Ulipristal acetate does not impact human normal breast tissue

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BACKGROUND: Antiprogestins are of growing interest for the development of new treatments in the gynecological field. Ulipristal acetate (UPA) is a progesterone receptor (PR) modulator considered for long-term administration in contraception and is currently being registered for the treatment of uterine fibroids. In light of the influences of hormonal dysfunction in breast pathologies, the secondary consequences of chronic UPA therapy need to be established. The aim of this study was to determine UPA actions mediated by PR and glucocorticoid receptor (GR) in normal and transformed breast.

METHODS: UPA, progesterone (P) and dexamethasone (DEX) effects were observed on PR and GR responsive genes and on proliferation and apoptosis of normal human breast epithelial (HBE) and breast cancer cells. Human normal breast tissue samples were xenografted in athymic mice and treated with estradiol (E2), or E2 + P, or E2 + P + UPA.

RESULTS: Analysis of PR and GR reporter gene transactivation and their respective endogenous target genes indicated that UPA exerted anti-progestational and anti-glucocorticoid activity in both types of cells with a more pronounced effect in cancer cells. When combined with P or DEX, UPA limits the proliferation of HBE cells but increases growth in breast cancer cell lines. UPA administration had no impact on the mitotic index on xenografted human breast tissue exposed to gonadal hormones at similar concentrations to those present in normal women.

CONCLUSIONS: Although further clinical trials are required to confirm that the results from our experimental models can be extrapolated to women treated with UPA, they suggest that such treatment would not be deleterious to normal breast tissue at least for a cycle (28 days) of continuous administration.

Key words: Ki67 / cyclin A / BCL2 / FASN / IEX-1

Introduction

Antiprogestins have provided a significant therapeutic benefit to women since their discovery and clinical exploitation. Antiprogestins, also known as selective progesterone receptor modulators (SPRMs), exert activities ranging from pure progesterone receptor (PR) antagonism to mixed PR antagonist/agonist actions. SPRMs may also display glucocorticoid receptor (GR) antagonist properties. The most well-known SPRM is mifepristone employed in medical termination of pregnancy. Mifepristone was also shown to be effective in myoma treatment (Eisinger et al., 2005; Esteve et al., 2012). However, because of its marked anti-glucocorticoid activity, long-term therapies of mifepristone were controversial and led to the developed of other SPRMs (Cadepond et al., 1997). Ulipristal acetate (UPA), also known as VA-2914 or CDB-2914, is an SPRM which efficiently binds and inhibits PR and exhibits PR in progesterone target tissues (Cook et al., 1994; Wagner et al., 1998; Wagner et al., 1996; Attardi et al., 2004). UPA is well characterized for its anti-fertility potency in various animal models (Reel et al., 1998; Hild et al., 2000; Gainer and Ulmann, 2003; Brenner et al., 2010) and in women (Blithe et al., 2003;
Chabbert-Buffet et al., 2007). In vitro studies have also determined its biological effects in normal myometrial and leiomyoma cells. UPA was shown to exert an anti-proliferative activity in leiomyoma cells (Xu et al., 2005, 2006; Maruo et al., 2010; Yoshida et al., 2010).

UPA has largely been employed in the gynecological field, because of its reduced anti-glucocorticoid activity (Wagner et al., 1999; Attardi et al., 2004). In addition to being marketed as a new generation of emergency contraceptive pill (ellaOne™), UPA is currently being considered for regular contraception. Preclinical and Phase II clinical trials investigating the contraceptive activity of locally delivered UPA are in progress (http://www.popcouncil.org/pdfs/factsheets/RH_ContraceptiveDev.pdf). UPA was registered as treatment for uterine fibroids, and associated with decreasing bleeding (Graham and Clarke, 1997; Levens et al., 2008; Nieman et al., 2011). Some evidence suggests that UPA might also exhibit benefits for endometriosis treatment, as well as ovarian and breast cancer therapies (Spitz et al., 1996; Cadepont et al., 1997; Klijn et al., 2000; Chabbert-Buffet et al., 2005; Goyeneche et al., 2012).

Long-term UPA exposure may affect hormone-responsive tissues, and particularly breast tissue. Because of the controversial effects of progesterone in the human normal breast tissue, the effects of SPRMs need further characterization (Graham and Clarke, 1997). A study on normal breast tissue collected by fine needle aspiration in women treated with mifepristone suggested a potent anti-proliferative effect of this SPRM (Engman et al., 2008). However, UPA treatment has not been explored in normal human epithelial breast cells and is limited to gene reporter transactivation studies in the T-47D breast cancer cell line (Attardi et al., 2002, 2004). As UPA has impending future clinical use, we investigated its PR- and GR-mediated activities on target gene expression, as well as on proliferation and apoptosis in primary cultures of normal human breast epithelial cells (HBE) and in breast cancer cell lines. We also evaluated the role of UPA in the context of ovarian hormones, using an in vivo experimental model, with normal human breast tissues xenographed in the nude mice. Our results show that treatment with UPA does not alter normal breast cell proliferation under these conditions.

**Materials and Methods**

**Steroids**

The SPRM UPA and its mono-N-demethylated metabolite CDB-3877 (CDB) were kindly provided by HRA-Pharma (Paris, France). 17β estradiol (E2), progesterone (P), dexamethasone (DEX) and mifepristone were purchased from Sigma (St Quentin Fallavier, France). T-47D cell line was derived from a human ductal breast carcinoma and constitutively expressed high amounts of PR and estrogen receptor (ER). Normal HBE cell primary cultures were obtained from 26 women breast tissues. The procedure used to culture HBE cells is described in detail by Gompel et al. (1986). HBE cells were maintained in HAM F10 medium (PAA Laboratory, Les Mureaux, France) containing hydrocortisone (5 ng/ml), Triiodo-L-thyronine (6.5 ng/ml), cholera toxin (10 ng/ml), transferrin (5 mg/ml), insulin (0.016 U/ml), epidermal growth factor (10 ng/ml) (Sigma, St Quentin Fallavier, France) and 5% human serum (Etablissement Français du Sang). We previously demonstrated that HBE primary cultures expressed epithelial markers as well as low levels of estradiol receptor (ER) and estradiol induced PR (Malet et al., 1991; Courtin et al., 2012).

**Steroid treatments**

After seeding, cells were cultured for 24 h in serum and phenol red free medium. Then treatments were carried out in a phenol red free medium containing 5% dextran–charcoal-stripped serum. Cells were treated with P or DEX (100 nM), alone or in combination with UPA, mifepristone (1 nM to 1 µM) or CDB (100 nM). Control cells were treated with ethanol, at 1:1000 final ethanol concentration alone as vehicle or E2 (10 nM).

**Reporter enzyme assays**

Cells were transfected with reporter gene plasmids containing glucocorticoid and progesterone responsive elements (GRE/PRE): (i) the MMTV-Luc is a Mouse Mammary Tumor Virus Long Terminal Repeat promoter containing one GRE/PRE palindrome and three GRE/PRE hemi-palindromes upstream to firefly luciferase gene in pFC31 vector (ii) the GRE-Luc contains six copies of GRE/PRE palindrome upstream to firefly luciferase gene in pB8 vector. When indicated, HBE cells were transfected with human PR isoforms hPR-A and hPR-B expression plasmids constructed in POP3 vectors. Rous Sarcoma Virus promoter upstream to beta galactosidase gene (pRSV-β-Gal) was transfected in each experiment as control. Transfections were performed according to the manufacturer instructions using Lipofectamine or Lipofectamine LTX Reagents (Invitrogen, Cergy-Pontoise, France) for breast cancer cell lines or HBE cells, respectively. After 24 h of transfection, breast cancer and HBE cells were treated with hormones for 24 or 48 h, respectively. At the end of the experiment, cells were lysed and luciferase activity was determined using the Luciferase Assay System (Promega, Charbonnières-les-Bains, France). Beta galactosidase activity was assessed using the Galacto Star kit (Applied Biosystems, Courtaboeuf, France) to normalize luciferase activity data. Each independent experiment was performed in triplicate.

**Real-time quantitative reverse transcription PCR**

Total RNA was extracted using Trizol Reagent (Invitrogen, Cergy-Pontoise, France). Two micrograms of total RNA were subjected to reverse transcription (RT) using random primers for 1 h at 37°C. Two microliters of RT product were diluted (1:10) and subjected to a quantitative PCR using sequence-specific primers (375 nM) and Brilliant SYBR GREEN QPCR master mix (Fermentas, Saint-Rémy-les-Chevreuse, France) on an Mx3000P apparatus (Agilent Technologies, Massy, France). Sequence primers for target genes were: 3684, upper, 5′-gattgtccacacagtcttgg-3′; lower, 5′-caggccgacgaccaa-3′; FASN, upper, 5′-tacacagctgcatcagcgc-3′; lower, 5′-gatactctctcgctgcatac-3′; ALPL, upper, 5′-tacttctcgagatgctggtg-3′; lower, 5′-ttcttcagggcgctctgc-3′; cyclin A, upper, 5′-gcagagctgctgcatg-3′; lower, 5′-ttgctctggctgctgaccc-3′; BCL2, upper, 5′-gactgattctgaatctg-3′; lower, 5′-agtctccaggcatcaca-3′; IEX-1, upper, 5′-cggctgagcatgtcactct-3′; lower, 5′-acacctgctcgccatcagca-3′; GOS8, upper, 5′-ttctctactctggaagc-3′; lower,
Thermocycling conditions were 1 cycle at 95°C for 10 min followed by 40 cycles at 95°C for 30 s, 60°C for 1 min and 72°C for 30 s. Gene expression values were normalized to the housekeeping gene 36B4. Time of steroid treatment was chosen to get the optimal stimulation for a given gene. ALPL and GOS8 mRNA were analyzed after 6 h of treatment. IEX-1, FASN, and BCL2 were analyzed after 24 h of treatment. Cyclin A mRNA was analyzed after 24 h for GR responses or 48 h for PR responses. Each independent experiment was performed in duplicate.

**Tritiated thymidine incorporation**

After 24 h of hormonal treatment, cells were incubated with [methyl-3H] thymidine (Perkin Elmer, Courtaboeuf, France) for 48 or 20 h for HBE or cancer cells, respectively, at 37°C. After incubation cells were washed twice with phosphate-buffered saline (PBS) 1X and once with 5% trichloroacetic acid (TCA). Cells were incubated in 5% TCA for 15 min at 4°C and lysed in NaOH 0.1 M for 30 min at 37°C. The total cell lysate was added to 5 ml of Ecolite scintillation liquid (MP biomedical, Illkirch, France) and radioactivity was counted with a β-counter HIDEX 300SL (ScienceTec, Courtaboeuf, France). Each independent experiment was performed in triplicate.

**Flow cytometry analysis**

After 96 or 48 h of hormonal treatment, respectively, for HBE or MCF-7 and T-47D cells, cells were washed in PBS, matrix dissociated with accurate enzyme (PAA laboratory, Les Mureaux, France) and centrifuged 5 min at 300 g. Cells were fixed and frozen at −20°C in 70% ethanol. Before analysis, cells were washed in PBS and stained with 10 μg/ml propidium iodide in PBS (containing 0.835 U/ml RNase A; Sigma, St Quentin Fallavier, France). For each sample at least 10,000 cells were counted in a BD LSR II flow cytometer (BD Biosciences, Le Pont de Claix, France). After gating out doublets and debris, cycle distribution was analyzed using the ModFit LT software (Venty Software House, USA). Each independent experiment was performed in triplicate.

**Human breast xenografts in mice and pellet treatments**

Four weeks old ovariectomized female NMR/immu athymic mice were purchased from Janvier laboratory (Le Genest Saint Isle, France). Breast tissue samples were obtained from six consecutive premenopausal women undergoing mammoplasty surgery and free of concomitant treatment. Their mean age was 36.5 ± 5.16 years (range: 29–42). The day of the menstrual cycle at the time of the surgery was Days 3, 7, 13, 21, 25 and 29 for patient 1–6, respectively. For each patient, human mammary gland structures were cut into 2 × 2 × 2 mm fragments, and four fragments were then subcutaneously xenografted onto the back of each mouse of the experiment. Four treatment groups were performed per experiment: control, E2, E2 + P and E2 + P + UPA. Each group contained four mice. Each patient was treated as a separate experiment. Therefore, for each patient, 64 fragments were grafted in 16 mice. Treatments were administered by subcutaneously grafting steroid pellets onto the neck of each mouse. The experimental conditions were initially determined by using a dose range of hormones mixed with cholesterol into the pellets. Blood sample assays were performed after 2 and 4 weeks of treatment to measure plasmatic hormonal concentrations. Finally, the dose of 0.3 mg per pellet for E2 and 20 mg per pellet for P and UPA were used, as it provides the appropriate blood concentrations. For the control groups, pellets containing only cholesterol were used. To reproduce menstrual cycle conditions, mice were grafted on the first day of experiment with cholesterol, E2 and/or UPA containing pellets and on the fourteenth day with cholesterol or P containing pellets in control and E2 groups or in E + P and E2 + P + UPA groups, respectively. Twenty-eight days after the start of experiment, mice were sacrificed. Blood was collected for each mouse, and serum was frozen at −20°C until hormone concentrations analysis. Breast tissue fragments were collected and immediately fixed in paraformaldehyde solution for immunohistochemical analysis. All study protocols and environmental conditions of the animal rooms were approved by the French Ethic committee for the care and use of laboratory animals Charles Darwin.

**Hormone concentration analysis**

Estradiol was measured by radioimmunoassay using Clinical Assays Estradiol-2 (Sonin Biomedica Diagnostics SpA, Saluggia, Italia). Progesterone levels were evaluated by UPLC-MSMS using Acquity UPLC and Quattro Premier XE (Waters, Milford, MA, USA). UPA concentrations were measured using LC-MS/MS technique by MPI Research (State College, PA, USA).

**Immunohistochemical analysis**

Mitotic index was calculated using the Ki67 antibody, and was determined for each breast tissue grafted into mice. Immunohistochemical analyses were performed using the BOND-MAX workstation (Leica, Nanterre, France). Paraffin sections of breast tissue xenografts were de-waxed and rehydrated before antigen retrieval using citrate retrieval solution (pH 6.0) for Ki67 antibody or EDTA retrieval solution (pH 9.0) for PR and ER alpha antibodies, for 30 min. Sections were then incubated with Ki67 at 1:100 (Novocastra, NCL-Ki67-M1); PR at 1:80 (Biogenex, MU-328-UC) or ER at 1:300 (Novocastra, NCL-ER-6F11) monoclonal antibodies. For signal detection, the Kit Bond Polymer Refine Detection kit was used. Reagents were purchased from Menanni-Diagnostic (Rungs, France). A negative control (omitting the first antibody) was included in each set. For each marker, determination of the ratio of positive cells was performed on a total of 1000 lobular and 1000 ductal luminal cells in the four breast tissue fragments grafted into each mouse. For each experiment, the final percentages for each treatment were the mean of percentages obtained in the four mice per group.

**Ethical permissions**

The breast samples were obtained from women having signed an informed consent according to the French law on clinical experimentation (L. 1243-3 and L. 1243-4), as a part of a biomedical project including the collection and conservation of cell cultures and xenografts of breast tissues obtained from women undergoing plastic surgery. The authorization number filed for this project is 11 826, from the French ethical committee ‘Comité de Protection des Personnes’.

**Statistical analysis**

Results were expressed as mean ± SEM and n represents the number of independent experiments. To determine the statistical significance of treatments, one-way analysis of variance and Tukey–Kramer multiple comparisons tests were performed to compare the relative efficiency of each treatment with the Instat 3 software (GraphPad, USA). When only two treatments were compared, an unpaired t-test was performed, with P < 0.05 considered as significant.
Results

UPA and the PR

**UPA effects on PR gene transactivation**

In order to analyze UPA antagonist properties on PR-induced gene transactivation, normal HBE cells and T-47D breast cancer cell line were transfected with the MMTV-Luc reporter gene (Fig. 1). In HBE cells which expressed low and variable amounts of PR among patients (Malet et al., 1991), P induced a significant luciferase expression (1.38 ± 0.11 control fold induction, P < 0.05; Fig. 1A). To better study the dose effects of UPA on PR, HBE cells were co-transfected with hPR-A and hPR-B expression plasmids (Fig. 1B). UPA and mifepristone inhibited P induced MMTV-Luc transactivation in a dose-dependent manner from 10 to 1 000 nM. HBE cells were then transfected with only hPR-A or hPR-B, to discriminate UPA actions between PR isoforms. UPA was an effective antagonist no matter which PR isoform was increased (Fig. 1C and D). In the T-47D cell line, UPA and mifepristone displayed similar and potent PR antagonist actions from 10 to 1000 nM (Fig. 1E). Partial antagonist responses were also detected at 1 nM (Fig. 1E). In both HBE and T-47D cells, UPA and mifepristone did not exhibit progestational agonist properties on reporter gene transcription (Fig. 1B and E). These results indicated that UPA acted as a potent P antagonist in normal and tumorous breast cells.

**UPA activity on mRNA expression of PR target genes**

A hundred nanomolar was the lowest concentration of UPA necessary to exert a complete antagonist activity in HBE cells. This concentration was, therefore, chosen to further study the effects of UPA on specific P target genes in HBE and T-47D cells. Estradiol (E2) was added to HBE cells, in order to increase PR expression as reported in several previous studies (Clarke et al., 1997; Anderson, 2001; Courtin et al., 2012).

Fatty acid synthase (FASN) is implicated in normal breast cell differentiation as well as in mammary tumor progression (Joyeux et al., 1990; Kuhajda, 2006). An up-regulation of FASN mRNA by progestins through PR was previously demonstrated in normal and tumoral breast cells in vitro and in vivo (Joyeux et al., 1990; Chalbos et al., 1992; Courtin et al., 2012). As shown in Fig. 2A, UPA was able to prevent P induction of FASN mRNA expression in both HBE and in T-47D cells. We observed that P down-regulated cyclin A mRNA expression in normal breast cells and in T-47-D cells (Fig. 2B), as previously reported in MDA-MB-231 breast cancer cells transfected by PR (Lin et al., 2003). UPA reversed P-induced cyclin A mRNA down-regulation in HBE and T-47D cells (Fig. 2B). We previously reported a decrease of the anti-apoptotic B-cell CLL/lymphoma 2 (BCL2) protein expression under progestin treatment in normal and tumoral breast cells (Kandouz et al., 1996; Gompel et al., 2000). As shown in Fig. 2C, we also observed a decrease of BCL2 mRNA expression by P in HBE and T-47D cells (Fig. 2C). Nevertheless, UPA did not reverse BCL2 mRNA down-regulation induced by P in HBE cells, whereas UPA antagonized the P inhibitory effect on this transcript in T-47D cells (P < 0.01; Fig. 2C). Tissue non-specific alkaline phosphatase (ALPL) is a P responsive gene implicated in metastasis of breast cancer (Di Lorenzo et al., 1993; Ritzke et al., 1998). In T-47D cells, UPA totally inhibited the strong P mediated induction of ALPL transcript (Fig. 2D). In HBE cells, ALPL mRNA expression was not modified by P treatment (data not shown). UPA did not display any PR agonist activity on these genes.

**UPA and the GR**

Due to the similarities in the structure of nuclear receptors PR and GR, antiprogestins also display anti-glucocorticoid properties. We therefore evaluated GR antagonist/agonist activity of UPA in normal and tumoral breast cells.

**UPA effects on GR gene transactivation**

The study was performed in HBE and MCF-7 cells, which expressed high amounts of GR as previously reported (Courtin et al., 2012). Cells were transfected with a GRE-Luc reporter gene and treated with DEX, UPA and its proximal mono-demethylated metabolite, CDB-3877 (CDB), known to exert less anti-glucocorticoid activity than UPA (Attardi et al., 2004). We confirmed the reduced anti-glucocorticoid activity of this metabolite, since UPA inhibited by 41.3 ± 5.8% the DEX-induced luciferase transactivation, whereas its metabolite failed to inhibit DEX activity in HBE cells (18.1 ± 11.8%; Fig. 3A). However, in MCF-7 cells, both UPA and CDB significantly antagonized by 59 ± 3.4 and 26.5 ± 9.1%, respectively, the DEX-induced luciferase transactivation (Fig. 3B). These results suggest a stronger anti-glucocorticoid potency of UPA in MCF-7 breast cancer cells than in HBE.

**UPA activity on mRNA expression of GR target genes**

In order to better define UPA anti-glucocorticoid effects in HBE and MCF-7 cells, mRNA expressions of various glucocorticoid responsive genes were analyzed after DEX and UPA treatment. Immediate early response 3 (IEX-1) and regulator of G-protein signaling 2 (G0S8) have been implicated in cell survival under stress conditions (Wu, 2003; Shen et al., 2006) and in G-protein signaling (Kehrl and Sinnarajah, 2002), respectively. IEX-1 and G0S8 were characterized as glucocorticoid responsive genes (Wan and Nordeen, 2002). As shown in Fig. 4A, IEX-1 mRNA expression was down-regulated by DEX and UPA did not antagonize this response in HBE and MCF-7 cells. On the other hand, UPA exerted a slight and non-significant antagonist effect on DEX-induced up-regulation of G0S8 mRNA in HBE cells, whereas its anti-glucocorticoid activity was strong in MCF-7 cells (P < 0.001; Fig. 4B). Cyclin A and BCL2 genes were previously shown to be regulated by DEX in vitro in osteoblasts and neuroblastomas, respectively (Sengupta et al., 2000; Gabet et al., 2011). DEX and UPA actions were therefore analyzed on cyclin A and BCL2 mRNA expression in HBE and MCF-7 cells. Cyclin A was differentially regulated by DEX in the two types of cells as mRNA expression was induced in HBE cells and repressed in MCF-7 cells (Fig. 4C; Courtin et al., 2012). In both cellular models, UPA partially inhibited DEX effect on cyclin A mRNA expression. BCL2 mRNA was down-regulated by DEX in HBE and in MCF-7 cells (Fig. 4D). However, UPA did not reverse the DEX down-regulation of BCL2 mRNA in HBE cells, whereas it partially antagonized this effect in MCF-7 cells. UPA did not display any glucocorticoid agonist activity on these genes (Fig. 4).
Figure 1 UPA effects on MMTV reporter gene transactivation. MMTV-Luc reporter gene was transfected in HBE cells: (A) alone, or in combination, (B) with human progesterone receptor (hPR)-A and hPR-B, (C) hPR-A or (D) hPR-B isoform plasmids. (E) T-47D cells were transfected with MMTV-Luc. Cells were treated with P at 100 nM and/or UPA, or Mifepristone (RU) from 1 to 1000 nM as indicated or with 100 nM when not specified. Results are corrected for changes in βGal expression and normalized to control values (mean ± SEM, n = 3 for HBE and T-47D). *P < 0.05, **P < 0.001.
UPA action on proliferation and apoptosis

P and glucocorticoid can induce different proliferative and survival effects on normal and tumoral breast cells. In order to evaluate the role of UPA on these cellular events, proliferation and apoptosis were measured, respectively, by tritiated thymidine incorporation (Fig. 5) and flow cytometry (Fig. 6) in the three cellular models. UPA effects on PR were studied in HBE and T-47D, but not in MCF-7 cells, because we previously demonstrated a lack of PR functionality in this cell line (Courtin et al., 2012). UPA actions on GR were only studied in HBE and MCF-7 cells as T-47D cells do not express GR (Courtin et al., 2012). In HBE cells, UPA did not inhibit the anti-proliferative effect induced by P (Fig. 5A). Conversely, UPA inhibited the strong proliferative activity induced by DEX (Fig. 5A). In T-47D cells, UPA reversed P anti-proliferative activity (Fig. 5B). In MCF-7 cells, UPA tended to prevent the weak anti-proliferative effect of DEX (Fig. 5C). UPA treatment alone had no effect on proliferation in any type of cells (Fig. 5).

Apoptosis was measured by quantification of sub-G1 percentage of cells by flow cytometry analysis. P exerted an intense pro-apoptotic effect in HBE cells, in contrast to DEX which displayed anti-apoptotic features (Fig. 6A). When combined with UPA, both hormone activities were reversed. Similarly, P increased the percentage of sub-G1 cells and UPA tended to inhibit this effect in T-47D cells (Fig. 6B). In MCF-7, DEX showed a pro-apoptotic ability which was totally inhibited by UPA (Fig. 6C). UPA had no apoptotic or survival properties.

UPA effects on PR target gene expression. In HBE and T-47D cells, mRNA expression was analyzed by Quantitative RT–PCR for (A) FASN, (B) cyclin A, (C) BCL2 and (D) ALPL. Cells were treated with P and/or UPA at 100 nM, and/or E2 at 10 nM. Results are normalized to control fold induction (mean ± SEM, n = 5 for HBE and n = 2 for T47D). *P < 0.05, **P < 0.01.
by itself, but counteracted most of the P and DEX effects in HBE, T-47D and MCF-7 cells.

**UPA action on breast tissue proliferation**

Due to the limitations of the in vitro models, we next developed an in vivo model in order to have a better appraisal of UPA in clinical use and of its impact on breast tissue. Human normal breast tissues samples were xenografted in athymic mice treated with E2, or E2 + P or E2 + P + UPA, or cholesterol (control) (see experimental procedure). In order to reproduce the chronology of E2 and P secretions occurring in human female menstrual cycle, E2 pellets have been grafted since the beginning of the experiment, whereas P pellets were grafted at the 14th day. UPA pellets were grafted at the beginning of the experiment to mimic a chronic treatment. E2, P and UPA concentrations in mice serum were measured to validate the treatment method. Average E2 concentrations in mice serum was $36.88 \pm 4.25$ pg/ml (mean $\pm$ SEM) corresponding to the low range of the physiological E2 levels reported in follicular phase. The P level was $13.05 \pm 1.14$ ng/ml (mean $\pm$ SEM), similar to the average P plasma levels in women during the mid-luteal phase. UPA concentration was $63.49 \pm 10.46$ ng/ml (mean $\pm$ SEM), which is the same range as that observed in clinical use (HRA-Pharma personal communication). Hormones levels were undetectable in control mice (E2 < 0.8 pg/ml; P < 0.4 ng/ml; UPA < 0.5 ng/ml). As shown in Fig. 7A, estradiol receptor (ER) and PR expression were maintained in the treated engrafted breast fragments at the end of the experiment compared with the original breast tissue before grafts. We analyzed the mitotic Ki67 expression marker in order to determine the breast tissue proliferative activity in glandular lobules and ducts according to the treatment (Fig. 7B and C). Proliferation rates could only be calculated in lobules of four out of six women. In the two others, this structure was not detectable. For the ducts, proliferation rates were calculated from the six patients. In the control group of grafted tissues, the rate of mitotic cells was low and homogeneous, at $1.7 \pm 0.4\%$ in the lobules and $1.8 \pm 0.6\%$ in ducts (data not shown). The proliferative activity was slightly but not significantly increased in lobules of E2 treated group when compared with the control group (Fig. 7B and C). However, in ducts the mitotic index was significantly elevated in E2 treated group (3.1 $\pm$ 0.7-fold induction compared with the control group, $P < 0.05$). No significant difference was observed in E2 + P and E2 + P + UPA groups when compared with E2 treatment in lobular and ductal structures (Fig. 7B and C). These results strongly suggest that proliferative activity in breast tissue is predominantly mediated by E2. We also showed that UPA does not significantly influence the proliferation rate of normal epithelial breast cells.

**Discussion**

In this study, we discriminated between GR and PR antagonist activity of UPA on specific target genes and cellular processes in normal and cancerous breast cells. The result of our investigation shows a more potent inhibition by UPA of PR and GR actions on cancer cells than in normal cells. The result of our investigation shows a more potent inhibition by UPA of PR and GR actions on cancer cells than in normal cells. This observation has previously been highlighted by the variable effects of UPA and other PR modulators observed in normal myometrial cells compared with leiomyoma cells (Yoshida et al., 2010). Consequently, the oncogenic context seems to
Figure 4  UPA effects on GR target gene expression. In HBE and MCF-7 cells, mRNA expression was analyzed by Quantitative RT–PCR for (A) IEX-1, (B) G0S8, (C) cyclin A, (D) BCL2. Cells were treated with DEX and/or UPA at 100 nM. Results are normalized to controls (mean ± SEM, n = 5 for HBE and n = 2 for MCF-7). *p < 0.05, **p < 0.01, ***p < 0.001.
influence the UPA antagonist potency that would modulate the clinical indication of UPA.

Drug metabolism efficiency and its rate are individually and genetically variable and are modified during the carcinogenesis process. For example, cytochrome P450 CYP34A activity is variable between individuals, and correlates with the cellular efficiency to metabolize steroids (Keshava et al., 2004). Its expression is also modulated with carcinogenesis process as CYP3A4 mRNA was found in 70% of normal breast tissues but only in 18% of tumor tissues (Huang et al., 1996). As it was shown that CYP34A is involved in UPA metabolism (Jang et al., 1996; Gainer and Ulmann, 2003), the catabolism of UPA into its less anti-glucocorticoid main metabolite, the CDB-3877, may vary between normal and tumoral cells. UPA could be rapidly metabolized in CDB-3877 in HBE cells, resulting in the lower anti-glucocorticoid activity observed in HBE cells compared with MCF-7 cells. A number of results argue for this hypothesis, as DEX induced G0S8 mRNA expression was diminished by a third in HBE cells and a two third in MCF-7 cells in the presence of UPA. BCL2 mRNA down-regulation, by DEX, was also partially antagonized in MCF-7 cells, but not in HBE cells.

GR and PR agonist and antagonist actions are also mediated through PR and GR specific co-activators and co-repressors. PR and GR associate preferentially with SRC-1 and SRC-2, respectively, both belonging to the main SRC-1/p160 family of co-activators (Onate et al., 1995; Voegel et al., 1996; Li et al., 2003). Conversely, when bound to an antagonist ligand, PR or GR recruited co-repressors, such as the nuclear receptor co-repressor (N-CoR) or the silencing mediator for retinoid and thyroid hormone receptor (SMRT) which induces the repression of signal transactivation (Chen and Evans, 1995; Horlein et al., 1995). Modulation of the cellular responses to PR and GR agonist and antagonist are linked to the amount and accessibility of co-activators and co-repressors. For example, difference in mifepristone agonist/antagonist action observed in T-47D cells compared with HeLA cells is modulated by the ratio SRC-1 and SMRT of these cells (Liu et al., 2002). In our case, the variability in co-activator/co-repressor expression ratio may explain the attenuated UPA antagonist responses in HBE cells, when compared with breast cancer cells models (Liu et al., 2002; Smith and O’Malley, 2004).

UPA agonist activity was previously shown in MDA-MB-231 cells overexpressing PR-A, PR-B or both PR isoforms. UPA inhibited cell

**Figure 5** UPA effect on hormone mediated cell proliferation. Cell proliferation was measured by [3H] thymidine incorporation in (A) HBE cells, (B) T-47D cells and (C) MCF-7 cells. HBE cells were treated 96 h and T-47D and MCF-7 cells were treated 48 h at the concentration of 100 nM for P, DEX, UPA and 10 nM for estradiol (E2). Results are expressed as percentage of control (mean ± SEM, n = 7 for HBE, n = 4 for T-47D and n = 3 for MCF-7). *p < 0.05, **p < 0.01, ***p < 0.001.
growth and regulated expression of genes involved in cell cycle regulation in the same manner than P through both isoforms (Leo and Lin, 2008). In contrast, we did not observe UPA agonist effects on the PR target genes in normal or cancerous cells endogenously expressing PR. Absence of agonist activity of UPA may be attributed in one part to a lower level of PR in our models, as transfected MDA contained about twice as much PR as in T-47D. Additionally, the ratio of co-regulators could be in favor to the co-activators in MDA-MB-231 cell line compared with HBE and T47D cells. As supporting information, the steroid receptor co-activator, amplified in breast cancer (AIB1) is strongly expressed in MDA-MB-231 cells whereas it is moderately expressed in T-47D cells and is absent in normal breast tissue (Hudelist et al., 2003; Li et al., 2008).

Numerous studies had previously highlighted the interest of using antiprogestins as breast cancer treatment (Klijn et al., 2000). Mifepristone and onapristone exhibited growth-inhibitory effects especially on PR-positive human breast cancer cell lines as well as on breast tumors in animal models (Bardon et al., 1985; Bakker et al., 1987; Schneider et al., 1992; Klijn et al., 1994; Dannecker et al., 1996; Poole et al., 2006). Administration of mifepristone or onapristone is also a second or third line of breast cancer treatment. The use of mifepristone or onapristone in post menopausal women with metastatic breast cancer (n = 123) was associated with a partial or complete response in 11% and absence of progression in 43% of the patients (Romieu et al., 1987; Klijn et al., 1989). Unfortunately, clinical studies were not sustained because of the anti-glucocorticoid and toxic liver side effects of mifepristone and onapristone, respectively. One of the most important findings of this study is the UPA anti-proliferative and pro-apoptotic effect in HBE cells when combined with either P or DEX. We examined UPA characteristics and antagonist potency in a more accurate in vivo hormonal environment, by grafting breast tissue into mice. This experimental procedure represents a complementary approach to the study of human epithelial cells in their structural and environmental integrities. In this model, the monitoring of steroid concentrations can be performed, whereas this remains more difficult in women because of the variation and the irregularity of the ovarian cycle. We designed hormonal treatments in order to

**Figure 6** UPA effects on hormone mediated cell apoptosis. Cell apoptosis was measured by flow cytometry quantification of sub-G1 phase in (A) HBE cells, (B) T-47D cells and (C) MCF-7 cells. HBE cells were treated 96 h and T-47D and MCF-7 cells were treated 48 h at the concentration of 100 nM for P, DEX, UPA and 10 nM for estradiol (E2). Results are expressed as a percentage of control (mean ± SEM, n = 9 for HBE, n = 3 for T-47D and n = 4 for MCF-7). *P < 0.05, **P < 0.001.
imitate human female hormonal physiology, as it was previously shown that changes in the levels of hormones impact breast tissue regulation (Clarke et al., 1997). Our results indicate that the proliferative activity in breast tissue, especially on duct structure, seems to be highly dependent on E2 signalization, even with E2 levels corresponding to the early follicular phase. This is in contrast with previous results reporting E2 proliferative effects occurring only at much higher E2 concentrations (341 pg/ml, range: 315.8–368 pg/ml) (Clarke et al., 1997). This discrepancy can be associated with our analysis which discriminates between lobules and ducts since our results point to a difference of the steroidal hormone activities between these structures.

This observation was previously made in an ER knockout mice model where duct development was impaired whereas PR knockout mice showed a lack of lobulo-alveolar structures (Lydon et al., 1995; Bocchinfuso and Korach, 1997). The luteal phase in women corresponds to the secreted P period in addition to E2 secretions, and is correlated to the higher proliferative state in the breast tissue (Longacre and Bartow, 1986; Soderqvist et al., 1997; Ramakrishnan et al., 2002). However, the effect of P on the proliferation of the luminal breast cells is still a controversy (Graham and Clarke, 1997). Studies which monitored the concentrations of P show a preponderant effect of E2 administration with a limited action of P on breast proliferation.

Figure 7 PR, ER and Ki67 expressions in breast tissue xenografts. ER, PR and Ki67 expression were analyzed by immunohistochemistry. (A) Representative image for ER and PR labeling in original breast tissue before and in treated grafts in mice. (B) Ki67 expression in C, E2, E2 + P, E2 + P + UPA treatment groups of grafted tissues. Magnification ×400. (C) Mitotic index (Ki67 positive cells percentage) control fold induction in lobules and ducts (mean ± SEM), lobule structures from four patients, duct structures from six patients. *P < 0.05.
during the luteal phase (Clarke et al., 1997; Foidart et al., 1998). In a recent study, E2 + P treatment was administered to post-menopausal women and did not increase epithelial cell proliferation whereas conjugated estrogens associated with the progestin medroxyprogesterone acetate (MPA) treatment was proliferative (Murkes et al., 2011). In the same vein, in mammary tissues collected from ovariectomized cynomolgus macaque female adults, E2 + MPA treatment was mitogenic and activated growth factor signalization. This effect was not seen with E2 + P treatment (Wood et al., 2007, 2009). Our results corroborate those results as we observed that addition of P or UPA, to E2 treatment did not markedly alter cell proliferation when compared with E2 alone. Nevertheless, our in vivo model offers some limitations, with the continuous deliverance of steroids through a pellet which would have a different release kinetic when compared with steroid secretion from the human ovary. In addition, to detect small amplitude of P on cell proliferation, a higher number of experiments should be performed with breast samples from women at the same period of the menstrual cycle.

The impact of SPRMs on normal mammary tissue especially in human is difficult to evaluate. Using fine needle aspiration, Engman et al. (2008) showed that mifepristone was a potent anti-proliferative compound on normal breast cells. The mechanism underlying these actions is still not clarified. A reasonable hypothesis is that the mifepristone anti-proliferative effect could be mediated through its antigonadotropic action (Marions et al., 2002; Leminen et al., 2005) rather than by or in addition to a specific anti-progesterone effect on breast cell proliferation. It is also known that mifepristone and MPA interact with GR. The influence of SPRMs on glucocorticoid activity should therefore be taken into account for the final mitogenic response as we showed the important impact of glucocorticoids on normal luminal cells proliferation (Courtin et al., 2012).

The experiment performed in this study used a rate of UPA similar to the steady-state concentration of UPA after clinical chronic administration in women (HRA pharma personal data). This level ensured a maximum potential to inhibit progesterone actions. Our results suggest that UPA does not exert growth stimulation on normal breast tissue and could be used as an anti-progesterin molecule targeting hormone-responsive organs, such as uterus, for long-term therapy. Further clinical studies in women are nevertheless necessary to confirm its non-detrimental effect in normal breast tissue.

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Authors’ roles

A.G.: conception of the study, data interpretation, manuscript writing and final approval of manuscript. L.C.: collection and assembly of data, data analysis and interpretation and manuscript writing. M.V.: collection of data, data analysis and interpretation and manuscript writing. J.H. and A.C.: collection of data, data analysis and interpretation and reviewing of the manuscript. N.M.: data analysis and interpretation and reviewing of the manuscript. N.L.: performing estradiol and progesterone dosages in the mice samples and reviewing of the manuscript. S.D.: collection and assembly of data. M.C.: access and collection of materials. P.F.: data interpretation, reviewing and editing of the manuscript.

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Conflict of interest

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