# Effects of 4-hydroxytamoxifen, raloxifene and ICI 182 780 on survival of uterine cancer cell lines in the presence and absence of exogenous estrogens

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Received June 30, 2006; Accepted August 10, 2006

**Abstract.** The impact of 17ß-estradiol and antiestrogens on uterine cancer cells is poorly understood. The aim of this study was to determine the impact of 17ß-estradiol, 4-hydroxytamoxifen, raloxifene and ICI 182 780 on the cell proliferation of six uterine cancer cell lines: HeLa, HEC-1-A, KLE, RL-95-2, Ishikawa and EN-1078D. The effects of these compounds on the cell proliferation of the six uterine cancer cell lines were studied in the presence and absence of estrogens (phenol red and serum deprivation of sex steroids). In a general manner, 17ß-estradiol and 4-hydroxytamoxifen showed similarities in their effects whereas raloxifene showed a different pattern of cell proliferation (agonistic and antagonistic) and ICI 182 780 had antagonistic activity. In the presence and absence of estrogens, we observed that each cell line had diverse expression of ERα, ERß, GPR30 and REA. GPR30 mRNA expression was significantly reduced in a serum/phenol-free medium. REA mRNA expression was not influenced by the media. Results demonstrated the importance of removing phenol red and the use of deprived serum when studying uterine cancer cells in relationship with 17ß-estradiol and antiestrogens. The affinity of each compound to the binding of ERα and ERß was very similar with the exception of raloxifene that had a preference for ERα binding. Akt phosphorylation/activity was reduced in cells cultured in a phenol red- and steroid-free culture medium indicating that the presence of steroids in the culture media can influence the activity of this survival pathway. Our results suggest that the expression of ERα, ERß and GPR30 are influenced by sex steroids and might play a role in the response of cells to 17ß-estradiol and antiestrogens but are not the only factors involved in this process.

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*Key words:* estrogen receptor, GPR30, REA, phenol red, Akt

## Introduction

Estrogens stimulate many biosynthetic processes in hormone-responsive cells such as in breast and uterus (1-3) and are known to play a major role in the etiology of breast and endometrial cancers (4,5). Worldwide, breast cancer is the most frequent of all female cancers whereas endometrial cancer is the fourth most frequent(6).

The importance of estrogens was extensively studied in the past two decades, in particular using breast cancer cell lines. Concerned about eliminating exogenous sources of estrogens in cell culture media, researchers deprived sera of steroid hormones (7,8) but cell proliferation seemed to be affected by other estrogenic sources. After an examination of the potential sources of estrogens, Berthois *et al* (9) demonstrated that phenol red, used in media as a pH indicator, had estrogenic properties at concentrations found in tissue culture media; it binds to estrogen receptors causing significant stimulation of cell proliferation and specific protein synthesis in cells responding to estrogens. Following this finding, it was suggested to consider the estrogenic activity of phenol red in any study of estrogen-responsive cells.

Estrogens can act in target cells by two mechanisms: a genomic or classical mechanism and a non-genomic or rapid mechanism. The first mechanism involves the estrogen receptors (ERs), proteins to which estrogen binds after its intracellular diffusion (10). Consecutive to its binding to estrogen, a conformational change appears in the ERs, permitting interactions with transcriptional co-regulators and regulatory DNA sequences of target genes (10,11). A physiological response within hours following the estrogen exposure takes place in consequence of synthesis in proteins and mRNAs that DNA sequences encode (11). The non-genomic mechanism gives response to estrogen within seconds or minutes by a direct effect on proteins and on certain signalling pathways, which suggests that this mechanism is membrane-mediated (11,12).

ERs belong to the nuclear receptor superfamily of transcription factors and have two isoforms, ERα and ERß (13,14). Human ERα was first cloned in 1986 (15-17) whereas, human ERß was cloned in 1996 (18,19). These two isoforms are expressed in breast and uterus tissues (13,20). Recently, a new receptor in the G protein-coupled receptor (GPCR) family was discovered, GPR30 (21). This receptor presented all

characteristics of a membrane estrogen receptor including the capacity to bind estrogens (22).

The ERs' activity is influenced not only by the binding of estrogen but also by coregulator proteins. These proteins can act like coactivators to enhance or, like corepressors, to repress transcriptional activity (13,23). A new repressor of estrogen receptor activity, REA, has been identified (24). This protein represses the transcriptional activity of ERs only and has no effect on other steroid or non-steroid nuclear receptors (24).

Antiestrogens are compounds that compete with estrogens to bind the ERs, but are unable to activate it (25,26). Tamoxifen (Nolvadex™) and raloxifene (Evista™) are selective estrogen receptor modulators (SERMs); they can exert an agonistic or antagonistic action to estrogens depending on the target tissue (27,28). Tamoxifen is widely used in the prevention and the treatment of breast cancer (29-32). However, the long term use of tamoxifen increases endometrial cancer risk (33,34). Raloxifene, developed originally for breast cancer therapy (35), is now given to patients in prevention and treatment of osteoporosis (36,37) and appears to be safe for the endometrium (36,38). ICI 182 780 (Faslodex™) is another type of anti-estrogen that has only an antagonistic action towards estrogens and is called a pure antiestrogen (39). The latter compound can be used in cases of localized advance or metastatic breast cancer in menopausal women (40) or in the development of resistance to tamoxifen treatment (41).

In the past, most attention has been directed towards breast cancer when studying the effects of estrogens and anti-estrogens. However, several studies showed that tamoxifen exerts estrogenic effects on uterine tissues. Thus, we used six uterine cancer cell lines to investigate and establish links between the expression of ERs and the impact of 17ß-estradiol and antiestrogens (4-hydroxytamoxifen, raloxifene and ICI 182 780) on cell proliferation in the presence and absence of estrogens (sex steroid-deprived serum and phenol red-free media).

In the present study, we have characterized the effect of 17ß-estradiol, 4-hydroxytamoxifen, raloxifene and ICI 182 780 on the cell proliferation of HeLa, HEC-1-A, KLE, RL-95-2, Ishikawa and EN-1078D cells. The affinity of each compound to ERs was determined by binding assay. The expression of the two ER isoforms at mRNA and protein levels was characterized. Messenger RNA abundance of GPR30 and REA was also determined. The presence of the active form of Akt (phospho-Akt) was also measured. All experiments were carried out in the presence and absence of estrogens. Finally, our results suggest that the presence of each ER isoform plays a role in the proliferative response of the uterine cancer cells to each compound in the absence of estrogens.

## Materials and methods

*Reagents*. 17ß-estradiol, 4-hydroxytamoxifen, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), charcoal activated and dextran were purchased from Sigma (St. Louis, MO). Raloxifene was obtained from Toronto Research Chemicals Inc. (North York, ON). ICI 182 780 was purchased from TOCRIS (Ellisville, MO). DMEM/F12 with and without phenol red containing HEPES, McCoy's, RPMI, BGS, FBS and PCR primers were obtained from Life Technologies (Burlington, ON). DMEM/F12 without phenol red was bought from Hyclone (Logan, UT). Anti-ERα Ab-15 (Clone AER611) and anti-ERß Ab-24 were purchased from Lab Vision (Fremont, CA). Akt and phospho-Akt antibodies were obtained from Cell Signaling Technology (Beverly, MA). HitHunter™ EFC Estrogen Fluorescence Assay Kit was obtained from Discoverx (Fremont, CA).

*Cell culture*. Human endometrial (HEC-1-A, KLE, RL-95-2) and cervical (HeLa) cancer cells were obtained from ATCC (Manassas, VA). Human endometrial Ishikawa cells were generously provided by Dr Sylvie Mader and EN-1078D was characterized by our group. Cells were cultured in 25- or 75-cm$^2$ flasks at 37˚C in an atmosphere of 5% $CO_2$. HeLa, KLE, Ishikawa and EN-1078D cells were maintained in DMEM/F12 containing phenol red supplemented with 2.438 g/l (1.34 g/l for KLE) of $NaHCO_3$, 2% BGS (10% FBS for KLE) and 50 µg/ml gentamycin. HEC-1-A cells were grown in McCoy's containing phenol red supplemented with 2.2 g/l of $NaHCO_3$, 5% BGS, and 50 µg/ml gentamycin. RL-95-2 cells were cultured in DMEM/F12 containing phenol red supplemented with 1.75 g/l of $NaHCO_3$, 10% of BGS, 5 µM HEPES, 2.5 µg/ ml insulin and 50 µg/ml gentamycin. After a minimum of two passages cells were transferred to another media. HeLa, KLE, Ishikawa and EN-1078D cells were maintained in DMEM/F12 supplemented with 2% FBS (10% for KLE) and 50 µg/ml gentamycin. HEC-1-A cells were grown in RPMI supplemented with 5% FBS and 50 µg/ml gentamycin. RL-95-2 cells were cultured in DMEM/F12 supplemented with 15 µM HEPES, 10% FBS and 50 µg/ml gentamycin. After a minimum of two passages in this new media, cells were used for MTT experiments. All experiments in the absence of estrogens were performed with these media but phenol red-free and supplemented with FBS depleted of sex steroids by dextran-charcoal extraction (FBS-DC).

*MTT proliferation assay*. Cells were plated in 96-wells plates in their respective media. At 60% confluence, media were changed for media without serum for 24 h (starvation). Cells were then cultured for 24 and 48 h in the presence of different concentrations of 17ß-estradiol, 4-hydroxytamoxifen, raloxifene or ICI 182 780 in their respective media without serum. Four hours before the end of the culture period, 10 µl of MTT (5 mg/ml) was added to each well. At the end of the culture period, 100 µl/well of solubilization solution was added (10% SDS in 0.01 M HCl). The microplate was incubated overnight at 37˚C in a 5% $CO_2$ atmosphere. The OD was read with the FluoSTAR OPTIMA (BMG Labtechnologies Inc.; Durham, CN) between 550 and 600 nm. The results are presented as a percentage of cell proliferation in comparison with control cells (e.g. untreated).

*Protein extraction and Western analysis*. Cells were trypsinized, lysed in RIPA lysis buffer (pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40 in PBS, complete mini; protease inhibitor cocktail (Roche, Indianapolis, IN), frozen/thawed three times, then centrifuged (13000 x g, 20 min at 4˚C) to remove insoluble material. The supernatant was recovered and stored at -20˚C pending analysis. Protein

concentration was determined with the Bio-Rad DC protein assay (Bio-Rad, Mississauga, ON). Protein extracts (50 $\mu$g) were heated at 95˚C for 3 min, resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred to nitrocellulose membranes at 15 V for 30 min using a semidry transfer system (Bio-Rad). The membranes were then blocked for 2 h at RT with PBS containing 5% milk powder and 0.05% Tween, then incubated with anti-ERα (1:500), anti-ERß (1:2000), anti-Akt (1:2000), or anti-pAkt (1:1500) overnight at 4˚C and subsequently with horseradish peroxidase (HRP)-conjugated anti-rabbit (1:4000) or anti-mouse (1:2500) secondary antibodies for 45 min at RT. Peroxidase activity was visualized with the Super signal® (West Femto maximum sensitivity substrate (Pierce, Arlington Heights, IL) according to the manufacturer's instructions.

*RNA extraction and semi-quantitative RT-PCR analysis*. Total RNA was extracted with TRIzol® reagent (Life Technologies) according to the manufacturer's instructions. Total RNA (0.4 $\mu$g) was used for preparation of the first strand cDNA by reverse transcriptase (RT). The RNA samples were incubated at 65˚C for 10 min with 2 $\mu$l oligo dT (deoxythymidine) primers in a final volume of 10 $\mu$l. Samples were then incubated at 37˚C for 60 min in 20 $\mu$l of reaction buffer (1X) containing 10 mM dithiothreitol (DTT), 0.5 mM deoxynucleotide triphosphates (dNTPs) and 10 U Muloney murine leukemia virus reverse transcriptase (MMLV-RT). After cDNA synthesis, the reaction volumes were brought up to 60 $\mu$l with RNAse-DNAse free water.

Human ER0.4 $\mu$g mRNA NM_000125; gi:62821793; 264 pb (1504-1768 pb) was amplified using sense primer 5'-GTGCCTGGCTAGAGATCCTG-3' and antisense primer 5'-AGAGACTTCAGGGTGCTGGA-3'. For ERß mRNA, the expression was determined by amplification of NM_001437; gi:10835012; 172 pb (1358-1530 pb) and the sequences of the primers were 5'-AAGAAGATTCCCGGCTTTGT-3' (sense) and 5'-TCTACGCATTTCCCCTCATC-3' (antisense). Expression of GPR30 was determined by amplification of NM_001505; gi:4504090; 187 pb (1633-1820 pb). Amplification was carried out using the sense upstream sequence 5'-CTCCAACAGCTGCCTAAACC-3; and the antisense downstream sequence 5'-TCTACACGGCACTGC TGAAC-3'. REA mRNA AF150962; gi:5020252; 396 pb (385-781 pb) was amplified using sense primer 5'-CGAAAA ATCTCCTCCCCTACA-3' and antisense primer 5'-CCTGCT TTGCTTTTTCTACCA-3'. Human ß-actin mRNA was amplified by the sense primer 5'-GAGGATCTTCATGAG GTAGTCTGTCAGGTC-3' and the antisense primer 5'-CAA CTGGGACGACATGGAGAAGATCTGGCA-3'. Each reaction mixture (final volume 50 $\mu$l) contains 5 $\mu$l RT template or negative control, 2.5 $\mu$l each primer, 5 $\mu$l ThermoPol buffer (New England Biolabs; Mississauga, ON), 2 $\mu$l dNTPs and 0.3 $\mu$l Taq polymerase (New England Biolabs). The PCR cycling conditions were: i) 30 sec at 94˚C, ii) 30 sec at 66˚C (ERα) or at 58˚C (ERß, GPR30 and REA), iii) 30 sec at 72˚C for 35 cycles (REA) or 40 cycles (ERα, ERß, GPR30). ß-actin was used as the control reaction. Reaction products were analyzed on 1.0% agarose gels. Bands were visualized by ethidium bromide or SYBR safe™ DNA gel stain (Molecular Probes, Eugene, OR) staining. Densitometry was determined with the Quantity One Software version 4.5.2 (Bio-Rad).

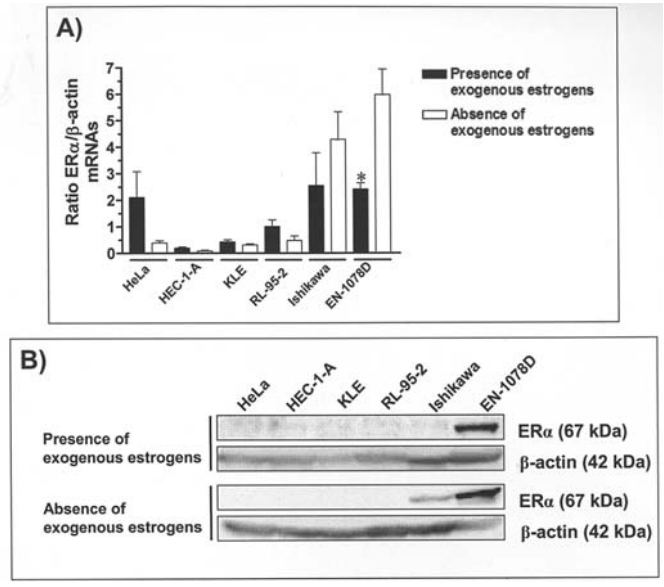


Figure 1. (A) ERα mRNA abundance in uterine cancer cell lines studied in the presence and absence of exogenous estrogens as determined by RT-PCR. Data analysis was performed by quantity one software and is presented as a ratio (value/ß-actin). Results represent the mean ± SEM of 3 independent experiments. *P<0.05 compared to the absence of exogenous estrogens. (B) ERα protein expression in each cancer cell line in the presence and absence of exogenous estrogens as determined by Western blotting. One representative experiment of 3 is shown. ß-actin was used as a control to correct for loading.

*ER binding assay*. The HitHunter™ enzyme fragment complementation (EFC) estrogen fluorescence assay kit was used according to the manufacturer's instructions. Briefly, the principle refers to the detection of a target of interest by controlling the complementation of two inactive ß-galactosidase enzyme fragments termed enzyme donor (ED) and enzyme acceptor (EA) with a binding protein. A target of interest can be covalently attached to ED, minimally affecting its structure and function. Unknown targets and ED conjugate compete for binding to a target specific binding protein. Unbound ED will recombine with EA to form active ß-galactosidase enzyme that is quantified by substrate hydrolysis. The signal generated is proportional to the concentration of the bound target.

*Statistical analysis*. All experiments were repeated a minimum of three times. Data were subjected to One-way ANOVA (PRISM software version 4.0; GraphPad, San Diego, CA) or Student's t-test. Differences between experimental groups were determined by Tukey's test.

## Results

*Expression of mRNA genes*. To determine the abundance of ERα, ERß, GPR30 and REA mRNAs in human uterine cancerous cell lines, RT-PCR was carried out using specific primers chosen from human DNA sequences and amplified with the PTC-100 thermal cycler (MJ Research, Waltham, MA). In the presence of estrogens, the ERα mRNA expression was detected in all cell lines, weakly in HEC-1-A cells and highly in HeLa, Ishikawa and EN-1078D cells (Fig. 1A). In the absence of estrogens, the presence of ERα was almost undetectable in HeLa, HEC-1-A, KLE and RL-95-2 cells

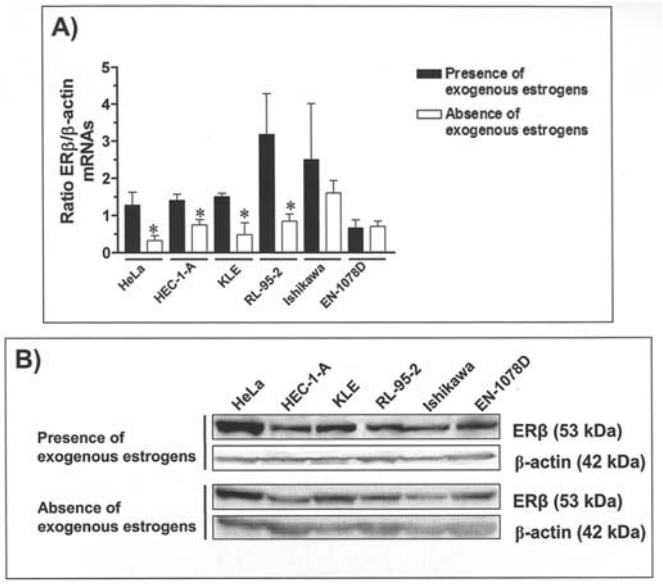


Figure 2. (A) ERß mRNA abundance in each cell line studied in the presence and absence of exogenous estrogens as determined by RT-PCR. Data analysis was performed by quantity one software and is presented as a ratio (value/ ß-actin). Results represent the mean ± SEM of 3 independent experiments. *P<0.05 compared to the presence of exogenous estrogens. (B) ERß protein expression in each cancer cell line in the presence and absence of exogenous estrogens as determined by Western blotting. One representative experiment of 3 is shown. ß-actin was used as a control to correct for loading.

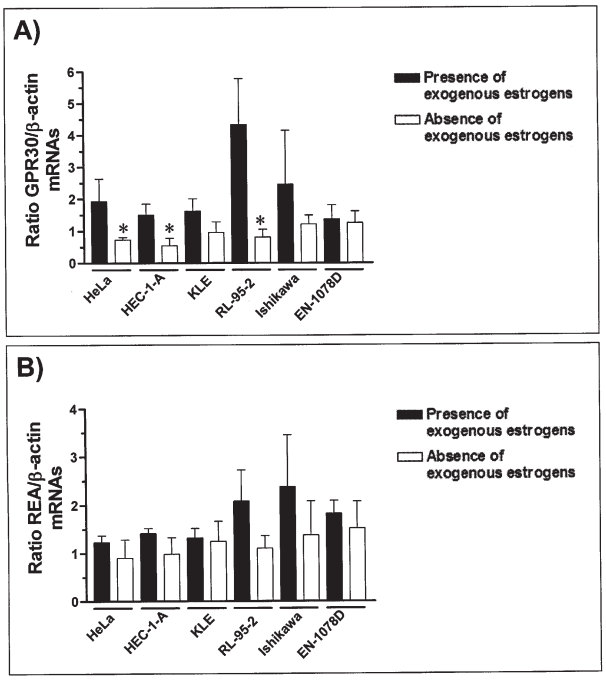


Figure 3. (A) GPR30 mRNA abundance in each cell line studied in the presence and absence of exogenous estrogens as determined by RT-PCR. *P<0.05 compared to the presence of exogenous estrogens. (B) REA mRNA abundance in each cell line studied in the presence and absence of exogenous estrogens as determined by RT-PCR. ß-actin was used as a control to correct for loading. Data analysis was performed by quantity one software and is presented as a ratio (value/ß-actin). Results represent the mean ± SEM of 3 independent experiments.

compared to Ishikawa and EN-1078D cells (Fig. 1A). A significant difference was observed in EN-1078D cells in the presence and absence of estrogens (P<0.05). ERß mRNA abundance was detected in the six cell lines in the presence of estrogens (Fig. 2A). In the absence of estrogens, the ERß mRNA expression was significantly reduced in HeLa, HEC-1-A, KLE and RL-95-2 and Ishikawa cells when compared with in the presence of estrogens (P<0.05). The expression of GPR30 was observed in all uterine cancer cells with no significant differences in the presence of estrogens (Fig. 3A). However, in the absence of estrogens, the basal mRNA level of GPR30 was significantly reduced in HeLa, HEC-1-A, KLE and RL-95-2 cells compared with in the presence of estrogens (P<0.05). REA mRNA, was detected in all cell lines without significant difference in the presence or in the absence of estrogens (Fig. 3B).

*Protein expression*. In order to confirm results obtained at the mRNA level for ERα and ERß, Western blot analyses were carried out to detect the presence of ERα and ERß proteins. Surprisingly, in the presence of estrogens, ERα protein was only detected in EN-1078D cells whereas all cell lines presented mRNA at different levels (Fig. 1B). In the absence of estrogens, this protein was observed in Ishikawa and predominantly in EN-1078D cell lines. ERß protein was present in all uterine cell lines but predominant in HeLa cells and this was observed in the presence and absence of estrogens (Fig. 2B). To verify if the levels of ER proteins could be modulated by 17ß-estradiol, time course experiments were carried out for 0, 5, 15, 60, 120 min and 24 and 48 h with 17ß-estradiol ($10^{-8}$ M) in the absence of estrogens in HeLa and EN-1078D cells. Results showed no significant variation

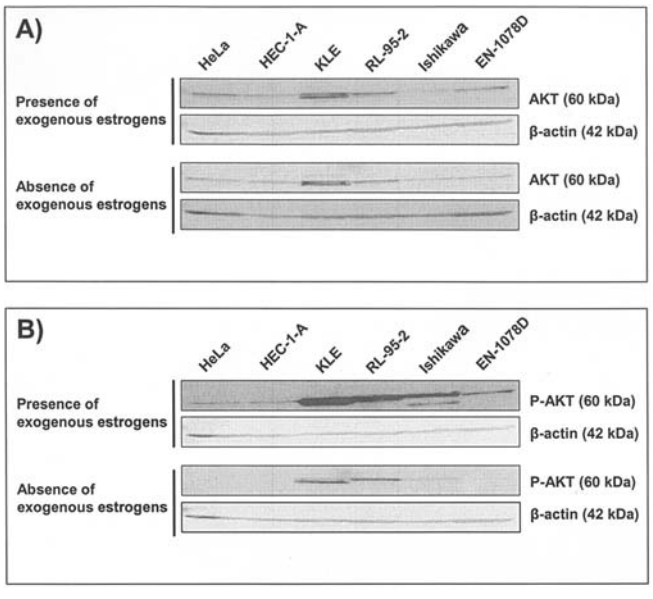


Figure 4. Total Akt (A) and phospho-Akt (B) protein expression in each cancer cell line in the presence and absence of exogenous estrogens as determined by Western blotting. One representative experiment of 4 is shown. ß-actin was used as a control to correct for loading.

in the ERα and ERß protein level expression (data not shown).

Akt has been shown to be regulated by estradiol in different systems and we sought to determine if the constitutive

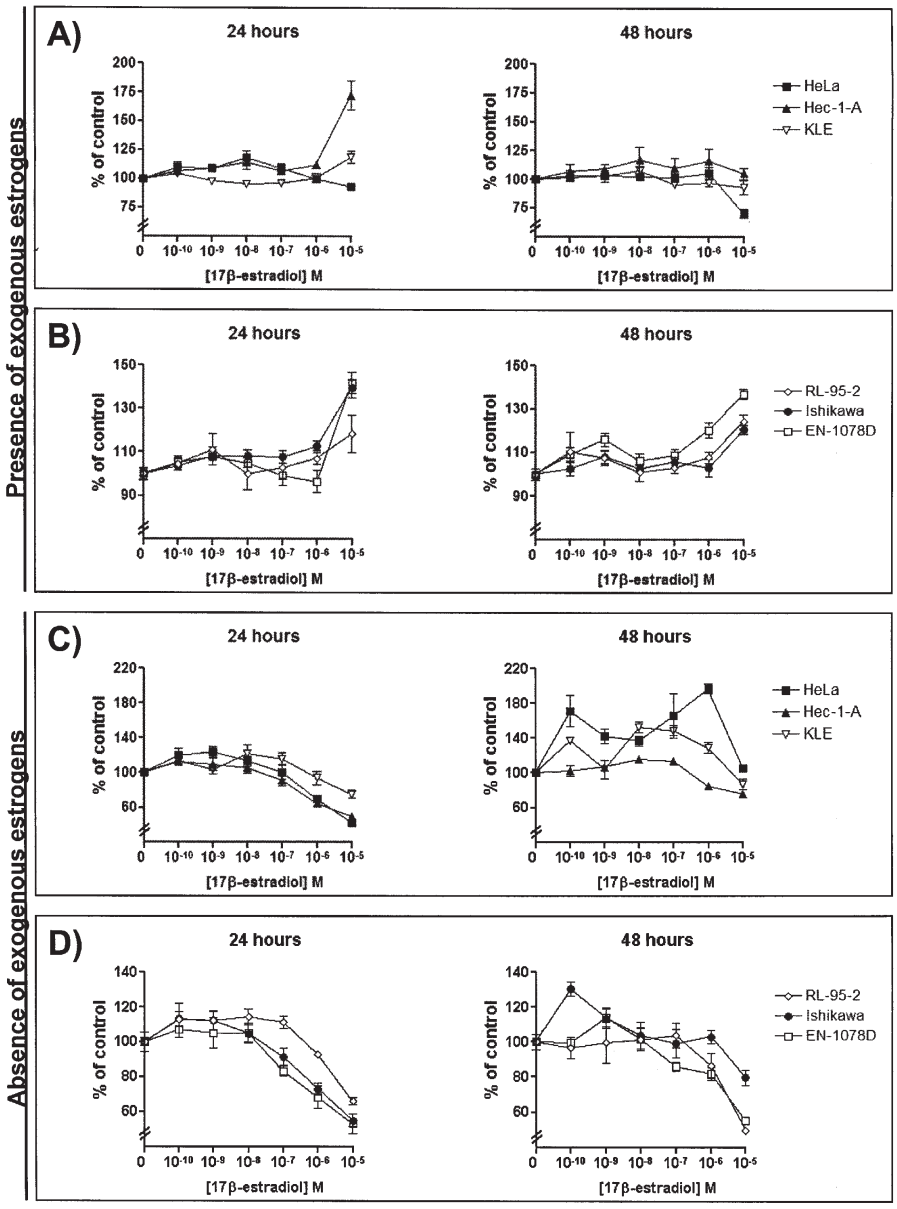


Figure 5. Effect of 17ß-estradiol on cell proliferation of HeLa, HEC-1-A, KLE (A and C) and RL-95-2, Ishikawa and EN-1078D (B and D) uterine cancer cell lines as determined by the conversion of MTT into a thiazolyl blue MTT-formazan. Treatments were performed as described in Materials and methods in media with (A and B) and without exogenous estrogens (C and D) for 24 and 48 h. Results represent mean ± SEM of 3 independent experiments performed in duplicate.

expression of phospho-Akt (the active form of Akt) in mutated PTEN cells (RL-95-2 and Ishikawa) might be influenced in the presence or absence of endogenous estrogens (Fig. 4). The results showed that total Akt protein is not influenced by estrogens but clearly showed a reduction of Akt phosphorylation in cells cultured in a phenol red- and steroid-free culture medium. In wild-type PTEN cells (Hela and HEC-1-A) the presence of phospho-Akt was weakly detectable in a culture medium containing serum and phenol red but is completely absent in a culture medium without phenol red containing steroid-free serum.

*Cell proliferation*. The conversion of MTT to formazan by metabolically active cells was used to measure the effect of 17ß-estradiol, 4-hydroxytamoxifen, raloxifene and ICI 182 780 on the proliferation of the six cell lines studied.

*In the presence of estrogens*. Results obtained by MTT assay showed that 17ß-estradiol, at low doses ($10^{-10}$-$10^{-8}$ M), slightly increased cell proliferation of all cell lines (Fig. 5A and B). At higher concentrations ($10^{-7}$-$10^{-5}$ M), 17ß-estradiol increased cell proliferation of RL-95-2, Ishikawa and EN-1078D cells whereas proliferation of HeLa cells decreased, HEC-1-A cells showed different patterns depending on the duration of treatments and KLE cells were not affected by the treatment. 4-hydroxytamoxifen caused a slight increase of cell proliferation in HeLa, HEC-1-A, RL-95-2 and EN-1078D cells but the major impact was observed at $10^{-5}$ M with a decrease of cell proliferation in all cell lines studied (Fig. 6A and B). Results obtained with the compound raloxifene showed a slight increase of cell proliferation in KLE, RL-95-2 and EN-1078D at low doses $10^{-10}$-$10^{-8}$ M whereas at a high dose an important augmentation of cell

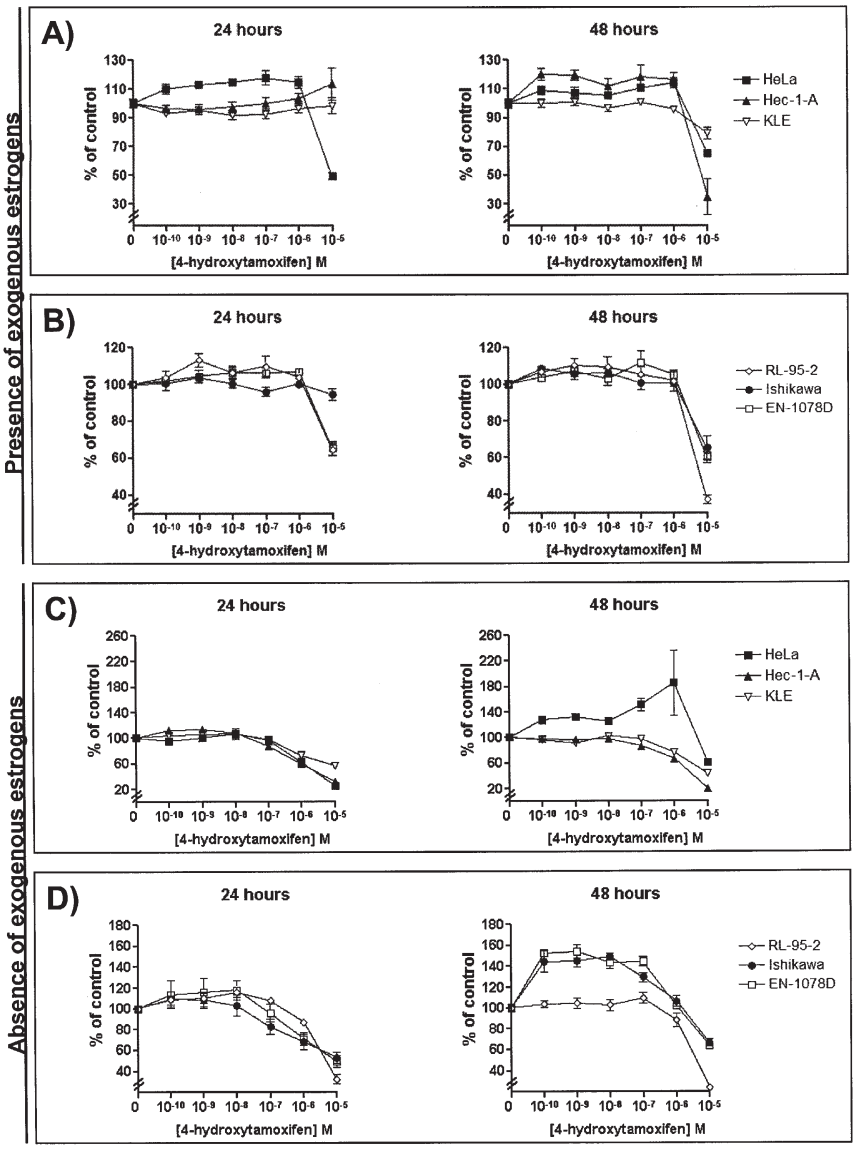


Figure 6. Effect of 4-hydroxytamoxifen on cell proliferation in HeLa, HEC-1-A, KLE (A and C) and RL-95-2, Ishikawa and EN-1078D (B and D) uterine cancer cell lines as determined by the conversion of MTT into a thiazolyl blue MTT-formazan. Treatments were performed as described in Materials and methods in media with (A and B) and without exogenous estrogens (C and D) for 24 and 48 h. Results represent mean ± SEM of 3 independent experiments performed in duplicate.

proliferation was observed in HeLa, HEC-1-A, KLE and Ishikawa cells (Fig. 7A and B). RL-95-2 and EN-1078D cell proliferation was reduced in the presence of raloxifene at a high dose. Cell proliferation of all cell lines was increased in the presence of ICI 182 780 (Fig. 8A and B).

*In the absence of estrogens*. Results obtained by MTT assay with 17ß-estradiol showed increased cell proliferation in most of the cell lines at low doses ($10^{-10}$-$10^{-8}$ M) and reduced cell proliferation at high doses ($10^{-7}$-$10^{-5}$ M) after 24 h (Fig. 5C and D). After 48 h, the same pattern was observed but the reduction of cell proliferation was delayed for a majority of the cell lines. HeLa and KLE cells were the exception showing an increase of cell proliferation at 48 h. Results with 4-hydroxytamoxifen at 24 and 48 h of treatments showed a biphasic effect similar to 17ß-estradiol at 24 h with all cell lines excepting HeLa cells at 48 h who presented the same pattern as their response to 17ß-estradiol (Fig. 6C and D). Treatments for 24 h with raloxifene demonstrated a relatively stable cell proliferation in all cell lines that highly increased in KLE, RL-95-2, Ishikawa and EN-1078D cell lines at $10^{-5}$ M (Fig. 7C and D). After 48 h in the presence of raloxifen, cell proliferation of RL-95-2 decreased compared to 24 h. KLE and EN-1078D cells showed a gradual increase of cell proliferation and proliferation of HeLa and Ishikawa cells was highly increased at high doses. Results obtained by MTT assay with ICI 182 780 at 24 and 48 h, showed a biphasic effect of cell proliferation with each cell line except at 48 h when HeLa cells constantly increased and Ishikawa cells demonstrated an alternation of augmentation and reduction of cell proliferation (Fig. 8C and D).

*ER binding assay*. To evaluate the affinity of 4-hydroxytamoxifen, raloxifene, ICI 182 780 for ERs, the ERα and ERß affinity assay was performed using the HitHunter™ EFC estrogen fluorescence assay kit (Discoverx) according

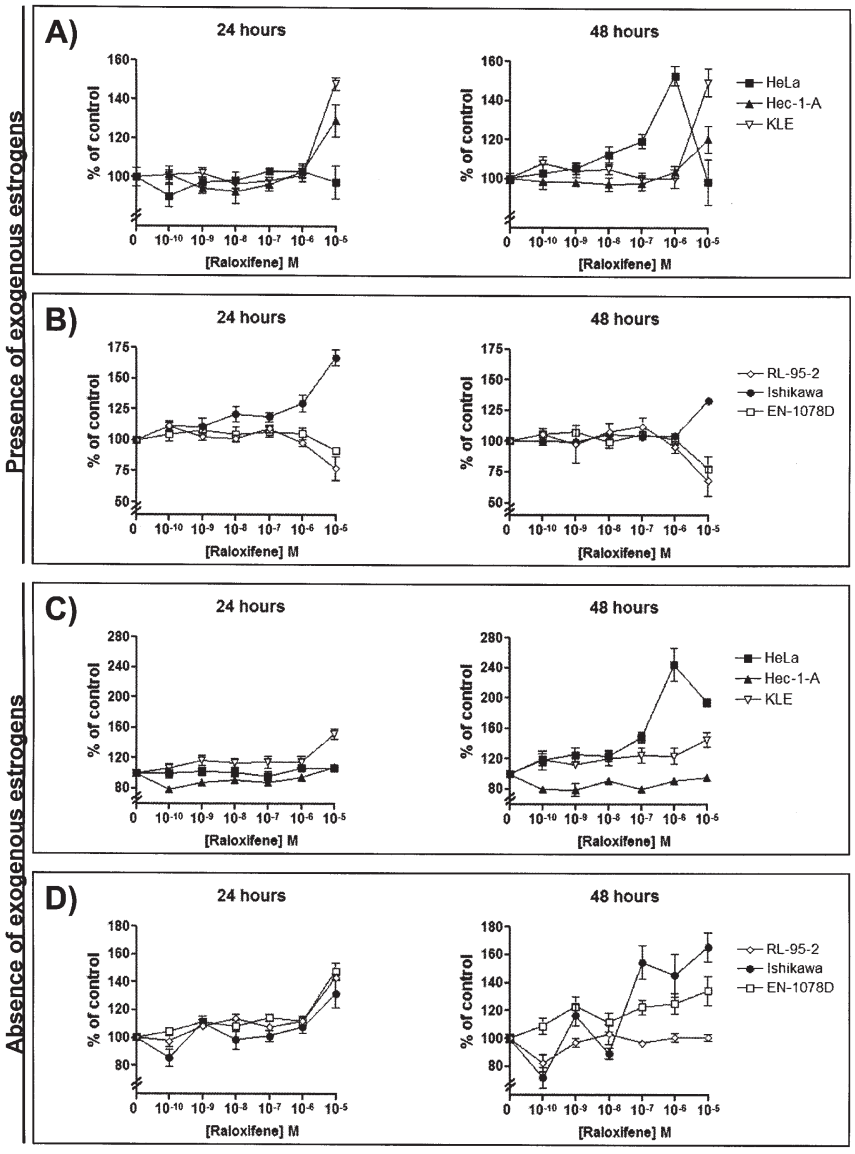


Figure 7. Effect of raloxifene on cell proliferation in HeLa, HEC-1-A, KLE (A and C) and RL-95-2, Ishikawa and EN-1078D (B and D) uterine cancer cell lines as determined by the conversion of MTT into a thiazolyl blue MTT-formazan. Treatments were performed as described in Materials and methods in media with (A and B) and without exogenous estrogens (C and D) for 24 and 48 h. Results represent mean ± SEM of 3 independent experiments performed in duplicate.

to the manufacturer's instructions. The ERα binding studies showed a strong affinity of the three antiestrogens for ERα (Fig. 9A). The $IC_{50}$ of the antiestrogens obtained was 0.98 nM with 4-hydroxytamoxifen, 0.66 nM with raloxifene and 0.80 nM with ICI 182 780 compared to 0.68 nM with 17ß-estradiol, the natural ligand. Results obtained for the ERß binding affinity demonstrated a high affinity of the anti-estrogens 4-hydroxytamoxifen and ICI 182 780 only (Fig. 9B). Corresponding $IC_{50}$s were 2.46 nM for 4-hydroxytamoxifen and 1.12 nM for ICI 182 780, compared to 1.01 nM with 17ß-estradiol. With raloxifene the $IC_{50}$ was not reached.

## Discussion

Although antiestrogens are widely used in the prevention and treatment of long-term effects of estrogen deficiency such as osteoporosis or estrogen-responsive cancers such as breast cancer, their effects on uterine cancer remain poorly understood. In the present study, we have examined the effects of 17ß-estradiol, 4-hydroxytamoxifen, raloxifene and ICI 182 780 on the proliferation of six uterine cancerous cell lines in the presence and absence of exogenous estrogens. Differences between cellular proliferation in the presence and absence of estrogens were readily observable. This is in accordance with the results obtained by Berthois *et al* (9) in breast cancer cells and also by David *et al* (42) in human endometrial cells. Our present results thus confirm the importance of considering phenol red from media and estrogens from serum in any investigation using estrogen-responsive cells and demonstrated this for the first time in uterine cancer cell lines. We have demonstrated that 17ß-estradiol and 4-hydroxytamoxifen showed similarities in their effects on uterine cancer cell proliferation; most of these cell lines proliferate at low doses (containing the physiological doses). This is consistent with

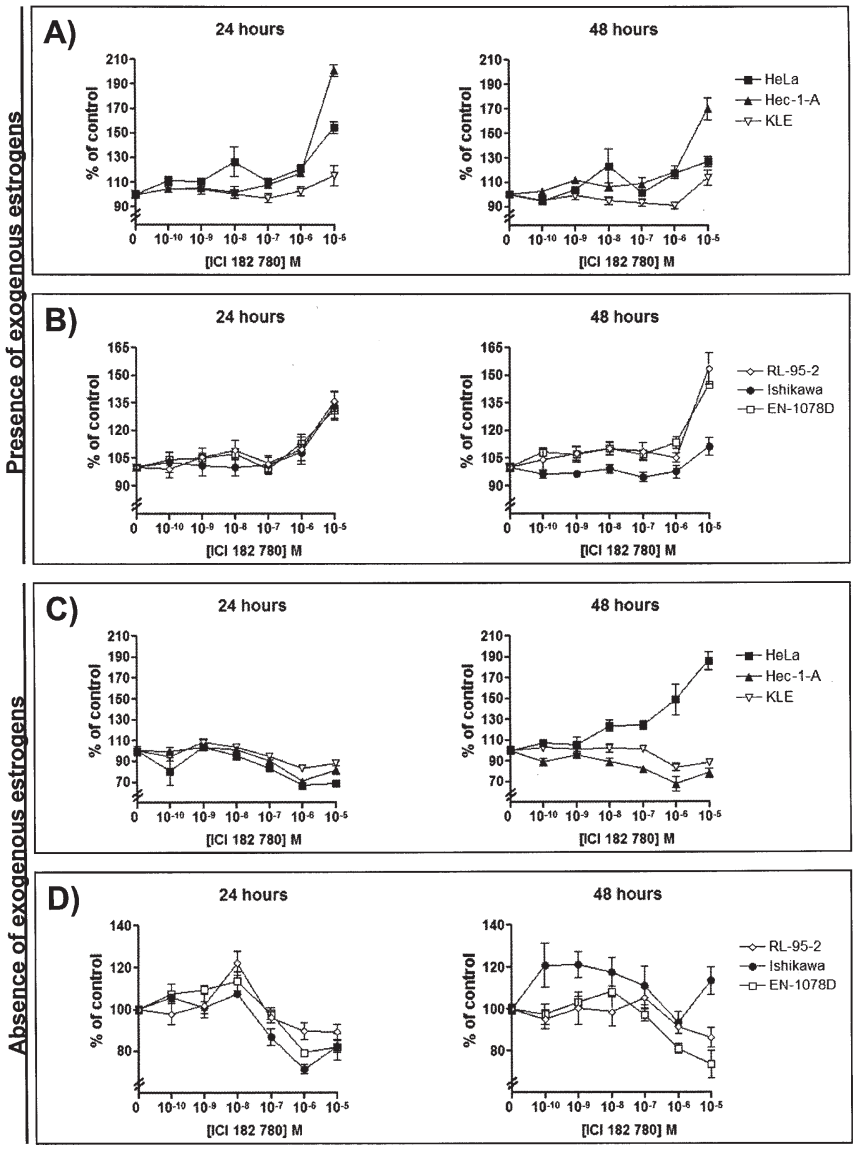


Figure 8. Effect of ICI 182 780 on cell proliferation in HeLa, HEC-1-A, KLE (A and C) and RL-95-2, Ishikawa and EN-1078D (B and D) uterine cancer cell lines as determined by the conversion of MTT into a thiazolyl blue MTT-formazan. Treatments were performed as described in Materials and methods in media with (A and B) and without exogenous estrogens (C and D) for 24 and 48 h. Results represent mean ± SEM of 3 independent experiments performed in duplicate.

the fact that tamoxifen acts like an agonist of estrogens on endometrium (33,34). Interestingly, raloxifene presented an agonistic/antagonistic pattern of cell proliferation depending on doses with the uterine cell lines whereas ICI 182 780 exerted mostly antagonistic action at low doses as expected for a pure antiestrogen.

The relative expression levels of the two estrogen receptor subtypes in a particular tissue were suggested to influence estrogen and selective estrogen receptor modulator (SERM) activities (43). To examine their implication on cell proliferation, mRNA/protein expression was measured in all cell lines in the presence and absence of estrogens. ERα was present in EN-1078D cells in the presence and absence of estrogens, and in Ishikawa cells only in the absence of estrogens whereas no other cell lines produced this isoform of the ER. ERß was produced in all cell lines evenly in the presence rather than in the absence of estrogens at a higher level in HeLa cells than others. The present results on ER subtypes showed differences in their level of ER expression compared to the results obtained by other studies with the same cell lines (44-47) demonstrating that the conditions of cell maintenance have an impact on ER expression as we have observed in the presence and absence of estrogens with Ishikawa cells.

REA mRNA was measured and no difference between the presence and absence of estrogens was observed. However, GPR30 mRNA expression was significantly higher in the presence of exogenous estrogens compared to cells cultured in a serum/phenol-free medium suggesting that GPR30 mRNA is up-regulated in the presence of exogenous estrogens in the culture media. Moreover, the GPR30 expression suggests that these cells permit non-genomic actions of estrogens whereas they already allow those that are genomic. A recent study, demonstrated that GPR30 participated in the mediation of cell proliferation induced by 17ß-estradiol and 4-hydroxy-tamoxifen in HEC-1-A and Ishikawa cell lines (48). Thus, it

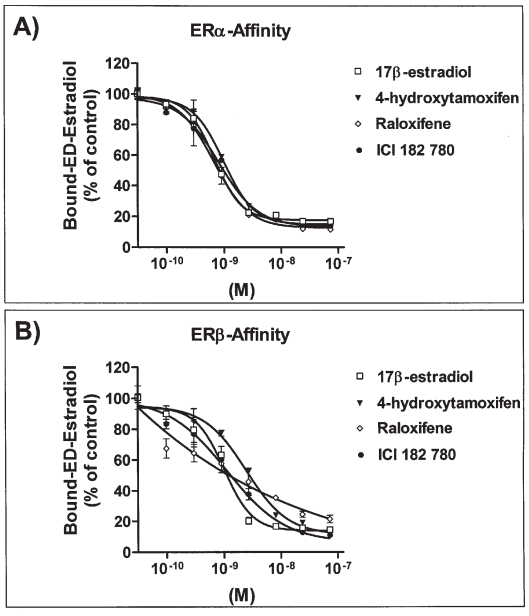


Figure 9. (A) ERα binding affinity of 17ß-estradiol, 4-hydroxytamoxifen, raloxifene and ICI 182 780. (B) Same as in A but with ERß. □, 17ß-estradiol; ▼, 4-hydroxytamoxifen; ◇, raloxifene; and •, ICI 182 780. Data represent the mean ± SEM of 3 independent experiments.

is important to determine the exact role and involvement of GPR30 following treatment with 17ß-estradiol, 4-hydroxytamoxifen, raloxifene and ICI 182 780 in the proliferation of uterine cancer cells. REA was never measured previously in the uterine cancer cell lines studied. The expression of REA by all cell lines suggests that REA could play a role in the mediation of 17ß-estradiol and effects of antiestrogens and further investigations are necessary to better define this role. REA action has been described to be via a competition with the co-activator of steroid receptors, SRC-1 (24), and consequently, the expression of this co-activator has to be measured in the future to clarify our comprehension of the mechanism of 17ß-estradiol and antiestrogen action. However, Akt activity was significantly reduced in all cell lines studied in the estrogen-free medium indicating that phenol red and steroids present in the serum influence the activity of this proliferation and survival pathway. Low levels of phospho-Akt were detected in cells expressing wild-type PTEN in the regular culture medium but were completely absent in the estrogen-free medium. These results show that it will be important in the future to consider the effect of exogenous estrogens when studying the PI 3-K/PTEN/Akt survival pathway.

In the presence of high levels of ERα, in EN-1078D cells for instance, we observed a rapid increase in proliferation with the four compounds studied in the absence of estrogens. Those cells were probably already stimulated for cell proliferation by phenol red in the presence of estrogens. The presence of phenol red was demonstrated to affect the basal level of hormone-stimulated responses and the degree to which exogenous estrogens are able to stimulate these responses above the basal level (9). In general, EN-1078D cells are the most affected cells by 17ß-estradiol, 4-hydroxytamoxifen, raloxifene and ICI 182 780 suggesting that ERα is important in the response of cell proliferation induced by the compounds studied. In comparison with EN-1078D cells, Ishikawa cells expressed ERα only in the absence of estrogens. Ishikawa cells showed differences in the effects of the compounds studied between the presence and absence of estrogens. The augmentation of ERα production in absence of estrogens could play a role in the response to the compounds studied. The production of ERα in Ishikawa cells only in the absence of estrogens probably results in a survival engagement of cells in response to the lack of nutriments to increase the chances of growth stimulation. An augmentation of ERα levels in absence of estrogens was also described in MCF-7 cells (49,50).

In the presence of high levels of ERß and not significant levels of ERα (e.g. HeLa, HEC-1-A and KLE cells), we observed a significant increase of cell proliferation in response to the four compounds studied. This suggests a relationship between ERß and cell proliferation. To date, the role of ERß is poorly defined in uterine cancer. In contrast to our observations in uterine cancer, as seen in the breast cancer cell line MCF-7, ERß inhibits cell proliferation (51) and the ERß level is reduced in the carcinogenesis of breast cancer suggesting that ERß is protective against cancer development and thus cell proliferation (52). Further studies concerning the involvement of ERß in uterine cell proliferation must be performed.

HEC-1-A, KLE and RL-95-2 cells are influenced by 17ß-estradiol, 4-hydroxytamoxifen, raloxifene and ICI 182 780 but appear to be more resistant than the other cell lines. These cells have in common the expression of GPR30, REA and ERß and the absence of ERα expression. The mechanism implicated in this resistance is unclear at present and further investigations are needed to understand this result. Certain signalling pathways could be the key of this mechanism of resistance. It was already demonstrated that Akt in the PI 3-K pathway was involved in the cisplatin resistance of the KLE cells (53). Thus, it is important to verify the implication of the PI 3-K pathway on cell proliferation in the presence of the four compounds studied.

Our results demonstrate that the affinity of the three anti-estrogens to ER is similar to that of 17ß-estradiol with each isoform except for raloxifene which has higher binding affinity for ERα, which is in accordance with results reported previously (28,39). Surprisingly, raloxifene had an impact on cell proliferation of all cell lines, not just those that express ERα. Oddly, in most of the cell lines studied, raloxifene at very high doses exerted an important increase in cell proliferation. It has also been demonstrated that cell proliferation was increased in Ishikawa cells in response to a high concentration of raloxifene (54,55). It could be interesting to investigate the binding affinity of the antiestrogens studied to GPR30 to verify if the effect of raloxifene on proliferation could be mediated through its binding to GPR30.

Taken together, these results suggest that ERα and ERß play a role in the response to 17ß-estradiol, 4-hydroxytamoxifen, raloxifene and ICI 182 780 but are not the only factors involved. GPR30 and REA could play a role in these effects, but this remains to be elucidated. 4-hydroxytamoxifen acts like an agonist of 17ß-estradiol on uterine cancer cells whereas raloxifene exerts partial agonistic/antagonistic activity and ICI 182 780 had mostly antagonistic action. Akt pathway

activity was directly influenced by the presence or absence of exogenous estrogens. Thus, in studying estrogens and antiestrogens in uterine cancer cell lines, phenol red and exogenous steroids have to be considered. Further studies are necessary to understand more precisely the mechanism of action of 17ß-estradiol, 4-hydroxytamoxifen, raloxifene and ICI 182 780 in uterine cancer cell lines.

## Acknowledgements

This work has been supported by a grant from CIHR (MOP-66987). Eric Asselin is a New Investigator of the Canadian Institute of Health Research of Canada (CIHR). Kim Leblanc is a recipient of a CIRH and FRSQ scholarship. Émilie Sexton and Marie-Claude Déry are recipients of NSERC scholarships. We thank Dr Celine VanThemsche and Mrs. Daphne Efford for reviewing the manuscript.

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