. Cancer Res 1993; 53: 5004-5011.
[54] Freund A, Chauveau C, Brouillet JP, Lucas A, Lacroix M, Licznar A, Vignon F, and Lazennec G. IL-8 expression and its possible relationship with estrogen-receptor-negative status of breast cancer cells. Oncogene 2003; 22: 256-265.
[55] Sathya G, Yi P, Bhagat S, Bambara RA, Hilf R and Muyan M. Structural regions of ERalpha critical for synergistic transcriptional responses contain co-factor interacting surfaces. Mol Cell Endocrinol 2002; 192: 171-185.
[56] Papoutsi Z, Kassi E, Mitakou S, Aligiannis N, Tsiapara A, Chrousos GP and Moutsatsou P. Acteoside and martynoside exhibit estrogenic/

antiestrogenic properties. J Steroid Biochem Mol Biol 2006; 98: 63-71.
[57] Kumar R, Wang RA, Mazumdar A, Talukder AH, Mandal M, Yang Z, Bagheri-Yarmand R, Sahin A, Hortobagyi G, Adam L, Barnes CJ and Vadlamudi RK. A naturally occurring MTA1 variant sequesters oestrogen receptor-alpha in the cytoplasm. Nature 2002; 418: 654-657.
[58] Kumar R. Another tie that binds the MTA family to breast cancer. Cell 2003; 113: 142-143.
[59] Levin ER. Cellular functions of plasma membrane estrogen receptors. Steroids 2002; 67: 471-475.
[60] Marino M and Ascenzi P. Membrane association of estrogen receptor alpha and beta influences 17beta-estradiol-mediated cancer cell proliferation. Steroids 2008; 73: 853-858.
[61] Norman AW, Mizwicki MT and Norman DP. Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. Nat Rev Drug Discov 2004; 3: 27-41.
[62] Evinger AJ 3rd and Levin ER. Requirements for estrogen receptor alpha membrane localization and function. Steroids 2005; 70: 361-363.
[63] Ohshiro K, Mudvari P, Meng QC, Rayala SK, Sahin AA, Fuqua SA and Kumar R. Identification of a novel estrogen receptor-alpha variant and its upstream splicing regulator. Mol Endocrinol 2010; 24: 914-922.
[64] Marczell I, Balogh P, Nyiro G, Kiss AL, Kovacs B, Bekesi G, Racz K and Patocs A. Membrane-bound estrogen receptor alpha initiated signaling is dynamin dependent in breast cancer cells. Eur J Med Res 2018; 23: 31.
[65] Darne J, Soutter WP, Ginsberg R and Sharp F. Nuclear and "cytoplasmic" estrogen and progesterone receptors in squamous cell carcinoma of the cervix. Gynecol Oncol 1990; 38: 216-219.
[66] Sun Q, Liang Y, Zhang T, Wang K and Yang X. ER-α36 mediates estrogen-stimulated MAPK/ERK activation and regulates migration, invasion, proliferation in cervical cancer cells. Biochem Biophys Res Commun 2017; 487: 625-632.
[67] Riera-Leal A, Ramírez De Arellano A, Ramírez-López IG, Lopez-Pulido EI, Dávila Rodríguez JR, Macías-Barragan JG, Ortiz-Lazareno PC, Jave-Suárez LF, Artaza-Irigaray C, Del Toro Arreola S, Montoya-Buelna M, Muñoz-Valle JF and Pereira-Suárez AL. Effects of 60 kDa prolactin and estradiol on metabolism and cell survival in cervical cancer: co-expression of their hormonal receptors during cancer progression. Oncol Rep 2018; 40: 3781-3793.
[68] Ramírez-López IG, Ramírez de Arellano A, Jave-Suárez LF, Hernández-Silva CD, García-Chagollan M, Hernández-Bello J, Lopez-Pulido EI, Macias-Barragan J, Montoya-Buelna M, Muñoz-Valle JF and Pereira-Suárez AL. Interaction between 17β-estradiol, prolactin and human papillomavirus induce E6/E7 transcript and modulate the expression and localization of hormonal receptors. Cancer Cell Int 2019; 19: 227.
[69] Hernandez-Silva CD, Riera-Leal A, Ortiz-Lazareno PC, Jave-Suárez LF, Ramírez De Arellano A, Lopez-Pulido EI, Macías-Barragan JG, Montoya-Buelna M, Dávila-Rodríguez JR, Chabay P, Muñoz-Valle JF and Pereira-Suárez AL. GPER overexpression in cervical cancer versus premalignant lesions: its activation induces different forms of cell death. Anticancer Agents Med Chem 2019; 19: 783-791.
[70] Spurgeon ME, den Boon JA, Horswill M, Barthakur S, Forouzan O, Rader JS, Beebe DJ, Roopra A, Ahlquist P and Lambert PF. Human papillomavirus oncogenes reprogram the cervical cancer microenvironment independently of and synergistically with estrogen. Proc Natl Acad Sci U S A 2017; 114: E9076-E9085.
[71] Sun J, Huang YR, Harrington WR, Sheng S, Katzenellenbogen JA and Katzenellenbogen BS. Antagonists selective for estrogen receptor alpha. Endocrinology 2002; 143: 941-947.
[72] Zhou HB, Carlson KE, Stossi F, Katzenellenbogen BS and Katzenellenbogen JA. Analogs of methyl-piperidinopyrazole (MPP): antiestrogens with estrogen receptor alpha selective activity. Bioorg Med Chem Lett 2009; 19: 108-110.
[73] Fertuck KC, Eckel JE, Gennings C and Zacharewski TR. Identification of temporal patterns of gene expression in the uteri of immature, ovariectomized mice following exposure to ethynylestradiol. Physiol Genomics 2003; 15: 127-141.
[74] Xu Q, Yang CH, Liu Q, Jin XF, Xu XT, Tong JL, Xiao SD and Ran ZH. Chemopreventive effect of epigallocatechin-3-gallate (EGCG) and folic acid on the N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced gastrointestinal cancer in rat model. J Dig Dis 2011; 12: 181-187.
[75] Pan MH, Chiou YS, Wang YJ, Ho CT and Lin JK. Multistage carcinogenesis process as molecular targets in cancer chemoprevention by epicatechin-3-gallate. Food Funct 2011; 2: 101-110.
[76] Yokoyama M, Noguchi M, Nakao Y, Pater A and Iwasaka T. The tea polyphenol, (-)-epigallocatechin gallate effects on growth, apoptosis, and telomerase activity in cervical cell lines. Gynecol Oncol 2004; 92: 197-204.
[77] Negri A, Naponelli V, Rizzi F and Bettuzzi S. Molecular targets of epigallocatechin-gallate (EGCG): a special focus on signal transduction and cancer. Nutrients 2018; 10: 1936.
[78] Khan N, Afaq F, Saleem M, Ahmad N and Mukhtar H. Targeting multiple signaling path-

ways by green tea polyphenol (-)-epigallocatechin-3-gallate. Cancer Res 2006; 66: 2500-2505.
[79] Park SB, Bae JW, Kim JM, Lee SG and Han M. Antiproliferative and apoptotic effect of epigallocatechin-3-gallate on Ishikawa cells is accompanied by sex steroid receptor downregulation. Int J Mol Med 2012; 30: 1211-1218.
[80] Chung SH. Targeting female hormone receptors as cervical cancer therapy. Trends Endocrinol Metab 2015; 26: 399-401.
[81] Martinkovich S, Shah D, Planey SL and Arnott JA. Selective estrogen receptor modulators: tissue specificity and clinical utility. Clin Interv Aging 2014; 9: 1437-1452.
[82] Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J, Daly M, Wieand S, Tan-Chiu E, Ford L and Wolmark N. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998; 90: 1371-1388.
[83] De Rosa N, Lavitola G, Giampaolino P, Morra I, Nappi C and Bifulco G. Impact of ospemifene on quality of life and sexual function in young survivors of cervical cancer: a prospective study. Biomed Res Int 2017; 2017: 7513610.
[84] Hsieh CJ, Hong MK, Chen PC, Wang JH and Chu TY. Antiestrogen use reduces risk of cervical neoplasia in breast cancer patients: a population-based study. Oncotarget 2017; 8: 29361-29369.
[85] Castle PE. Do selective estrogen receptor modulators treat cervical precancer and cancer? Time to pool data from relevant trials. Int J Cancer 2011; 128: 997-998.
[86] Munger K. Are selective estrogen receptor modulators (SERMs) a therapeutic option for HPV-associated cervical lesions and cancers? Am J Pathol 2014; 184: 358-361.
[87] Chauhan N, Maher DM, Hafeez BB, Mandil H, Singh MM, Yallapu MM, Jaggi M and Chauhan SC. Ormeloxifene nanotherapy for cervical cancer treatment. Int J Nanomedicine 2019; 14: 7107-7121.
[88] Chauhan N, Maher DM, Yallapu MM, B Hafeez B, Singh MM, Chauhan SC and Jaggi M. A triphenylethylene nonsteroidal SERM attenuates cervical cancer growth. Sci Rep 2019; 9: 10917.