**Identification and Biological Characterization of Progestins from Botanicals *In Vitro* and *In Vivo***

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THESIS
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## DEDICATION

This dissertation is dedicated to my parents and my husband, whose love and continuous support enabled my scientific aspirations to become a reality.

## ACKNOWLEDGEMENTS

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*Contribution of authors*

The emphasis of this Chapter I was to provide an overview on progesterone biology, its implications in various physiological processes, challenges and the current movement of the field. Chapter II represents the various materials and methods utilized and employed in this dissertation. With the exception of Figure 11, Chapter III represents a published manuscript (Toh MF, et al., 2012. Biological characterization of non-steroidal progestins from botanicals used for women's health. *Steroids* **77**:765–773), in which, I was the first author and the primary driver of the majority of the work. The co-authors listed in this publication contributed to various aspects of this research, allowing the successful completion of this study. Ping Yao and Shao-Nong Chen assisted with experiments shown in Figures 6 and 7. My graduate advisor, Dr. Joanna Burdette and our collaborator Dr. Judy Bolton contributed to the editing of the manuscript. Chapter IV represents a series of unpublished experiments directed at investigating kaempferol progestogenic effects *in vivo*. This work is currently under review and I anticipate that it will soon be published, wherein I am the primary author. Dr. Emma Mendonca generated Figure 15 and Table VII. Dr. Sharon Eddie and Dr. Michael Endsley played a significant role in editing this manuscript. Chapter V describes the search for novel progestins from Hops (*Humulus Lupulus*). This line of research will continue upon securing further funding in the form of grants. Chapter VI is a comprehensive summation of the results in this dissertation and its overall contribution to this field of study. The implication of these results to future investigation is discussed.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **AR** | Androgen receptor |
| **$E_2$** | Estrogen |
| **ER** | Estrogen receptor |
| **GR** | Glucocorticoid receptor |
| **HRT** | Hormone replacement therapy |
| **MPA** | Medroxyprogesterone acetate |
| **$P_4$** | Progesterone |
| **PR** | Progesterone receptor |
| **PGRMC1/2** | Progesterone receptor membrane component 1/2 |
| **SPRM** | Selective progesterone receptor modulator |

## SUMMARY

While extensive studies have been conducted on botanicals for the presence of estrogenic molecules, very little has been done to identify plant-based progestins. This underexplored area is troubling especially since there is a body of scientific evidence supporting the presence of progesterone receptor (PR) modulators in plant material that may affect endocrine function. For instance, some botanicals have been identified to contain progestogenic compounds as demonstrated by *in vitro* and *in vivo* assays (1-5). In particular, Zava *et. al* screened over 150 commonly consumed botanicals and identified oregano, verbena, tumeric, thyme, red clover and damiana as the six highest PR-binding herbs (3). Zand *et. al* assessed the steroidal activity of 72 flavonoids and identified 7 with progestogenic activity. At the same time, others have corroborated these results revealing that some plant-derived compounds can function as PR modulators by acting as weak agonists or antagonists (2, 6, 7). **Taken together, we hypothesize that natural progesterone ($P_4$)-like compounds from botanical extracts can be identified and biologically characterized for the improvement of women's health.**

The purpose of this dissertation was to identify whether botanical dietary supplements currently being used for women's health contain compounds with $P_4$-like activity. This project is important for two reasons: 1) the identification of novel, naturally occurring progestins will provide an avenue for new therapeutics with fewer side effects affecting women's health in terms of menopause, contraception, endometriosis, uterine disorders and breast cancer, and 2), women are already consuming many of these botanical extracts and should be aware of their potential progestogenic activities.

Red clover, hops, angelica, black cohosh, kudzu, dogwood, and chaste-tree berry were investigated for their ability to interact with purified PR, to activate PRE-luciferase transcription

in human breast cancer cells, and for tissue specific regulation of $P_4$ inducible genes. Kaempferol was identified as having $P_4$-like activity and may function in a cell-specific manner. *In vivo* studies revealed that kaempferol exhibited $P_4$-like effects in ovariectomized Sprague-Dawley rat model. Since genistein is a phytoestrogen that was previously demonstrated to increase uterine weight and proliferation (8), the ability of kaempferol to block genistein action in the uterus was investigated. Analyses of proliferation, steroid receptor expression, and induction of well-established PR-regulated targets *Areg* and *Hand2* were completed. In addition, kaempferol *in silico* binding analysis was completed for PR, as was the activation of ER and AR signaling *in vitro* in order to determine receptor specificity. The data from this dissertation suggest that kaempferol interacts with PR, activates the receptor without stimulating its degradation, induces known PR target genes *in vitro* and *in vivo* and blocked genistein-induced proliferation in the luminal epithelial cells in Sprague-Dawley rat uteri.

The toxicity of hops extracts in cell-based assays precluded further investigation *in vitro*, but an initial bioassay screen suggested that natural progestins can be identified from hops extracts (9). Biological activity of hops extracts made from pellets was confirmed in cell-based assays. These molecules activated a reporter gene in two major $P_4$-responsive cell-lines (breast and uterus), were blocked by receptor antagonists, activated an endogenous PR regulated gene, and accumulated in polar solvents. Further chemical and biological analyses are warranted to identify and characterize progestins from hops.

The comprehensive framework outlined in this thesis will provide a promising avenue for the identification of potentially better and safer compounds capable of activating PR signaling from botanical extracts used for women's health. In addition to the identification of kaempferol

as a potentially novel progestin, this volume may also serve as a useful resource to facilitate future investigation of additional botanicals for new sources of PR modulators. Knowledge of the progestogenic components will be essential for the standardization of these botanicals to produce a safer therapeutic.

# I. INTRODUCTION: PROGESTERONE AND GYNECOLOGICAL DISEASES

## A. Progesterone Biology and Mechanism of Action

Progesterone ($P_4$) is an ovarian steroid hormone that plays a vital role in regulating female reproductive physiology (10). $P_4$ is chiefly synthesized and secreted by a mass of cells developed from an ovarian follicle after ovulation known as the corpus luteum (10). The effects of $P_4$ are widespread and these effects have implications in multiple reproductive tissues, such as the breast, uterus, ovary and cervix (10). $P_4$ also affects non-reproductive tissues, in particular, the cardiovascular system, bone, and central nervous system and demonstrates the importance and prevalence of this hormone in normal physiology (11).

The physiological functions of $P_4$ are mediated by interacting with the progesterone receptor (PR), a member of the nuclear hormone receptor family (12, 13). Ligand occupied PR can function as transcription factors by binding to progesterone responsive elements (PREs) within the target gene or by binding to other transcription factors (14). PR is expressed in two major isoforms, PRA and PRB (14). Both isoforms are transcribed from the same gene by two distinct promoters, and have identical ligand-binding (LBD) and DNA-binding domains (DBD) (12). They differ in size being that PR-B has an additional 164 amino acids at the amino terminus not seen in PRA (12, 15, 16). Evidence has suggested that the different activities and function of the two PR isoforms are in part due to the different adopted conformations upon ligand binding (13, 17). Furthermore, PRB has an additional 164aa, a third activation domain, at the N-terminal, which may explain the stronger activation potential of PRB not seen in PRA (18). The ligand-bound PR then serves as a platform for the recruitment of other coregulatory proteins such as SP1, AP1, FOXO1, p65 and Src kinase (13, 19-21). The different PR conformations lead to

variation in PR/cofactor tethering interactions leading to differential expression of steroid-responsive genes and PR target tissues. In mice, PRA plays an important role in regulating ovarian function and protects the uterus against uncontrolled proliferation, whereas, PRB is involved in normal mammary gland development and function (22, 23). Interestingly, the selective roles of PRA and PRB are unique to murine tissues and have not been confirmed in humans (10). PRC, a recently identified third functional isoform of PR has shown to regulate the early events of parturition (24). Multiple studies have confirmed the presence of non-classical promoters termed progesterone receptor membrane component 1/2 (PGRMC1/2) (25, 26). PGRMC1 localizes to the plasma membrane, cytoplasm as well as the nucleus in rats and is required to regulate human ovarian function by mediating $P_4$'s actions in granulosa cells (27-29). PGRMC2 is most abundantly expressed in uterine tissue and is conserved between species, thus, it is deemed the main non-classical PR in the uterus (30). The signaling of PGRMC proteins has been shown to attenuate apoptosis and promote cell growth and survival of ovarian cancer cells and is linked to uterine diseases (30, 31). Additionally, the overexpression of PGRMC1 has been shown in multiple types of cancer, including breast, thyroid, colon, ovary, and lung cancer (31-33). The existence of various PR isoforms adds to the specificity and versatility of $P_4$ actions in different target tissues.

Structural biology studies of $P_4$-PR complex have shed light on the different binding modes adopted by PR ligands that elicit a wide range of biological responses. The mechanism of PR signaling is dependent on binding to the receptor, followed by dimerization and nuclear translocation to function as a transcription factor to regulate gene expression (13). PR ligands exhibit different binding modes depending on their antagonistic or agonistic nature (34, 35). In the case of an agonist conformation, helix-12, which is the substructure most dependent on ligand binding, is oriented toward the LBD (35). The key mode of action of receptor function

appears to be mediated by hydrogen bond interactions between the Met909 residue located within helix-12 and the ligand (35). The destabilization of the agonist conformation of helix-12 results in reduced response (35). Other molecular modeling studies revealed that PR function is dependent on mutually supported hydrogen bonding, Van der Waals interactions between the ligand and highly conserved residues within the LBD, and hydrophobic interactions with the steroid ring (**Figure 1**) (36). Therefore ligand-based molecular modeling techniques could serve as important tools for the development of novel PR modulators.

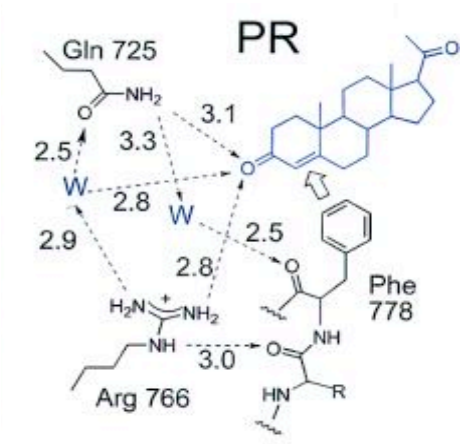


**Figure 1.** Network of important hydrogen bonds, Van der Waals and hydrophobic interactions between $P_4$ and conserved residues at PR LBD. (36)

Historically there have been three main areas of PR ligand development: agonists, antagonists and selective progesterone receptor modulators (SPRM), which interact with PR to activate or repress gene expression. Medroxyprogesterone acetate (MPA), norethindrone acetate and megestrol acetate are examples of PR agonists used in contraceptives, hormone replacement therapy (HRT), infertility treatments, fibroids, endometrial cancers and endometriosis (**Figure 2**) (37-39). To prevent pregnancies, progestins are used in oral contraceptives to block ovulation and increase the viscosity of the cervical mucus to reduce sperm mobility (40). Although MPA and norethindrone are effective PR agonists, they can promiscuously bind to the androgen and glucocorticoid receptor (AR/GR) leading to deleterious side effects such as blood clots, thrombosis, stroke, heart attack, and increased risk for breast cancer (37, 41). In humans, the administration of MPA and norethindrone at standard dosage levels significantly reduced the beneficial effects of estrogen ($E_2$) on endothelial function by inhibiting endothelium-dependent vasodilation in the brachial artery, which may increase the risk of atherosclerosis (42, 43). In contrast, the administration of progesterone, which has less androgenic activity compared to MPA and norethindrone did not adversely affect endothelial function, suggesting that the androgenic properties of MPA may be responsible (43). Population studies and a randomized trial conducted by the Women's Health Initiative (WHI) and A Million Women Study in the UK have demonstrated a link between the use of MPA when combined with estrogens and an elevated risk for breast cancer, not seen with estrogen only therapy (44-46). In animal studies, MPA administration resulted in high mitogenic activity in the mammary gland, reflected by enhanced BrdU incorporation and cyclin D1 expression (41). One explanation for MPA's proliferative response could be that MPA has significant GR activity, especially since different mouse models support the role of GR signaling as an activator of mammary epithelial cell proliferation (47-49). In light of these studies describing the detrimental effects of non-specific

activation of MPA signaling on AR and GR, it is critical to identify novel progestins with fewer off target effects.

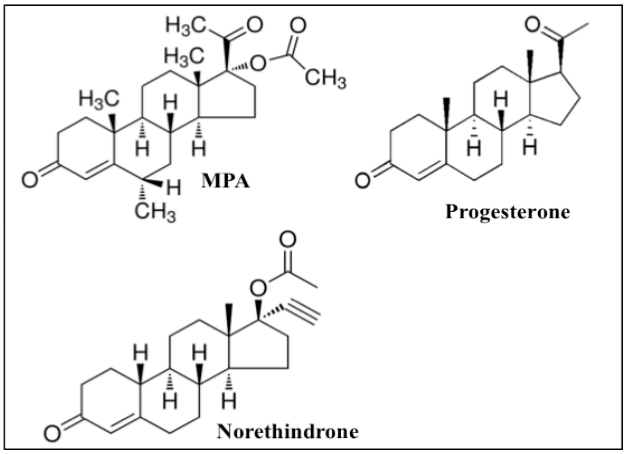


**Figure 2.** Structures of commonly used clinical progestins

Due to the failure of progestin analogs to mimic the actions of $P_4$ in terms of receptor specificity, the development of micronized 'bio-identical' $P_4$ has been attempted as a novel therapeutic approach. From a safety standpoint, natural $P_4$ is favored over most synthetic derivatives because it is devoid of unwanted interactions with non-specific targets (GR and AR) (50). Unfortunately, $P_4$ has poor oral bioavailability and a rapid clearance rate, which significantly limits its clinical use (51). For this reason, the reduction of $P_4$'s particle size through micronization has been proposed to improve its pharmacokinetic profile (51). Unlike oral $P_4$ which demonstrates a maximum mean plasma concentration observed after 4 hours of ingestion,

micronized $P_4$ remained significantly elevated after 6-7 hours (51). Contrary to MPA, micronized $P_4$ is therapeutically ideal, as it is bioavailable, has fewer reported cardiovascular side effects and is not mitogenic in breast tissues (45, 50-52). Although multiple studies have supported micronized $P_4$ in combination with transdermal $E_2$ as the optimal HRT regimen in women with an intact uterus (45, 52), it should be noted that micronized $P_4$ treatment alone is not as effective as progestins, such as lynestrenol (norethindrone), in the treatment of endometrial hyperplasia (53). These observations suggest that the endometrial protection of micronized $P_4$ is dependent on the treatment regimen and can only be secured when administered in combination with $E_2$ (52). Consequently, there remains a strong need for novel progestins, particularly those that are effective against endometrial diseases unrelated to HRT.

The adverse safety profile of clinical progestins and the need for more effective pharmaceutical agents led to the development of novel selective progesterone modulator (SPRMs). SPRM is a relatively new area of drug discovery with promising therapeutic utility for the treatment of uterine disorders such as endometrial fibroids and endometriosis (54). SPRMs are a class of PR ligands that function as either an agonist, antagonist, or mixed agonist/antagonist with clinically relevant tissue selectivity (54), therefore having the potential to provide beneficial progestogenic effects in the uterus, while avoiding their drawbacks in the breast (55). The growing evidence that SPRMs have a critical role in gynecologic therapies led to the discovery of a number of promising candidates with pharmacologic properties such as Asoprisnil, Proellex and ulipristal (56, 57). Unfortunately their applications did not live up to expectations, as reflected by clinical trials that were discontinued due to observed endometrial thickening and alterations in vascular and glandular architecture (58, 59). On a positive note, Tanaproget, a novel nonsteroidal PR agonist demonstrated tissue selective effects in the uterus but not in other sites such as the central nervous system, liver and vaginal tissues in preclinical

research (60). Additionally, Tanaproget was well tolerated and had an acceptable safety pharmacokinetic profile in healthy cycling women and has been recommended as a once-daily oral contraceptive (60).

| PROGESTIN | BRAND NAME | CLINICAL APPLICATION |
|---|---|---|
| Medroxyprogeterone acetate (MPA) | Depo-provera | HRT, dyfunctional uterine bleeding, induction of secretory endometrium, amenorrhea, contraceptive, endometriosis |
| Levonorgestrel | Plan B | Endometrial hyperplasia (IUD), contraceptive |
| Norethindrone acetate | Aygestin | HRT, amenorrhea |
| Norethindrone | Micronor | Contraceptive |
| Norgestrel | Ovrette | Contraceptive |
| Micronized progesterone | Prometrium | HRT, amenorrhea |

**TABLE I**. EXAMPLES OF PROGESTIN USED IN CLINICAL PRACTICE. 'IUD' INTRAUTERINE RELEASING SYSTEM

## B. The Role of Progestins in Women's Health

As described, $P_4$ regulates a variety of biological functions in a woman's body. The level of PR expression and its activation by various ligands are both important for the formation of disease and an opportunity for intervention. The biological implications of $P_4$ actions in normal physiological functions and gynecologic diseases are discussed.

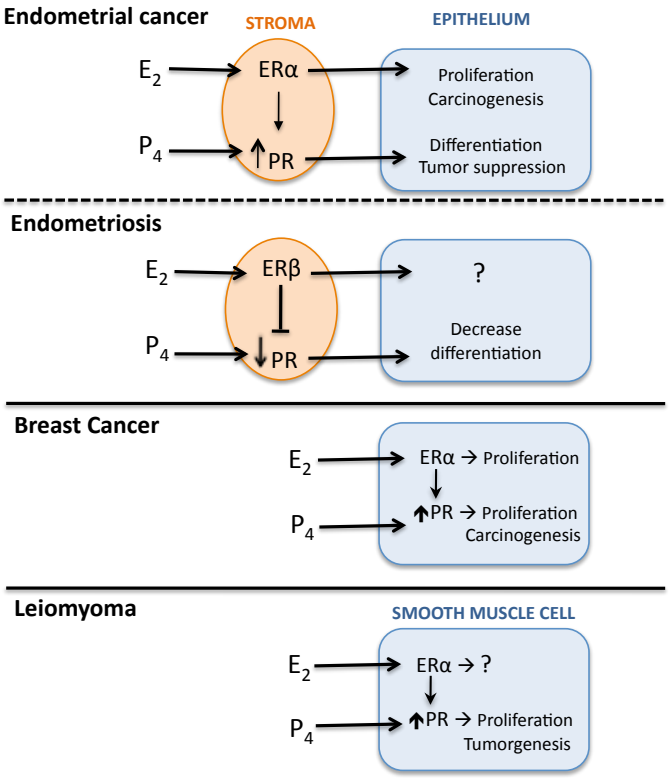


**Figure 3.** Epithelial and stromal $P_4$ signaling in various gynecological diseases. The proliferative effects of $E_2$ mediated via ERα are critical for endometrial carcinogenesis. $E_2$ enhances PR expression via ERα, promoting stromal PR action to oppose the negative effects of $E_2$ on the epithelium. High levels of stromal ERβ in endometriosis halt stromal PR expression. The lack of $P_4$ response (or progestin therapy resistance) leads to epithelial proliferation. $P_4$ acts directly in malignant breast epithelial cells and leiomyoma smooth muscle cells. Adapted from reference (10).

1. *Endometrial function*

The endometrium is the lining of the uterus and is one of the most highly responsive tissues in the body to ovarian steroid hormones (10). Endometrial cancer is one of the most common gynecological malignancies in the United States and the fifth most common cancer among women in the world (10). An estimated 49,560 women in the United States will develop

cancer of the endometrium and 8,190 deaths are predicted to result from the disease this year (61). Estrogen signaling plays a critical role in inducing endometrial hyperplasia and its heightened levels are a well-established risk factor for endometriosis (10, 14). $P_4$ inhibits endometrial epithelial growth through PR action in the stroma (**Figure 3**) (10). $P_4$ induction of 17β-hydroxysteriod dehydrogenase Type 2 (17β-HSD-2) expression is one of the molecular mechanisms underlying its ability to protect the uterus (62). 17β-HSD-2 metabolizes the more potent estradiol ($E_2$) to inactive estrone ($E_1$), halting $E_2$ induced proliferation of the endometrial layer (62). During the first half of the menstrual cycle, $E_2$ induces uterine proliferation via wnt/β-catenin signaling (63). However during the second half of the menstrual cycle, $P_4$ induces the expression of wnt/β-catenin signaling inhibitors DKK and FoxO1, thereby counterbalancing $E_2$ driven proliferation (63). Additionally, studies have shown that $P_4$ induction of Hand2 expression in the stroma blocks the production of paracrine mediators such as the fibroblast growth factor ligands, which control $E_2$ induced proliferation of the uterine epithelium (10). While many studies support the role of $E_2$ in promoting endometrial tumorigenesis, its actions are crucial to prime the endometrial stroma for $P_4$ responsiveness, as PR is an $E_2$ regulated protein (10). These observations further emphasize the importance of the sequential exposure to $P_4$ and $E_2$ in maintaining homeostasis in the endometrium. Since $P_4$ antagonizes $E_2$-induced endometrial hyperplasia by acting on the stromal cells, synthetic progestin MPA is often used clinically to treat advanced and recurrent endometrial cancers (10). Many investigators have shown that progestins are effective in reversing changes resulting from endometrial hyperplasia in 15% to 40% of patients (10). Unfortunately, the emergence of progestin resistance, due to PR down-regulation, limits the duration of progestin treatment and effectiveness (10). However, a study conducted by the Gynecological Oncology Group demonstrated that only 17% of women treated with 200 mg/d oral MPA demonstrated a complete response rate (10). Interestingly, better

overall response rates were observed in patients treated with alternating weekly cycles of MPA with added tamoxifen (64). The reason for tamoxifen incorporation was based on studies showing that tamoxifen is an estrogen receptor (ER) agonist that upregulates PR levels, resulting in the re-sensitization of the endometrium to progestin therapy.

2. *Endometriosis*

Another uterine disorder that can be treated with PR modulators is endometriosis. In the United States, 10-15% of women of reproductive age suffer from endometriosis and it is the third leading cause of gynecologic hospitalization in developed countries (10). Endometriosis is defined by the presence of uterine tissue outside of its normal location and is the most common cause of infertility and chronic pelvic pain (10, 14, 65). As a result, symptoms induced by endometriosis can have a debilitating impact on a woman's quality of life. Research findings indicate that ovarian steroid imbalance and retrograde menstruation are important factors underlying the pathology of endometriosis (10, 65, 66). Clinical studies revealed that progestin therapy is effective in 50% of patients in the alleviation of endometriosis-related pelvic pain (10). Unfortunately, progestin therapy resistance has been observed in patients with extremely low levels of PR expression in the endometrial tissue leading to a blunted $P_4$ response (10). Even though sizeable efforts have been invested in the development of more effective treatments for endometrial diseases, the overall response rates have not improved substantially. Therefore there is a great importance to develop novel, more efficient therapeutic strategies for the treatment of endometriosis and endometrial cancer.

3. *Uterine leiomyoma*

Uterine leiomyoma (fibroids) is another endometrial disorder that is influenced by changes in $P_4$ levels (10, 14). Some of the symptoms of leiomyomas include recurrent miscarriages, irregular uterine bleeding, urinary frequency, constipation and in some instances infertility (10, 14). 30-70% of women of reproductive age and up to 80% of women over 50 suffer from fibroids, which accounts for approximately 240,000 hysterectomies performed annually (10, 67-70). As is the case for endometriosis and endometrial cancer, $E_2$ has been deemed the main proliferative factor in leiomyoma. Despite experimental evidence (*in vitro* and in animal studies) suggesting that $E_2$ is the major driver in fibroids pathogenesis and $P_4$ inhibits fibroids development, clinical evidence revealed a more complicated mechanism (10, 71). For example, in human studies, MPA was able to dose-dependently induced fibroid proliferation in postmenopausal women taking combination HRT, not seen with $E_2$ only therapy (10, 72). Furthermore, fibroid xenograft models demonstrated that progestin alone is insufficient to drive fibroid formation, instead, $E_2$ stimulation of PR expression was vital in order to support $P_4$ action on fibroid cells (10). Clinical studies continue to support the therapeutic use of antiprogestins in leiomyoma treatment, further confirming the unfavorable effects of $P_4$ on fibroid growth (10). The eccentric role of $P_4$ between different endometrial diseases is heavily influenced by the underlying tissue microenvironment and external cues that signal through paracrine interactions (10). Attributed to the high cost and risk associated with surgical intervention, there is a clear need for therapeutic PR modulators that could be used as long-term therapeutic regimens and possibly as a prevention method against uterine fibroids.

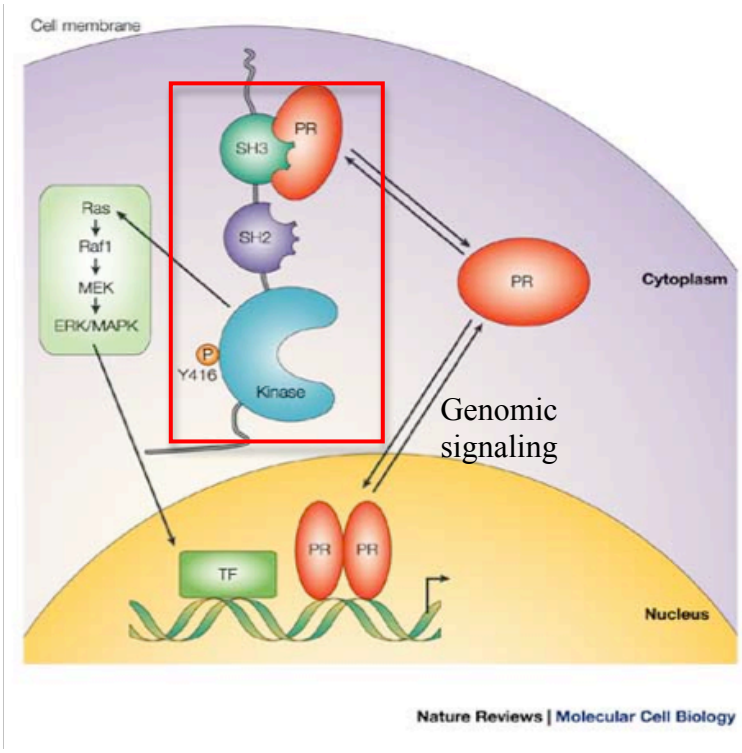


**Figure 4.** This schematic shows the interaction of the classical progesterone receptor (PR) with Src-homology-3 (SH3) domains and the initiation of the extracellular-signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) cascade. The amino-terminal proline-rich domain of PR interacts with the SH3 domain of Src. This activates the Ras–Raf–ERK/MAPK pathway, which then influences the activity of transcription factors (TFs) in the nucleus. The more conventional (genomic) action of the PR is depicted on the right of the figure; PR functions as a dimer in the nucleus to mediate transactivation and subsequent transcriptional changes. Non-genomic signaling is highlighted in the red box. SH2, Src-homology-2. Adapted from reference (73).

4. *Breast cancer*

A wealth of experimental and epidemiologic evidence has supported $E_2$ as one of the major risk factors in breast cancer (73-77). However, there have been controversial observations when it comes to the role of $P_4$. Research findings indicated that $P_4$ elicit biphasic effects on breast cancer regulation (10). Animal models with the BRCA1 gene deleted in the mouse

mammary epithelial cells displayed an increase in PR levels and $P_4$ mediated hyperproliferation (78). In addition to the genomic functions of PR (**Figure 4**), it can facilitate proliferative responses in breast cancer cells through non-genomic signaling (**Figure 4**, red box) (79). For example, PR interacts with c-SRC, subsequently turning on a downstream MAPK signaling cascade (**Figure 4**), which may explain how $P_4$ promotes breast cancer progression (10, 13, 79). PR-knockout mice had a decrease in carcinogen-induced mammary tumors compared to wild-type mice with normal PR expression (10). Furthermore, the treatment with PR antagonist RU486 inhibited tumor development (10). Although research with human breast cancer cell lines, human tumor samples, and clinical trials have associated $P_4$ with increased breast cancer risk, interestingly, some contradicting results have emerged indicating that PR halted proliferation in human breast cancer cells by inactivating MAPK through MAPK phosphatase I (MKP-1) mediated dephosphorylation (10). In advanced breast cancer, PR function has been shown to inhibit tumor proliferation, invasion, metastasis and inflammatory response (10, 80). These seemingly disparate observations support the biphasic role of $P_4$ on mammary tumorigenesis, which is dependent on the various stages of breast cancer, cell context and tissue microenvironment. In the case of HRT, there is a wealth of evidence supporting the role of progestins in driving proliferation when $E_2$ is present. In other words, combined progestin- $E_2$ regimes are associated with enhanced breast cancer risk not seen with estrogen monotherapy (37, 44, 81).

5. *Infertility*

Progesterone administration is the most common treatment used to assist patients with infertility. Infertility affects 6.7 million women ages 15-44 in the U.S. (82). Endometrial

remodeling is essential for uterine receptivity to embryonic implantation and the maintenance of pregnancy (83). Uterine remodeling relies solely on the duration and adequate progesterone exposure, given that there is sufficient estrogen priming of the uterus during the follicular phase (51, 83), and this highlights the vital role of $P_4$ in pregnancy. During this process the endometrium undergoes dynamic morphological and biochemical changes, releasing important regulatory factors such as metalloproteinases, cytokines, integrin growth factor binding protein-1 (IGFBP) and prolactin (84). These factors in turn lead to the remodeling of the extra cellular matrix, cytoskeleton, the development of endometrium-specific angiogenesis and ultimately the formation of the placenta (85). In some cases, the lack of/low levels of endogenous $P_4$ expression may cause infertility. As a result, vaginal $P_4$ administration has been used in infertility treatments to increase pregnancy rates.

6. *Ovarian cancer*

Ovarian carcinoma is one of the most lethal gynecological malignancies in the world and approximately 14,000 women diagnosed with ovarian cancer die each year (86). Ovarian cancer is called the "silent" killer due to the lack of symptoms and early detection methods (86). Roughly 75% of ovarian cancers will have metastasized by the time of detection and most patients will die within 5 years (87). Other factors that contribute to the lethality of this disease include the failure of current therapeutics and the lack of early detection and preventive methods (88). There is strong evidence supporting the increased risk of ovarian cancer with repeated ovulation without pregnancy-induced rest periods and excessive gonadotropin (LH/FSH) stimulation (86). In addition to these hypotheses, it appears that there is speculation concerning the role of hormonal stimulation on the etiology of ovarian cancer. More specifically, ovarian

cancer risk has shown to increase with excess androgen signaling of the ovarian epithelial cells (OSE) and decreased $P_4$ stimulation (86). For example, patients with PR overexpression are associated with favorable prognosis, and high-dose progestin oral contraceptive formulations are more effective against the disease than low-dose formulations (89). Additionally, women with multiple pregnancies tended to have reduced risk of ovarian cancer suggesting that the increased maternal circulating $P_4$ levels may explain the protective aspect of pregnancy (86). Furthermore, various studies have demonstrated the growth inhibitory effects of $P_4$ on the induction of apoptosis via the caspase 8/FasL signaling pathway in the OSE (90). Collectively, these observations correlate with a positive role of $P_4$ in ovarian cancer protection. Owing to the protective effects of $P_4$ on the development of ovarian cancer, $P_4$ can be used clinically for the treatment of ovarian carcinoma (91). Unfortunately, side effects associated with the use of progestin therapy have deterred the long-term use of progestins as a chemopreventive agent. Therefore the discovery and development of novel progestins for use in ovarian cancer patients without side effects is of great importance.

7. *Hormone replacement therapy*

Menopause is an inevitable predicament that most woman will eventually experience and most will seek alleviation of symptoms, which include depression, mood swings, fatigue, hot flashes, menstrual irregularities, cardiovascular disease, osteoporosis, coronary artery disease, and strokes (92). HRT is the most commonly prescribed treatment for the alleviation of menopausal symptoms. However, estrogen-only replacement increases the risk of endometrial cancer by 120% for every 5 years of use (44). Therefore, women taking HRT must take a combination of $E_2$ and a progestin (MPA) to prevent uterine hyperplasia and cancer (44).

Unfortunately, clinical evidence provided by the WHI and A Million Women Study demonstrated that a heightened risk of breast cancer and cardiovascular complications were seen in women treated with $E_2$ and MPA, compared to $E_2$ only treatment (45, 46, 81).

## C. Botanical Dietary Supplements

### 1. *The importance of combining phytoestrogenic and phytoprogestogenic botanicals*

The fear of the HRT-associated side effects from clinical studies fueled the popularity of alternative treatments for menopausal symptoms. As a result, more and more women are seeking safer estrogen/progesterone alternatives in the form of botanical extracts and dietary supplements (92), essentially taking responsibility for their health through self-medication. Some of these remedies are characterized and standardized to contain specific compounds, and most of the formulas to treat menopausal symptoms contain botanical-derived estrogenic compounds, such as genistein and 8-prenyl-naringenin that bind and activate ER (4, 8). The number of women choosing botanical dietary supplements for amelioration of menopausal symptoms has increased dramatically in recent years, but most manufacturers and women are only aware of the estrogenic activity of these extracts (92, 93). More alarmingly, due to limited oversight, the active compounds in these botanicals are frequently not known. Therefore, the use of botanicals containing only plant-derived estrogens in the absence of progestins might increase the risk of endometrial cancer similar to taking $E_2$ therapy alone. If botanicals are to be standardized in the future to contain estrogens to alleviate menopausal symptoms, then progestins should be added to protect against undesirable side effects in the uterus. Further confounding this issue is that botanical dietary supplements are generally self-prescribed; as a result, patients often do not

disclose herbal use to their physicians. This lack of transparency may potentially lead to negative interactions with prescription drugs or other substances (92).

2. *Botanicals commonly consumed for menopausal symptoms*

Red Clover (*Trifolium pratense)*

Shown below are the eight commonly used botanicals in women's health (**Table II**). Some of these botanicals are preferred over others because they have been used traditionally (92). For example, red clover has ethnomedical use for its antispomadic and anticancer properties (92). However, more recently, red clover has emerged as a popular herb for the treatment of menopausal symptoms. In the 1950's, the discovery of isoflavones in red clover were suspected to affect the reproductive system of sheep grazing heavily on red clover (94, 95). Some subterranean forage crops demonstrated high levels of phytoestrogens, such as genistein, that was thought to cause infertility and a prolapsed uterus in livestock (94, 95). In addition to genistein, red clover also contains daidzein, formononetin, coumesteral, and biochanin A, which are established phytoestrogens that can interact and activate the ER (4, 96). The estrogenic properties of red clover resulted in the development of Promensil, a dietary supplement marketed for the treatment of menopausal symptoms (97, 98). Unfortunately, data supporting their benefit on menopausal symptom relief have been inconsistent (92, 99, 100). Most studies showed minimal overall effect in the relief of hot flashes between participants taking red clover and those given placebo (92, 99, 100). Only one clinical trial, in which patients treated with higher dose of red clover (82mg) received significant faster relief of hot flashes compared to the placebo and a group treated with a lower dose (57mg) (92, 101). Other applications of red clover include the prevention against osteoporosis, heart disease and potentially cognitive functions (92). As with

hot flashes, there is disappointing evidence supporting the use of red clover for age related health issues. Nonetheless, it is relevant to note that the duration of most botanical studies tend to only last three months, just about the time one would expect the placebo effects to diminish (92, 100).

Hops (*Humulus lupulus)*

Hops are best known for their antimicrobial properties and aromatic flavors for use in the beer brewing process (102). However, the use of hops for women's health concerns remains controversial. Intriguingly, females harvesting hop flowers for an extended time in Germany experienced menstrual disturbances (103). Additionally, women in Germany would take hops baths to treat gynecologic disorders, pointing to its potential endocrine effects (104). The most potent phytoestrogen in hops, 8-prenynaringenin (8-PN), was confirmed using an ER ligand screening assay and animal models have shown hops or purified 8-PN to have estrogenic effects in the uterus and mammary gland (4, 103). Collectively, these discoveries have led to the suggested therapeutic use of hops to address menopausal symptoms. A double-blinded randomized placebo controlled study of 67 menopausal women, demonstrated that administration of hops containing 250 μg of 8-PN for 6 weeks was significantly superior to placebo (92). Although these effects diminished after 12 weeks, there was a more rapid decrease in menopausal discomfort compared to placebo, particularly in the hot flash score (105). In addition to the pharmacologic effects on the endocrine system, hops are approved for the treatment of anxiety, restlessness, as a mood enhancer, sedative and sleep aid by the German Commission E (92). Owing to the presence of 8-PN and its supposed proliferative effects on breast tissue, hops are widely advertised as breast enlargement supplements (106). However, since $P_4$ can also increase breast size by inducing the growth and development of milk producing cells, it is possible that progestogenic constituents may be found in hops (107).

| Common Name | Binomial name | World population | Ethnobotanical | Current application |
|---|---|---|---|---|
| * Red Clover | *Trifolium pratense* | North America, Europe, Australia, Asia | Anticancer treatment, antispasmodic | Hot flashes, osteoporosis, lipid profiles |
| Black Cohosh | *Cimicifuga racemosa* | North America | Native Americans, menstrual problems and childbirth. | Serotonergic effects, hot flashes and mood improvements |
| Valerian | *Valeriana officinalis* | Europe, America and Asia | Insomnia in Greece and Rome | Menopause |
| * Chaste-Berry | *Vitex agnus-castus* | Europe, Mediterranean region | Early physicians believe they had an effect on the female reproductive system, reduced sexual desire | PMS, menstrual irregularities, breast tenderness, hot flashes. |
| Dong Quai | *Angelica sinensis* | China, Japan, Korea | Traditional medicine to treat gynecological ailments, abnormal menstruation, menopausal symptoms, "female tonic" | Serotonergic effects, Merck, Eumenol. Menstrual disorders |
| * Hops | *Humulus lupulus* | Asia, North America, Europe | Anti-bacteria, sedative | Depression, mood swings, sleep disturbances, menopause |

**TABLE II**. COMMON BOTANICALS USED IN WOMEN'S HEALTH. Highlighted red are plant extracts with potential estrogenic activity.

Black cohosh (*Actaea racemosa*)

Black cohosh was traditionally used by Native Americans to induce labor, treat menstrual problems and pain associated with childbirth (92). Although black cohosh does not display any estrogenic action, it was able to improve bone density, hot flashes, depression and mood swings perhaps through serotonergic effects (92, 108). Since black cohosh is not estrogenic and has a positive safety profile, it has been suggested for the relief of vasomotor symptoms in breast cancer patients treated with tamoxifen (92).

Dong quai (*Angelica sinensis*)

Dong quai is one of the most popular Chinese herbs prescribed for the treatment of abnormal menstruation, pre-menstrual syndrome and menopausal symptoms (92). However, a double-blinded, placebo-controlled trial did not support significant effects of dong quai for menopausal symptoms such as hot flashes and vaginal dryness, and human studies did not identify any estrogenic mechanisms of action (92). Nonetheless, dong quai still has the reputation as a "female tonic" and is widely used in combination with other herbs to promote women's health (92).

Chastetree (*Vitex agnus-castus*)

Chastetree is consumed by women as a dietary supplement for the treatment of menopausal symptoms. Despite their popular use, there is lack of rigorous randomized controlled trials to justify their relevance in the context of menopause (92). The bulk of research has been confined to menstrual disorders, therefore, information regarding their benefits on menopause-related complaints is limited (92). Nonetheless, in a clinical study of 52 peri- and postmenopausal women treated with chastetree oil (1.5% solution of essential oil, on the skin, one time per day 5-7 days per week for 7 months), 33% of patients reported significant improvement in symptoms related to emotional problems and hot flashes (92, 109). Unfortunately, the study had no placebo or comparison group. Experimental evidence showed competitive binding of chastetree extract to ER and was able to activate ligand driven effects in a dose dependent manner (110). Further evaluations identified the apigenin and linoleic acid as possible estrogenic components in chastetree (110, 111), which supports its role in the alleviation of menopausal symptoms. Hyperprolactinaemia has been linked to irregular or lack of ovulation, resulting in infertility (112). Interestingly in a human study involving 52 women with hyperprolactinaemia revealed that a daily dose of 20 mg capsule of chastetree was able to reduce

and normalize plasma prolactin levels, supporting its therapeutic application for the treatment of this disease (112). Additionally, German health authorities have approved the use of chastetree for PMS, breast tenderness and menstrual irregularities suggesting that hormonal signaling may underlie the pharmacologic effects of chastetree (92).

The use of botanical dietary supplements continues to gain popularity despite the lack in scientific backing. Although women find botanical supplements appealing due to their perceived safety, there is very limited data on the actual benefits and potential dangers associated with their use. The precise mechanism of action of these alternative therapies is a 'black box' and much remains to be determined. Therefore, it is essential for human health to characterize and study the different components in these new therapies so that more informed regulatory decisions can be made.

### D. Phytoprogestins: A New Alternative for Women

While extensive studies have been conducted on botanicals for the presence of estrogenic molecules, very little has been done to identify plant-based progestins. This underexplored area is troubling especially since there is a body of scientific evidence supporting the presence of PR modulators in plant material that may affect endocrine function. For instance, some botanicals have been identified to contain progestogenic compounds as demonstrated by *in vitro* and *in vivo* assays (1-5). In particular, Zava *et. al* screened over 150 commonly consumed botanicals and identified oregano, verbena, tumeric, thyme, red clover and damiana as the six highest PR-binding herbs (3). Zand *et. al* assessed the steroidal activity of 72 flavonoids and identified 7 with progestogenic activity. At the same time, others have corroborated these results revealing that some plant-derived compounds can function as PR modulators by acting as weak agonists or

antagonists (2, 6, 7). Namely, apigenin, naringenin and syringic acid stimulated PSA levels in breast cancer cell lines, and these effects were attenuated in the presence of RU486 (6). In a different study, luteolin at physiologic levels displayed potent PR antagonistic activities and was able to destabilize helix-12 from assuming a favorable conformation for coactivator binding in docking studies (113). Collectively, these observations indicate that natural compounds with PR modulating activities are present in plant matter and may affect signal transduction pathways and cellular processes in PR responsive gynecologic tissues. **Taken together, we hypothesize that natural progesterone-like compounds from botanical extracts can be identified and biologically characterized for the improvement of women's health.**

## E. The Importance of Using New Assays on Old Structures

"One of the major challenges in medicinal use of natural products is understanding the mechanism of action for novel compounds [without overlooking potential novel mechanisms of known compounds]" (114). "When trying to understand how a botanical extract functions in the human body, an investigator often predicts a particular biological activity based on ethnobotanical human consumption or based on a chemical structure present in the plant extract" (114). "Finding new phytochemicals from botanicals simply to recapitulate existing biological activity might fail at identifying novel mechanisms of action and might fail to take advantage of unique biology to improve safety" (114). "The challenge comes in using either the known biological endpoint or the known chemical structure to identify the target of interest. Alternatively, libraries of extracts and compounds are tested against a series of enzymes or cells, and this approach can provide a challenge in later experiments when trying to determine if the compound is properly moved across the cell membrane, metabolized to something active or

inactive, or toxic to alternative enzymes or cell types when introduced *in vivo*. Mechanisms need to be evaluated at the enzyme or receptor, cell, and whole animal levels because many aspects of drug activation or elimination can only be studied in a living organism. Finally, looking at single target assays might miss complementary relationships between compounds in plant extracts. The power of botanical medicine might reside in identifying mechanisms by evaluating beyond model compounds and existing structures"(114). "Therefore, when looking for new biological mechanisms of action, it is critical to consider that new targets and new compounds might be present in botanical extracts and these might ultimately provide a new molecule that is safer and more efficacious than an existing therapy." (114)

## F. Phytoprogestins: A New Endocrine Disruptor in Botanicals?

Exposure to various environmental chemicals that interact with and modify endocrine systems can give rise to adverse effects on reproductive health (113, 115). These chemicals include natural compounds (phytoestrogens), environmental pollutants (pesticides), drugs (tamoxifen, ethinyl estradiol) and industrial chemicals and are known as endocrine disruptors (115). The notion that natural progestins can be identified illustrates that a new type of "endocrine disruptor" may be present which adds to the field of estrogens and androgens that humans are exposed to through diet and possibly the environment. The disturbance in endocrine function especially during critical developmental periods can lead to profound and lasting adverse effects on the regulation of essential physiological and morphological processes. This is a critical issue because women are already consuming plant-based therapies for a variety of conditions, such as infertility, breast enhancement, irregular menstruation, menopausal and premenstrual symptoms (92). Whether sufficient levels of phytoprogestins exist in the ambient environment and in our diet still remain a critical gap in our knowledge. Studying the effects and

mechanisms of these phytoprogestins will help identify if progesterone signaling is being altered and whether this might contribute to the safety profile of red clover and hops in the uterus of women consuming estrogens or whether this might increase dangerous progesterone signaling, which impacts reproduction and development.

## G. Scope of Study

The emphasis of this chapter was to provide an overview on progesterone biology, its implications in various physiological processes, challenges and the current movement of the field. On the basis that there is still a medical need for safer progestin alternatives and the documented discovery of naturally occurring progestins, the goal of this project was to bridge this gap through the identification and characterization of progestins from botanicals for the improvement of women's health. This project is important for two reasons: 1) the identification of novel, naturally occurring progestins will provide an avenue for new therapeutics with fewer side effects affecting women's health in terms of menopause, contraception, endometriosis, uterine disorders and breast cancer, and 2) women are already consuming many of these botanical extracts and should be aware of their potential progestogenic activities. In order to support and launch the idea that plant extracts are a viable source for identifying new PR modulators, the following experimental strategies were proposed:

1. *Determine if botanical extracts contain PR modulators responsible for progesterone-like activity and evaluate phytoprogestins PR signaling specificity.*

Crude extracts were screened with well-established cell-based assays. The activation of a canonical $P_4$ responsive element fused to a luciferase reporter gene (PRE-luc) and qPCR served as useful tools to measure cell-based progestogenic activity. In order to evaluate tissue/cell-type selectivity and allow for direct comparison of responses between uterus and the mammary gland, multiple breast cancer and endometrial cell lines were used. PR status provides a 'molecular marker' for $P_4$ and $E_2$ action; therefore, western blot was used as an additional strategy to determine progestogenic effects and hormonal responsiveness. Furthermore, to assess PR interaction, fluorescence polarization receptor binding assay and in silico molecular modeling docking experiments were completed (**Figure 5**). Activation of the androgen receptor (AR), estrogen receptor (ER) and the glucocorticoid receptor (GR) by clinical progestins has been associated with an increased risk of breast cancer and cardiovascular events (81, 116, 117). Hence, an ideal progestin should demonstrate comparable properties as $P_4$ and display no mixed interactions with non-specific targets.

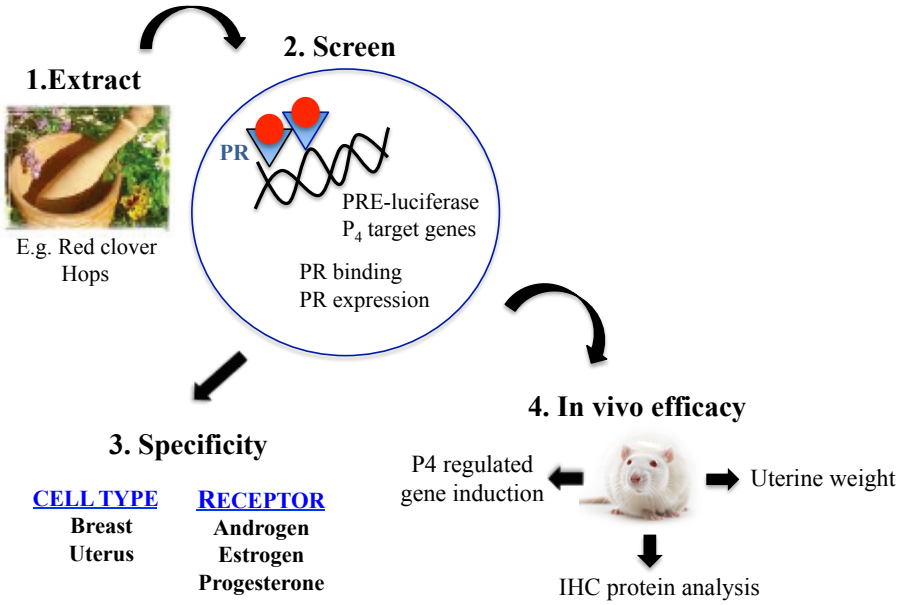


**Figure 5.** Schematic of phytoprogestin experimental strategies *in vitro* and *in vivo*

2. *Progestogenic effects of phytoprogestins in vivo*

Phytoprogestins are likely subjected to extensive pharmacokinetic and metabolic events, highlighting the importance for further studies *in vivo* to determine their endpoint signaling in target tissues and to better elucidate their role in women's health. Once kaempferol was identified and characterized as a phytoprogestin from *in vitro* systems, an ovariectomized rat model was used to assess its physiologic relevance *in vivo*. Since genistein and 8-PN are phytoestrogens that were previously demonstrated to increase uterine weight and proliferation (8), the ability of natural progestins to block phyto-estrogenic action in the uterus was

investigated. Furthermore, botanical formulations containing both a phytoestrogen and a phytoprogestin should minimize the adverse events in the uterus. This section of the study aimed to investigate if oral administration of kaempferol was progestogenic and could block proliferation induced by genistein in the uterus. As a quantitative measure of progestogenic activity, proliferation, expression of PR regulated transcriptional targets and steroid receptor status was analyzed. The data acquired from both *in vitro* and *in vivo* studies will contribute to a more predictive and an accurate reflection of physiological events in humans.

3. *Identify pure compounds responsible for progesterone-like activity in hops*

The purpose of this aim was to fractionate progestogenic compounds from hops for bioactivity testing with previously established progesterone bioassays. Fractions of metabolites from hops extracts were screened initially in the T47D PRE-luciferase assay. The fractions with biological activity were analyzed using an HPLC-based a biochromatogram technique to reduce the complexity of the fractions and to pinpoint the exact location of the progestogenic activity within the simpler mixture. In addition, the collected spectroscopic data of the active component(s) provided valuable information on the chemical composition of the samples. Bioactive molecules will be further separated using HPLC and will be characterized using various one- and two-dimensional NMR experiments (1H, 13C, 1D-selective TOCSY , COSY , HSQC, HMBC, HSQMBC, NOE, etc) and high-resolution MS analysis. Through these efforts, the establishment of an optimized experimental methodology will facilitate large-scale extraction to provide enough material for thorough characterization of hop-derived progestins from various sources *in vitro* and *in vivo*.

The comprehensive framework outlined in this thesis will provide a promising avenue for the identification of potentially better and safer compounds capable of activating PR signaling from botanical extracts used for women's health. In addition to the identification of novel progestins in this current study, this volume may also serve as a useful resource to facilitate future investigations of additional botanicals for new sources of PR modulators. Knowledge of the progestogenic components will be essential for the standardization of these botanicals to produce a potentially safer therapeutic.

# II. MATERIALS AND METHODS

## A. Reagents

All chemicals and reagents were purchased from Fisher (Hanover Park, IL) or Sigma–Aldrich (St. Louis, MO) unless otherwise indicated. All media for cell culture was purchased from (Life Technologies, Inc., Carlsbad, CA). Fetal bovine serum (FBS) and charcoal stripped serum was purchased from Atlanta Biologicals (Norcross, GA). Genistein, daidzein, biochanin A, formononetin, kaempferol, naringenin, apigenin were purchased form Indofine Chemical Co. (Belle Mead, NJ) and Sigma–Aldrich (St. Louis, MO). Desmethylarzoxifen (DMA) was provided by Dr. Gregory Thatcher (Department of Medicinal Chemistry, University of Illinois at Chicago).

## B. Extraction of plant extracts

All extracts were kindly prepared by the UIC/NIH Center for Botanical Dietary Supplements Research. *Angelica sinensis* (roots) was purchased from Yin Wall City, Inc., Chicago, IL (2001). *Pueraria lobata* (kudzu-flowering parts) was collected in Evanston, IL. *Actaea racemosa* (black cohosh-rhizomes and roots), *Cornus officinalis* (dogwood-fruits), *Valeriana officinalis* (valerian-roots), and *Vitex agnus-castus* (chaste-tree berry-berries), were provided by PureWorld Botanicals, now known as NATUREX (South Hackensack, NJ). A previously described $CO_2$-extracted nugget cultivar of *Humulus lupulus* (hops) was provided by Yakima Chief, Inc. Sunnyside WA (118). A *Trifolium pratense* (red clover) 30% isoflavones extract was provided by NATUREX (Hackensack, NJ). All voucher specimens have been deposited at the Pharmacognosy Field Station, Department of Medicinal Chemistry and

Pharmacognosy, College of Pharmacy, University of Illinois at Chicago. All plant extracts were prepared as described previously (119).

## C. Cell culture and cell lines

Human breast cancer cell line T47D American Type Culture Collection (Manassas, VA) was maintained in phenol red free RPMI 1640 medium (Life Technologies, Inc., Carlsbad, CA) containing penicillin/streptomycin (0.1 mg/ml), 10%(v/v) fetal bovine serum (FBS) (Life Technologies, Inc., Carlsbad, CA) and 4.5 g/L glucose in 24-well plate. An immortalized human endometrial stromal cell line (HESC), established by Krikun et al. (120), was kindly provided by Dr. Asgerally Fazleabas (Department of Obstetrics, Gynecology, and Reproductive Biology, Michigan State University, Grand Rapids, MI). HESC were cultured in DMEM/F12 1:1 (Life Technologies, Inc. Carlsbad, CA) supplemented with 10% dextran charcoal stripped FBS (Life Technologies, Inc. Carlsbad, CA) and 1% penicillin/streptomycin in 12-well plate. The human breast cancer cell line, MDA-MB231, was cultured in DMEM/F12 supplemented with 5% dextran charcoal stripped FBS, 1% penicillin/streptomycin, 1% L-glutamine and 20 ng/mL insulin in 24-well plate.

## D. Luciferase assay

T47D cells were grown in phenol-red free RPMI media in 24 well plates at 50,000 cells per well and endometrial stromal cells were grown in DMEM/F12 in 12 well plates until 80% confluent. Plasmid containing progesterone responsive element (PRE), prostate-specific antigen (PSA) or estrogen responsive element (ERE) fused to firefly luciferase (121) was transfected into T47D, MDA-231 cells (0.1 μg/well) and HSEC (0.5 μg/well) respectively, using Lipofectamine

2000 (Life Technologies, Inc., Carlsbad, CA) in Opti-MEM according to the manufacturer's protocol (Life Technologies, Inc., Carlsbad, CA). Treatment of human endometrial stromal cells is administered in combination with 10 nM $E_2$ to allow for stronger expression of the PR. Luciferase transfection efficiencies were normalized to an independent control plasmid expressing beta-galactosidase (β-gal) or renilla luciferase (0.1 or 0.5 μg), a kind gift of Dr. William T. Beck, (University of Illinois, Chicago, IL), cotransfected simultaneously. After transfection for 24 or 4 h, cells were treated with phytoprogestins for 24 or 48 h. Cell lysates (50 μL) were aliquoted into 96 well plates. The luciferase activity in assay buffer (25 mM glycyl glycine, 15 mM MgSO4, 4 mM EGTA, 100 mM potassium phosphate, 200 mM ATP, 1 M DTT) with 1 M d-luciferin (Life Technologies, Inc., Carlsbad, CA) was quantified. Luciferin substrate was injected followed by 12 s read by a POLARstar OPTIMA (BMG LabTech, Offenburg, Germany). The results are presented as the average fold induction of treated over untreated cells (DMSO) after correcting for transfection efficiency from triplicate experiments. Dose response curves were fitted to Gaussian distribution on prism with the equation, Y = amplitude * exp(−0.5 * ((X − mean)/SD)2).

**E. Progesterone receptor competitive binding assay**

The progesterone receptor competitive binding assay kit was purchased from (Life Technologies, Inc., Carlsbad, CA). Experiment was completed according to previously published literature (122). PR ligands (fluoromone green PL; 4 nM); $P_4$ (1 nM), plant extracts, or compounds were incubated in PR screening buffer with 4 mM dithiothreitol (DTT) in a total volume of 100 μL for 1 h at room temperature as described previously (122, 123). Each sample was analyzed in triplicate using POLARstar OPTIMA (BMG LabTech, Offenburg, Germany)

(122, 123). "An average of three samples containing only buffer and PR-LBD-GST with no fluorescent PL was used as the blank to eliminate background signal from the protein or buffer. A sample with no competitor was used to determine 100% binding capacity of the PR-LBD-GST for the PL ligand" (122).

**F. Cytotoxicity assay**

The sulforhodamine B (SRB) assay was used to measure cell viability. Cells were plated at 1000 cells per well in a 96-well plate and treated with DMSO or compounds for 24 h. Plates were processed according to protocols from previously published studies (124, 125).

**G. Western blot analysis**

T47D cells were incubated in serum free media with DMSO or compounds for 1.5 or 24 h. HESC cells were incubated in serum free media with various agents for 48 hours. Cells were lysed in 1X RIPA buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1% (v/v) Triton X-100, 0.1% (w/v) SDS) and Roche protease inhibitor (Roche, Madison, WI). Protein concentrations were measured using BCA protein assay reagent (Fisher, Rockford, IL). Protein was separated on denaturing 7% SDS–PAGE gels and transferred to PVDF membranes using iBlot (Life Technologies, Inc., Carlsbad, CA). Membranes were blocked in 3% milk in Tris buffered saline-Tween (1 M NaCl, 2% 1 M Tris, 3% Tween 20). Membranes were probed overnight at 4°C with antibody against PR-A/B (Cell Signaling, Danvers, MA or Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:500 in 5% milk in TBS-T. Membranes were washed and probed with HRP-linked secondary antibody (Cell Signaling, Danvers, MA). Chemiluminescent was detected using SuperSignal West Femto Chemiluminescent Kit, (Thermo Scientific, Hanover Park, IL)

according to the manufacturer's protocol and imaged on a Syngene G:Box P20111247 (Imgen Technologies, Alexandria, VA). Membranes were reblotted for actin (Cell Signaling, Danvers, MA) as loading control. Densitometry analysis was performed using Image-J from NIH and the average fold change from three blots is reported.

## H. Molecular modeling

The crystal structure of the human PR in complex with the agonist $P_4$ (PDB: 1A28; 1.8 Å) was used in the docking procedure (34). The protein model was analyzed using the protein structure preparation module in MOE (126) . All ligands and water molecules were removed and hydrogen atoms were added using Protonate3D. This structure was saved as a PDB file. The 3D structures of the ligands were built and inspected with VIDA and AM1-BCC (127) partial atomic charges were calculated with Molcharge (126) and minimized using OMEGA (126). All ligands were docked into the binding pocket of PR using GOLD (version 5.1, CCDC, Cambridge, UK) (128). The active site was defined as all protein atoms within 6 Å of $P_4$. The scoring function used to rank the docked poses was Chem-PLP. "A maximum of twenty docking solutions were generated for each structure, with early termination of the process if the respective RMSDs of the three highest ranked docking solutions were within 1.5 Å RMSD of one another (GOLD default 1 setting: 100,000 Genetic Algorithm (GA) Operations, 5 islands)" (129). "Flipping of ring free corners, amide bonds, [protonated carboxylic acids] and planar [or pyramidal] nitrogen atoms were allowed" (129). MOE was also used to analyze the docking results and generate figures. The top-ranked poses were further co-minimized using MOE LigX module utilizing AMBER12HT forcefield for optimization and calculation of affinity score (130). Emma Mendonca performed molecular modeling experiments under the direction of Dr. Pavel Petukhov.

## I. Rat study

All animal studies were approved by the UIC Animal Care and Use Committee. Sprague-Dawley ovariectomized (OVX) rats were utilized for this study to eliminate endogenous hormone production. Twenty-four animals weighing 160-180g were purchased for the study (n=8/group) (Harlan Laboratories, Madison, WI). All rats were housed at 21°C in 12 h light:12 h dark cycles and were fed 7% corn diet (Harlan Laboratories, Madison, WI) devoid of phytoestrogens. Two weeks post ovariectomy, 5.625 mg kaempferol or genistein was dissolved in a DMSO/corn oil mixture and given via oral gavage daily for 8 days based on a previous study demonstrating estrogenic action of genistein at this dose and duration (8). Control animals were given DMSO/corn oil only. Animals were sacrificed 24 h after the last injection.

## J. Immunohistochemistry

Uteri were carefully excised, weighed, and fixed in 4% paraformaldehyde overnight. The tissues were dehydrated in a series of alcohols of increasing concentrations, embedded in paraffin, and sectioned at a thickness of 0.5 μm. Three sections per animal were placed on slides, deparaffinized, and rehydrated. To facilitate antigen detection, slides were placed in boiling 10 mM sodium citrate buffer (pH 6.0) for 2 min on high followed by 5 min twice on low, and then cooled to ambient temperature. Endogenous peroxidase activity was quenched by incubation with 3% $H_2O_2$ for 15 min. Primary antibodies utilized in this study included Ki67 (Abcam, Cambridge, MA), PR, ERα and Hand2 (Santa Cruz Biotechnology, Santa Cruz, CA) (131) (**Table III**). For PR, ERα and Ki67, a biotinylated horseradish peroxidase-conjugated anti-rabbit IgG was used as the secondary antibody (1:200, Vector, Burlingame, CA). For Hand2 detection a biotinylated horseradish peroxidase-conjugated anti-goat IgG was used as the secondary

antibody (1:200, Vector, Burlingame, CA). Secondary antibody incubation was followed by ABC peroxidase detection enhancement (Vector, Burlingame, CA) and detected by DAB as the chromogen (Vector, Burlingame, CA). Slides were counterstained with haemotoxylin and photomicrographs of sections were obtained using Nikon Eclipse E600 microscope. To assess proliferation in the luminal epithelial cells, a minimum of 300 cells were quantified (2 sections per animal) and the average taken. Data are represented as percentage of positive cells. In the stroma, the entire endometrial section was examined, and the number of proliferating stromal cells were categorized as 0, no staining; fewer than 5, low; and more than 5, high. High, low and absent Ki67 expression was classified in endometrial stroma for all four groups.

| Peptide/protein target | Name of Antibody | Manufacturer, catalog #, and/or name of individual providing the antibody | Species raised in; monoclonal or polyclonal | Dilution used |
|---|---|---|---|---|
| PR-A/B | Anti-Pgr | Santa Cruz Biotech (sc-7208) | Rabbit polyclonal | 1:500 |
| actin | Anti-Actin (20-33) antibody produced in rabbit | Sigma Aldrich, Cat A5060 | Rabbit polyclonal | 1:2000 |
| ERα | ERα | Santa Cruz Biotech (sc-7207) | Rabbit polyclonal | 1:500 |
| Ki67 | Anti-Ki67 antibody | Abcam (ab15580) | Rabbit polyclonal | 1:200 |
| HAND2 | dHAND Antibody | Santa Cruz Biotech (sc-9409) | Goat polyclonal | 1:100 |

**TABLE III**. INFORMATION ON THE ANTIBODIES USED IN THIS STUDY.

## K. Quantitative PCR (*in vitro*)

Quantitative PCR was used to examine the modulation of zinc finger and BTB domain-containing protein 16 (ZBTB16), prolactin (PRL) and cannabinoid receptor 1 (CNR1) by phytoprogestins in T47D cells or HESC using SYBR green fluorescence. To demonstrate feasibility, RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed using RevertAid first strand cDNA synthesis kit (Fermentes, Glen Burnie, MD) according the manufacturers' protocols. Each reaction consisted of 100 ng cDNA, 10 μL SYBR Green PCR Master Mix (PE Applied Biosystems, Carlsbad, CA), and 0.5 μM forward and reverse primers (Sigma, St. Louis, MO) for 40 cycles (95°c for 15 s, 65 °C for 1 min). The fold changes in all genes were analyzed with the ΔΔCt method, with GAPDH or H3F3 as an internal control. Data reported are the mean fold change ± SEM for three replicates over negative control DMSO.

## L. Quantitative PCR (*in vivo*)

Uterine RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) reagent as per manufacturer's instructions. The quality of total RNA was determined spectrophotometrically. Complementary DNA was reverse transcribed using RevertAid first strand cDNA synthesis kit (Fermentes, Glen Burnie, MD) in a total volume of 20 μl. Each real-time PCR consisted of 100 ng cDNA, 10 μl FastStart SYBR Green PCR Master Mix (Roche, Madison, WI), and 0.5 μM forward and reverse primers (IDT, San Jose, CA). PCR analyses were conducted using the following set of primers; *Gapdh* 5'- CATGGCCTTCCGTGTTCCTA-3' (forward) and 5'-CCTGCTTCACCACCTTCTTGAT-3' (reverse), *Rpl1* 5'- CTGTGAGGGCATCAACATTTC-3' (forward) and 5'-GTTGGTGTTCATCCGCTTTC-3' (reverse), *ERα* 5′-

AATTCTGACAATCGACGCCAG-3' (forward) and 5'-GTGCTTCAACATTCTCCCTCCTC-3' (reverse), *PR* 5'- CCCGACACTTCCAGCTCTTT-3' (forward) and 5'-TGTGGGATTTGCCACATGGT-3' (reverse), *Hand2* 5'-AAGAGGAAGAAAGAGCTGAATGAGAT-3' (forward) and 5'-CGTTGCTGCTCACTGTGCTT-3' (reverse), *Areg* 5'-AACTGAACTTCTGGAGCCTTC-3' (forward) and 5'-CATGCCATAGCCTAGCTGAT-3' (reverse). Fold change in mRNA expression was determined via the ΔΔCt method, with *Gapdh* as an internal control for *Areg and Rlp1* for *Hand2*, *PR* and *ERα.* Data reported are the mean fold change ± SEM for three replicates compared to vehicle control.

# III. IDENTIFICATION AND BIOLOGICAL CHARACTERIZATION OF PROGESTINS FROM BOTANICALS *IN VITRO*

## A. Introduction

Due to the side effects associated with hormone therapy and the perceived safety of natural remedies, millions of menopausal women are seeking alternatives in the form of botanical extracts and dietary supplements. Unfortunately, the use of botanicals containing only plant-derived estrogens in the absence of progestin-like molecules might increase the risk of developing endometrial cancer similar to taking estrogen alone (*7*). For instance, red clover, hops, and angelica are common botanicals which contain phytoestrogens that bind and activate estrogen receptors (ER) and are used for the treatment of menopausal symptoms (119). Interestingly, when hops and red clover were given orally to ovariectomized rats, uterine weights were not significantly increased in animals treated with a crude extracts as compared to pure estrogenic compounds alone, suggesting the presence of progestins capable of opposing $E_2$ activity (4, 119).

The purpose of this study was to identify if botanical dietary supplements currently being used for women's health contain compounds with $P_4$-like activity. The following representative botanicals currently being sold as components of women's health formulations were tested as 75% ethanolic extracts: red clover, hops, angelica, black cohosh, kudzu, dogwood, and chaste-tree berry. Extracts were investigated for their ability to interact with purified PR, to activate PRE-luciferase transcription in T47D breast cancer cells, and for tissue specific regulation of $P_4$ inducible genes. Red clover, kaempferol, naringenin, and apigenin were identified as having $P_4$-like activity and may function as non-steroidal SPRMs.

## B. Results

*Plant extracts bound to PR and induced PRE-luciferase in T47D breast epithelial cells*

Eight ethanolic botanical preparations commonly used for women's health were tested for their ability to interact with purified progesterone receptor (PR) and for induction of a progesterone responsive element (PRE) linked to luciferase in T47D breast cancer epithelial cells (**Table IV**). The T47D cell line was used due to its high endogenous expression of PR (132). Four plant extracts demonstrated a significant dose-dependent ability to interact with purified PR in a receptor binding assay: the 75% EtOH extracts of valerian, dong quai, dogwood, and red clover (**Table IV**). The 75% EtOH extracts of hops and kudzu could not be measured for receptor binding due to interference of the crude plant extract with changes in fluorescence polarization. To determine if botanical extracts induce expression of a progesterone reporter gene, T47D cells were transiently cotransfected with the PRE-luciferase plasmid and used to measure activation of the functional PR-PRE complex in response to treatment with botanical extracts. Only red clover (20 μg/ml) significantly activated PRE-luciferase induction (**Table IV)**. The hops extract was cytotoxic in T47D cells and could not be evaluated in this assay (**Table IV**). Dogwood bound to the PR but did not induce PRE-luciferase activity (**Table IV**). Therefore, extracts were incubated with progesterone to determine if they bound the receptor and functioned as antagonists. In the presence of 100 nM $P_4$, only dogwood and black cohosh extracts significantly inhibited $P_4$-induced activation of luciferase in T47D cells indicating that they function as receptor antagonists (**Figure 6**).

| Plant Extracts 75% Ethanolic (20μg/mL) | PRE-luciferase fold increase | PR Binding $IC_{50}$ μg/mL | Toxicity, T47D $LC_{50}$ μg/mL |
|---|---|---|---|
| ***Humulus lupulus*** **(Hops)** | 2.2 ± 1.2 | N/D | 2.5 |
| ***Actaea racemosa*** **(Black Cohosh)** | 1.5 ± 0.1 | > 1 mg | > 20 |
| ***Cornus officinalis*** **(Dogwood)** | 1.3 ± 1.1 | 15 ± 1.4 | > 20 |
| ***Pueraria lobata*** **(Kudzu)** | 2.1 ± 1.4 | N/D | > 20 |
| ***Valeriana officinalis*** **(Valerian)** | 1.4 ± 0.7 | 97 ± 17 | > 20 |
| ***Vitex agnus castus*** **(Chaste-Tree Berry)** | 1.5 ± 1.0 | > 1 mg | > 20 |
| ***Angelica sinensis*** **(Dong Quai)** | 1.6 ± 0.7 | 106 ± 21 | > 20 |
| ***Trifolium pratense*** **(Red Clover)** | 4.7 ± 1.2* | 34 ± 20 | > 20 |

**TABLE IV.** PROGESTERONE RESPONSIVE ELEMENT (PRE)-LUCIFERASE INDUCTION, PROGESTERONE RECEPTOR (PR) BINDING, AND CYTOTOXICITY OF PLANT EXTRACTS IN HUMAN BREAST CANCER EPITHELIAL CELLS (T47D). Botanical extracts (20μg/mL) were tested at a single concentration in luciferase assays and at five doses in PR binding assays. N/D indicates that the plant extract interferes with measuring polarization. * $p < 0.05$. $P_4$ has an $IC_{50}$ of 25 nM in the PR binding assay and at 100 nM induces a 54 fold change over basal.

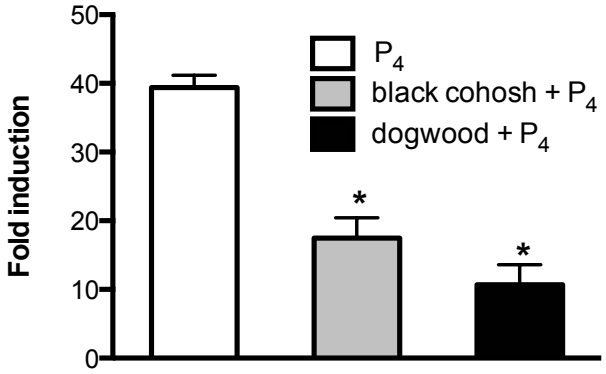


**Figure 6.** Botanicals that bind to and do not activate PRE-luciferase function as antagonists when combined with progesterone ($P_4$). PRE-luciferase expression in T47D cells treated with $P_4$ (1 μM) and black cohosh or dogwood (20 μg/mL). Data represent average +/- SEM fold change of relative light units normalized to β-gal in three independent experiments. (∗$p < 0.05$), significance against DMSO control, as determined by one-way ANOVA test.

*Identification of pure compounds from botanicals with progestegenic activity that dose dependently bound to PR, induced PRE-luciferase and are inhibited by the PR antagonist RU486 in T47D cells.*

Since a 75% ethanolic extract of red clover significantly induced PRE-luciferase activity (**Table IV**), a library of 26 compounds (**Table V**) previously isolated from red clover (133) were tested for their ability to bind to PR and activate the PRE-luciferase reporter gene (**Table VI**). Genistein, daidzein, biochanin A, and formononetin are isoflavonoids from red clover that were previously reported to interact with and activate ER signaling (134). First, the isoflavones with estrogenic activity were investigated to confirm that these compounds could not also interact with and activate PR. None of the isoflavones with estrogenic activity significantly interacted with purified PR in a receptor binding assay or induced PRE-luciferase expression. From the library, kaempferol was identified from red clover as a ligand for PR. Apigenin was investigated based on its similar structure to kaempferol and is a known constituent of chaste-tree berry (135). Kaempferol and apigenin have conjugated A and B ring systems and kaempferol has a 3' hydroxyl group not seen in apigenin. Both flavonoids were determined to significantly activate PRE-luciferase expression and bind to purified PR (**Table VI**). Naringenin, also found in the red clover library, bound to PR, but did not significantly activate PRE-luciferase at 10 μM (**Table VI**). In order to determine if kaempferol, apigenin, and naringenin could activate PRE-luciferase in a dose-dependent manner, five increasing doses of compounds were tested in T47D cells. $P_4$, kaempferol, apigenin and naringenin all dose-dependently activated PRE-luciferase (**Figure 8**). Naringenin at 10 μM was not significantly different than solvent control (**Table VI**), but at 20 μM reached significance. Based on dose response curves, kaempferol was the most active at 7.5

μM (**Figure 8B**) followed by apigenin at 5 μM (**Figure 8C**). To further confirm if these transcriptional effects were PR mediated, cells were treated with and without RU486, a well-characterized PR antagonist. RU486 was able to significantly abrogate $P_4$, kaempferol, apigenin and naringenin-induced PRE-luciferase activity (**Figure 8**). 100 nM $P_4$ induces a 54 fold change over basal. MPA demonstrated comparable activity (data not shown).

| | |
|---|---|
| 1 | Tyramine |
| 2 | Fraxidin |
| 3 | Xanthotoxol |
| 4 | Fisetin |
| 5 | Calycosin |
| 6 | Quercetin |
| 7 | Naringenin |
| 8 | Pratensein |
| 9 | Kaempferol |
| 10 | Daphnoretin |
| 11 | Pseudobaptigenin |
| 12 | Maackiain |
| 13 | Irilone |
| 14 | Dihydrobiochanin A |
| 15 | 6,7,4'-trihydroxyisoflavone |
| 16 | Prunetin |
| 17 | Daidzein |
| 18 | Genistein |
| 19 | Hyperoside |
| 20 | Sissotrin |
| 21 | Caffeic acid |
| 22 | Chlorogenic acid |
| 23 | Biochanin A |
| 24 | Formononetin |
| 25 | Coumestrol |
| 26 | Scopoletin |

**TABLE V**. COMPOUNDS IN RED CLOVER

| ***Progestegenic Compounds*** | ***PRE-luciferase Fold increase (10 μM)*** | ***PR Binding $IC_{50}$ μM*** | ***Toxicity $LC_{50}$ μM*** |
|---|---|---|---|
| Kaempferol | 5.5 ± 1.8* | 1.5 ± 0.4 | > 20 |
| Apigenin | 6.5 ± 1.9* | 1.0 ± 0.7 | > 20 |
| Naringenin | 1.9 ± 0.4 | 6.9 ± 1.8 | > 20 |

| ***Estrogenic Compounds*** | ***PRE-luciferase Fold increase (10 μM)*** | ***PR Binding $IC_{50}$ μM*** | ***Toxicity, $LC_{50}$ μM*** |
|---|---|---|---|
| Genistein | 2.2 ± 1.1 | >250 | > 20 |
| Daidzein | 1.0 ± 0.4 | >250 | > 20 |
| Biochanin A | 1.0 ± 0.1 | >250 | > 20 |
| Formononetin | 1.5 ± 0.6 | >250 | > 20 |

**TABLE VI**. PRE-LUCIFERASE INDUCTION, PR BINDING, AND CYTOTOXICITY OF ACTIVE PURE PLANT-DERIVED ESTROGENIC AND PROGESTIGENIC COMPOUNDS. Botanical compounds (10 μM) were tested for luciferase induction in T47D cells and for binding to the purified PR. * $p < 0.05$. Positive control progesterone ($P_4$) had a PR binding $IC_{50}$ of 25 nM. $P_4$ (100 nM) induces a 54 fold change over basal.

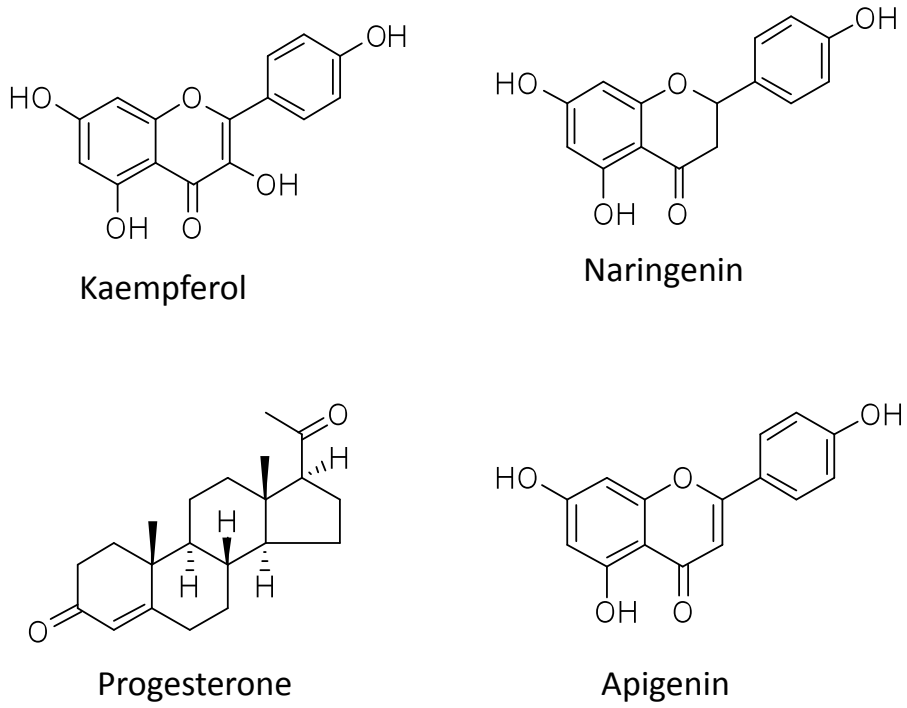


**Figure 7.** Chemical structures of flavonoids that bound to the progesterone receptor.

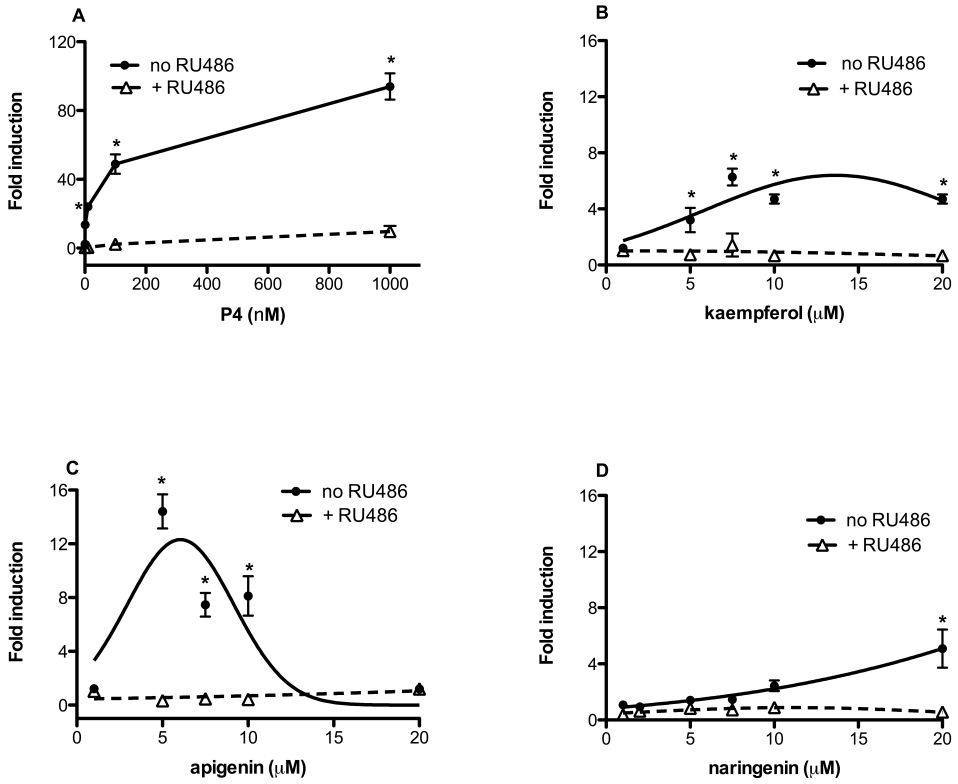


**Figure 8.** Kaempferol, apigenin and naringenin, dose-dependently activate PRE-luciferase and can be antagonized by the PR antagonist, RU486. T47D cells were transiently transfected with PRE-luciferase and treated with increasing concentrations of pure compounds with and without PR antagonist (1 μM RU486). Data represent average +/- SEM fold change of relative light units normalized to β-gal in triplicate experiments. Mean ± SEM (∗$p < 0.05$), significance against DMSO control, as determined by one-way ANOVA test.

*Kaempferol, apigenin, and naringenin induced the $P_4$-regulated gene ZBTB16 in T47D breast epithelial cells*

To further evaluate the effects of kaempferol, apigenin, and naringenin on progesterone signaling in breast epithelial cells, induction of the $P_4$ responsive gene ZBTB16 was measured using SYBR green real-time PCR (**Figure 9**). Kaempferol (100 μM) significantly induced

ZBTB16 mRNA after 24 h, while apigenin and naringenin did not induce ZBTB16 at this concentration (**Figure 9**). However, when cells were treated at higher doses of the purified compounds (250 μM), the induction of ZBTB16 was significant for all three flavonoids (**Figure 9**). The presence of the PR antagonist, RU486, significantly inhibited $P_4$ or phytoprogestin-induced ZBTB16 expression (**Figure 9**). The positive control (100 nM) was much more potent compared to phytoprogestins even when used at higher concentrations (100 μM and 250 μM).

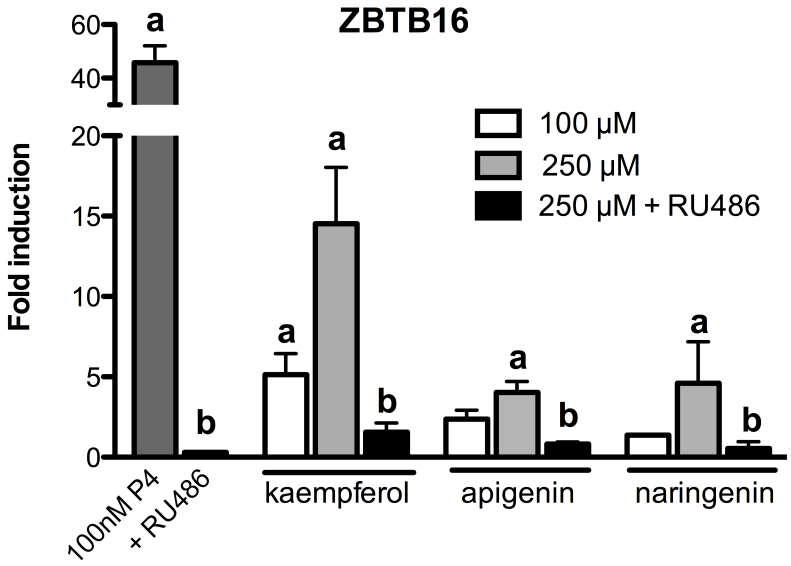


**Figure 9.** Expression of ZBTB mRNA in response to kaempferol, apigenin and naringenin in T47D cells. T47D cells were treated with 100 μM or 250 μM for 24 hours. Expression of ZBTB16 was measured using SYBR green real-time PCR. Data are expressed as the average fold mean ± SEM (∗$p < 0.05$), significance against DMSO control, as determined by one-way ANOVA test.

*Kaempferol, apigenin, and naringenin dose-dependently induced PRE-luciferase in human endometrial stromal cells (HESC) that is antagonized by RU486.*

The cell and tissue context is critical when investigating $P_4$ signaling because the actions of $P_4$ can be both tissue and cell type specific (136). In the uterus, cell type specific signaling is especially important since progesterone blocks estrogen-induced proliferation by acting on the stromal cells, a phenomenon not seen in breast epithelial cells, such as T47D, suggesting that progestins may have differential biological activity and potency in the breast as compared to the uterus (123). Because $P_4$ signals in the stromal cells of the uterus, the ability of kaempferol, apigenin, and naringenin to induce PRE-luciferase activity at different doses was investigated in a human endometrial stromal cell line (HESC) (**Figure 10A**). Medroxyprogesterone acetate (MPA), kaempferol, and apigenin, but not naringenin, dose-dependently activated PRE-luciferase in HSEC (**Figure 10A**). MPA was the most active compound followed by kaempferol and apigenin (**Figure 10A**). However, at higher concentrations, kaempferol and MPA had similar potency (**Figure 10A**). Purified compounds at 20 μM significantly upregulated PRE-luciferase expression in HESC and the presence of 1 μM RU486 significantly inhibited MPA, kaempferol, and apigenin induced PRE-luciferase at 20 μM (**Figure 10B**). In HESC cells, the positive control and kaempferol had similar biological activities indicating that purified compounds exert more potent transcriptional activity in HESC compared to T47D cells.

*Kaempferol was more active in the endometrial cell lines compared to breast epithelial cells.*

The progestogenic effects of kaempferol were compared in breast epithelial cells and endometrial epithelial cells (**Figure 11**). The outcome of this experiment showed that not all progestins exert similar effects; there are clear differences in PRE-luciferase activity in two $P_4$-

responsive cells lines. Consistent with HESC, kaempferol was more active in endometrial epithelial cells compared to breast epithelium (**Figure 11**).

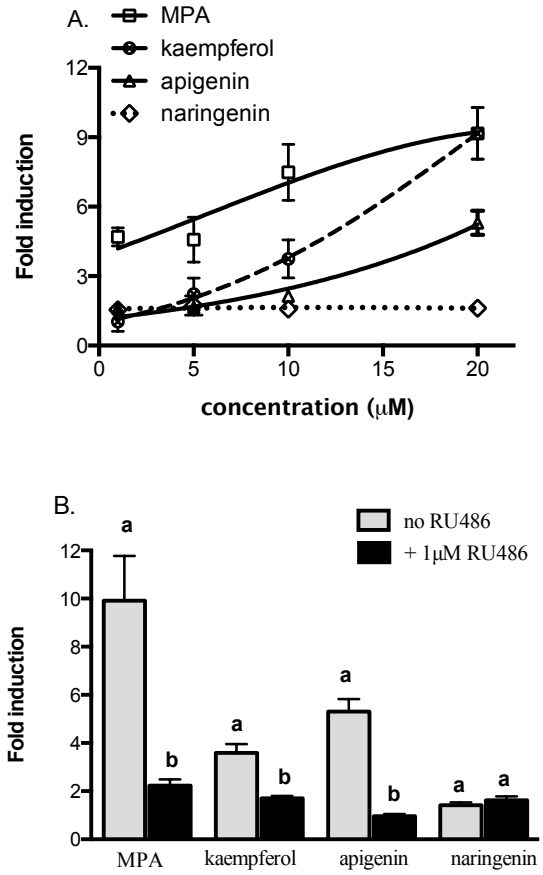


**Figure 10.** Kaempferol, apigenin and naringenin activate PRE-luciferase and can be antagonized by RU486 in human endometrial stromal cells (HESC). HESC were transiently transfected with PRE-luciferase and treated with (**A**) increasing concentrations of the pure compounds, (**B**) 20 μM of pure compounds with and without PR antagonist (RU486) for 48 hours. Data represent average +/- SEM fold change of relative light units normalized to β-gal in triplicate experiments. "**a**" indicates significant luciferase induction compared to basal DMSO; "**b**" indicates significant downregulation of luciferase induction by RU486.

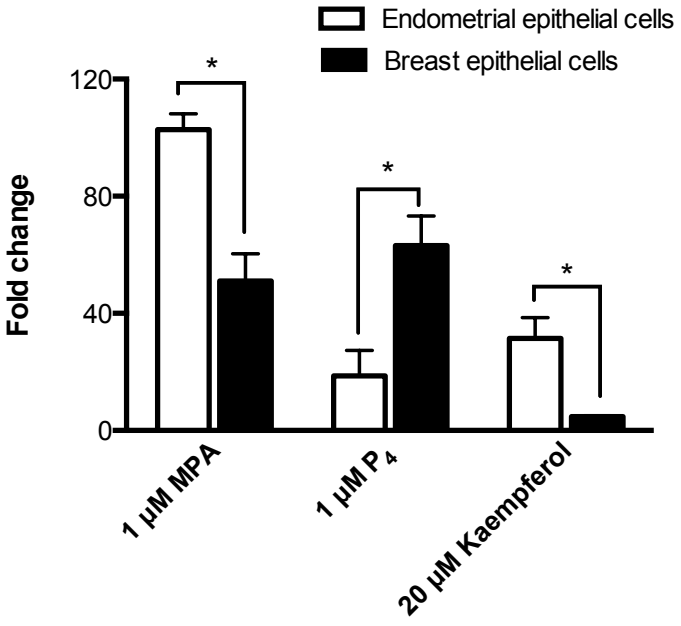


**Figure 11.** Kaempferol, $P_4$, and MPA exert different PRE-luciferase activities in human breast cancer epithelial cells and human endometrial Ishikawa cells. Cells were transiently transfected with PRE-luciferase and treated with kaempferol, $P_4$, and MPA for 24 hours. Data represent average +/- SEM fold change of relative light units normalized to β-gal in triplicate experiments. * $p < 0.05$ indicates significant difference in PRE-luciferase induction between the two $P_4$-responsive cell lines.

*Kaempferol, apigenin, and naringenin induced decidualization genes, PRL and CNR1, in HESC human endometrial stromal cells*

Decidualization is the morphological and biochemical change of the endometrial stroma during embryonic implantation, a process that critically relies on the end point of $P_4$ signaling. During this process, new proteins such as prolactin (PRL) and cannabinoid receptor 1 (CNR1) are expressed due to $P_4$ signaling (137). To evaluate the ability of kaempferol, apigenin, and naringenin to induce endogenous progestogenic signaling in endometrial stromal cells, genes activated during decidualization were measured using SYBR green quantitative PCR. In contrast to T47D cells, 100 μM kaempferol, apigenin and naringenin induced PRL expression in HESC cells after 48 h (**Figure 12A**). The induction of decidualization genes was attenuated in the presence of 1 μM RU486 (**Figure 12A**). A similar trend was observed when CNR1 expression was measured, with the exception of naringenin, which did not induce CNR1 expression (**Figure 12B**).

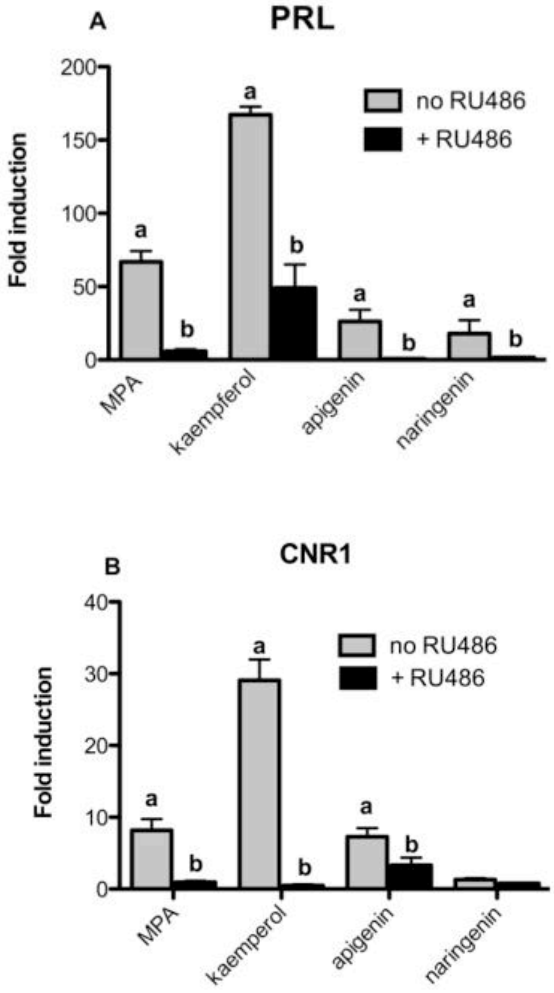


**Figure 12.** Expression of decidualization specific mRNA (PRL, CNR1) in response to kaempferol, apigenin and naringenin. Human endometrial stromal cells (HESC) were treated with phytoprogestins (100 μM), MPA (20 μM) in the presence of decidualization inducing estrogen ($E_2$) and 8-Br-cAMP, with and without PR antagonist for 48 hours. Expression of decidual genes was measured using SYBR real-time PCR. Data are expressed as the average fold change +/- SEM over basal (DMSO) normalized to GAPDH or H3F3. "**a**" indicates significant decidual gene expression compared to basal DMSO; "**b**" indicates significant downregulation of decidual gene expression by RU486.

*Kaempferol, apigenin, and naringenin did not downregulate PRA or PRB expression in T47D breast epithelial cells*

Progestin agonists autoregulate PR gene expression via a negative feedback loop (24). Proteasomal downregulation stimulated by $P_4$ binding of PR could be partially responsible for the progestin therapy resistance seen in endometriotic patients (138). If phytoprogestins can activate progesterone signaling without simultaneously downregulating PR, then an alternative therapeutic approach could be attempted using these natural ligands. The regulation of PRA and PRB expression by kaempferol, apigenin, and naringenin in breast epithelial cells was analyzed by western blot analysis. Cells exposed to 1 μM $P_4$ for 1.5 h (**Figure 13a**) or 16 h (**Figure 13b**) exhibited downregulation of PRB and more noticeably PRA, which was not observed with 100 μM kaempferol, apigenin, and naringenin treatments.

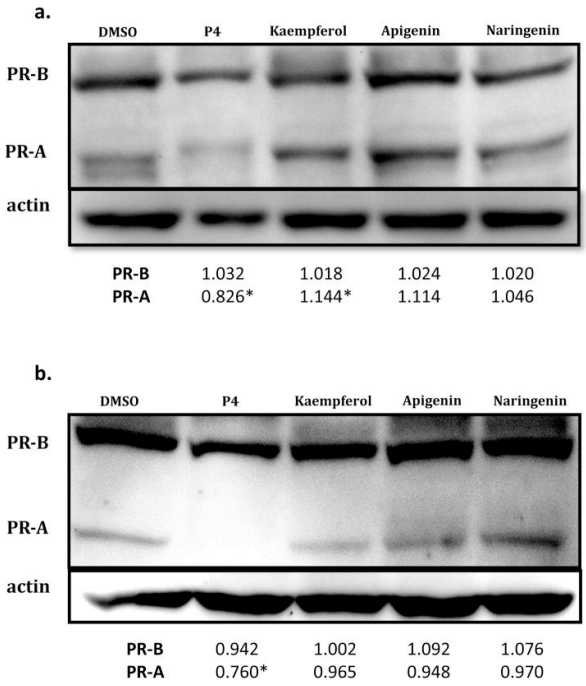


**Figure 13.** Progesterone induced downregulation of progesterone receptor in T47D cells. Levels of PRA and PRB protein in T47D cells after treatment (100 μM) with kaempferol, apigenin, and naringenin for 1.5 (**a**) or 16 (**b**) hours. Membranes were reblotted for actin as loading control.

*Kaempferol and apigenin antagonized $P_4$-induced PRE-luciferase activity in T47D breast epithelial cells*

Analyses of phytoestrogens like genistein have demonstrated their biphasic behavior, acting as agonists when no other hormone is present but functioning as antagonists in the presence of more potent estrogens such as estradiol. In addition, apigenin was recently reported to prevent MPA-induced mammary tumors in rats, suggesting that apigenin is a weak agonist in the presence of the more potent progestin, MPA (139). To investigate if purified compounds can elicit similar functions *in vitro*, T47D cells were incubated in combination with 100 nM $P_4$ plus 10 μM kaempferol, apigenin, or naringenin for 24 h. Kaempferol and apigenin, but not naringenin, significantly downregulated $P_4$-induced PRE-luciferase (**Figure 14**).

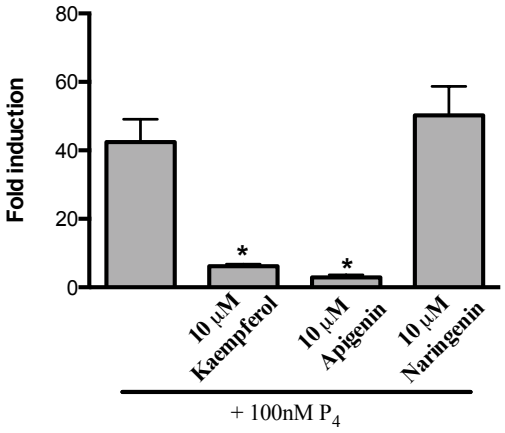


**Figure 14**. Purified phytoprogestins are weak PR agonist and function as antagonists when combined with $P_4$ in T47D cells. PRE-luciferase expression in T47D cells treated with $P_4$ (100 nM) and kaempferol, apigenin, and naringenin (10 μM). Data represent average +/- SEM fold change of relative light units normalized to β-gal in triplicate experiments. * $p < 0.05$ indicates significant antagonism of $P_4$ luciferase activity.

## C. Discussion

In this study, the progestogenic activity of eight commonly used botanicals for women's health and a library of purified compounds from red clover were evaluated using several *in vitro* progestogenic assays. Despite equivocal results in several double-blinded placebo controlled clinical trials evaluating botanicals as alternative therapies for the alleviation of hot flashes, women continue to utilize botanicals, emphasizing the importance of continued research into their safety and biological mechanism(s) of action (99). Published literature demonstrate that red clover contains estrogenic compounds, which explains its therapeutic use for the alleviation of menopausal symptoms (134), but the unknown presence of progestins in the crude extract might improve its safety in terms of uterine cell proliferation. The competitive binding of purified PR, induction of transiently transfected PRE-luciferase, and the up-regulation of $P_4$ inducible genes, in both T47D breast cancer epithelial and HESC cell lines suggests that an ethanolic extract of red clover contains natural ligands that can activate signaling through PR. More specifically, results from this study show that natural progestins kaempferol and naringenin were identified from a library of compounds isolated from red clover. Apigenin, a structurally similar flavonoid, demonstrated similar progesterone-like biological activity to naringenin and kaempferol. Progestogenic and estrogenic components found together in red clover may provide the benefits of $E_2$ for mitigating menopausal symptoms and the progestins necessary to combat the formation of estrogen-induced uterine cancers.

In the current study, the pure compounds identified in red clover (kaempferol, naringenin) and a structurally related flavonoid from chaste-tree berry (apigenin) bound to the PR and activated canonical and endogenous $P_4$ signaling in breast epithelial T47D and uterine stromal cells. These "phytoprogestins" showed considerably lower progestegenic activity

compared to positive control $P_4$, suggesting that they are weak PR agonists in the breast epithelial cells (Phytoprogestins are more than 50 fold weaker than $P_4$). The disparity in doses used between the in vitro binding and the cell based transcription assays could reflect the more complex cell based environment, specifically the ability or inability to cross the cell membrane, the importance of transcription factor comodulators, or interaction with other protein targets in the cell. Due to the sensitivity of transfected cells, the treatment concentrations in the luciferase reporter assays had to be maintained at doses lower than 20 μM. As seen with apigenin and kaempferol, the activity of PRE-luciferase started to decrease at concentrations higher than 5 μM and 10 μM likely due to cell death. Gene induction experiments allowed for higher treatment concentrations (100 μM) since these cells were not subjected to transfection prior to treatment. Interestingly, when PRE-luciferase reporter assay and gene induction experiments were repeated in the endometrial stromal and epithelial cells, kaempferol demonstrated higher progestogenic potency in relation to MPA, which was used in lieu of $P_4$ based on existing literature (63, 137, 138, 140-142). The discrepancies between the two cell types indicate that phytoprogestins may have the potential to confer the beneficial effects of progestins in the uterus while avoiding their drawbacks in the breast by functioning like a natural SPRM (selective progesterone receptor modulator). The molecular basis responsible for cell selectivity of phytoprogestins could be in part due to the different expression levels of coactivator and corepressor proteins found in the breast epithelial and endometrial stromal cells (54), subsequently regulating transcription of downstream gene targets. In addition, the expression ratios of the two PR isoforms (PRA, PRB) vary in reproductive tissues depending on the cell type (143), therefore, phytoprogestins may differentially mediate their effects depending on the tissue or cell target. Although the overall total level of PR is higher in T47D, the ligands were more active in HESC cells demonstrating

that additional factors beyond receptor binding impact transcription. Zhao et. al (140) showed that the PR concentration in the endometrial stromal cells was only 10% of that detected in T47D breast epithelial cells, whereby PRB is the dominant isoform. Phytoprogestins were able to activate $P_4$ signaling in T47D cells without downregulating PR expression, suggesting that these natural ligands could be used to overcome progestin therapy resistance. However the lack of PR degradation by these phytoprogestins could also be attributed to their low $P_4$ activity in this cell line. Unfortunately, low PR expression levels in the endometrial stromal cells due to the lack of prior estradiol priming stifled the investigation of phytoprogestin regulation of PR by western blotting expression in this cell type.

Zand et. al provided the first evidence that apigenin and naringenin may have progestogenic activity (2). Kaempferol was not investigated in these experiments. In previous reports, only breast cancer cell lines were used to study flavonoids, which does not account for cell type specific activity (2). Despite not having been previously characterized as active ligands for the PR *in vivo*, Stroheker et. al (144) demonstrated that kaempferol and apigenin are not estrogenic. Several pieces of data in the literature further support the idea that kaempferol and apigenin are biologically active as progestins *in vivo*. For example, Hyder et. al (139, 145) recently showed that apigenin prevents the development of MPA-induced mammary tumors in rats. Our *in vitro* results are consistent with these conclusions based on the observations that apigenin and kaempferol acted as weak agonists in breast epithelial cells when no other hormones were present, but in the presence of $P_4$, functioned as antagonists. The antagonistic effect induced by kaempferol and apigenin in the presence of $P_4$ could be due to PR binding prior to heterodimerization with $P_4$-PR complex. The interaction of the heterodimers at the response element of the target genes may be weaker, leading to silencing of PRE-luciferase gene

transcription. Nonetheless, further evaluations of phytoprogestins are necessary to explore their chemopreventive properties against breast cancers that develop in response to progestins such as MPA used in hormone replacement therapy.

Kaempferol inhibits the activation of nuclear factor-κB (NF-κB), implicating its potential as an anti-inflammatory agent (146, 147). Interestingly, steroids such as progestins can also signal through the PR to elicit similar anti-inflammatory mechanisms by forming a transcriptionally inactive complex between $P_4$ -bound PR with NF-κB in the cytoplasm, blocking downstream NF-κB signaling (148). Furthermore, studies have shown that kaempferol or $P_4$ can repress lipopolysaccharide (LPS)-induced interleukin-8 (IL-8) and cyclooxygenase 2 (COX-2) expression (147, 149). In a different study, kaempferol inhibited ovarian cancer tumorigenesis and angiogenesis in an animal model (150, 151) and in human observational studies, kaempferol intake was found to significantly decrease (40%) ovarian cancer incidence (152). These published data further support that kaempferol may function as a progestin, especially since progestins are one of the only established chemopreventive agents against the development of ovarian cancer (153). Although kaempferol was successfully identified as an active compound from a red clover compound library, other undiscovered active compounds might be present in the extract, which remains to be explored.

The notion that natural progestins can be identified illustrates a new type of "endocrine disruptor" that could interact with and modify endocrine systems. Endocrine disruption is a critical issue because women are already consuming plant-based therapies for a variety of conditions, such as infertility, breast enlargement, menopausal, and premenstrual symptoms (154, 155). Studying the effects and mechanisms of these phytoprogestins will help identify if progesterone signaling is being altered and whether this might contribute to the safety profile of

botanical supplements. Promiscuous binding of MPA to GR or AR initiates many side effects (156). Therefore, defining phytoprogestin binding to alternative biological targets is an important future goal to avoid known negative side effects associated with existing progestins. The cell and tissue selectivity of these phytoprogestins, and the ability to signal through the PR without reducing the receptor expression suggest that future *in vivo* studies are warranted to further validate their progestegenic nature in an animal model. Identification and characterization of natural progesterone-like molecules in plants might allow for informed decisions regarding their use as alternatives to progesterone therapies.

# IV. KAEMPFEROL EXHIBITS PROGESTOGENIC EFFECTS IN OVARIECTOMIZED RATS

## A. Introduction

Selective PR modulators (SPRMs) are a class of PR ligands that function as either an agonist, antagonist, or mixed agonist/antagonist and have clinically relevant tissue selectivity (54). Pharmaceutical development of novel SPRMs offers promising new therapeutic options, as SPRMs have the potential to provide beneficial progestogenic effects in the uterus, while avoiding their drawbacks in the breast (55). *In vitro* studies (Chapter III) demonstrated kaempferol is a non-steroidal phytoprogestin that functions in a cell-specific manner *in vitro* (9). Kaempferol is a widely distributed dietary flavonoid found in fruits and vegetables (157) that also has anti-oxidant and anti-inflammatory properties (157). The anti-inflammatory properties of kaempferol appear to be meditated by nuclear factor-κB ($NF_KB$) (146, 147). In animal studies, kaempferol inhibited ovarian cancer tumorigenesis and angiogenesis (150, 151). Moreover, in human epidemiological studies, kaempferol intake significantly decreased (40%) ovarian cancer incidence (151, 158). The biological activities demonstrated by kaempferol in these previous studies are consistent with kaempferol functioning as a progestin, especially considering progestins are known to inhibit $NF_KB$ and are well known to protect against ovarian cancer (86, 159, 160).

The objective of this study was to investigate if kaempferol exerts progesterone-like effects *in vivo* using the ovariectomized Sprague-Dawley rat model. Since genistein is a phytoestrogen that was previously demonstrated to increase uterine weight and proliferation (8), the ability of kaempferol to block genistein action in the uterus was investigated. Analyses of

proliferation, steroid receptor expression, and induction of well-established PR-regulated targets *Areg* and *Hand2* were completed. In addition, kaempferol *in silico* binding analysis was completed for PR, as was the activation of ER and AR signaling *in vitro* in order to determine receptor specificity. The data from this study suggest that kaempferol interacts with PR, activates the receptor without stimulating its degradation, antagonizes genistein-induced endometrial proliferation, and induces known PR target genes *in vivo*.

## B. Results

*Molecular modeling of kaempferol, MPA and $P_4$ in PR Ligand Binding Domain (LBD)*

Kaempferol was previously reported to bind to PR, activate PRE-luciferase in a concentration-dependent manner, and it was antagonized by RU486 in T47D and human endometrial stromal cells (HESC) (9). In order to further characterize the ability of kaempferol to bind the PR, a molecular docking study was used to highlight and compare the binding interactions of kaempferol with those of RU486, $P_4$, and MPA at active site residues. Kaempferol fits into the ligand binding domain (LBD) (**Figure 15**) and has an affinity score comparable to those of RU486, $P_4$, and MPA **(Table VII).** Consistent with previous reports, RU486 is a stronger PR binder as compared to $P_4$ and demonstrated the highest affinity score (13). Top poses of MPA and kaempferol are shown in **Figure 15**. The 4'-, 5-, and 7-hydroxyl groups of kaempferol form hydrogen bonds with Gln725, Thr894, Asn719, respectively. The binding pose of MPA is very similar to $P_4$ and RU486. In addition to the hydrogen bond between the 3-keto group and the side chain amide moiety of Gln725, the acetate group in kaempferol extends into the pocket formed by Leu715, Leu718 and Phe794, which is occupied by the 17α-propynyl

group in RU486. Kaempferol's interaction with the LBD of PR is driven by a combination of hydrogen bonding and hydrophobic contacts commonly observed for all PR ligands.

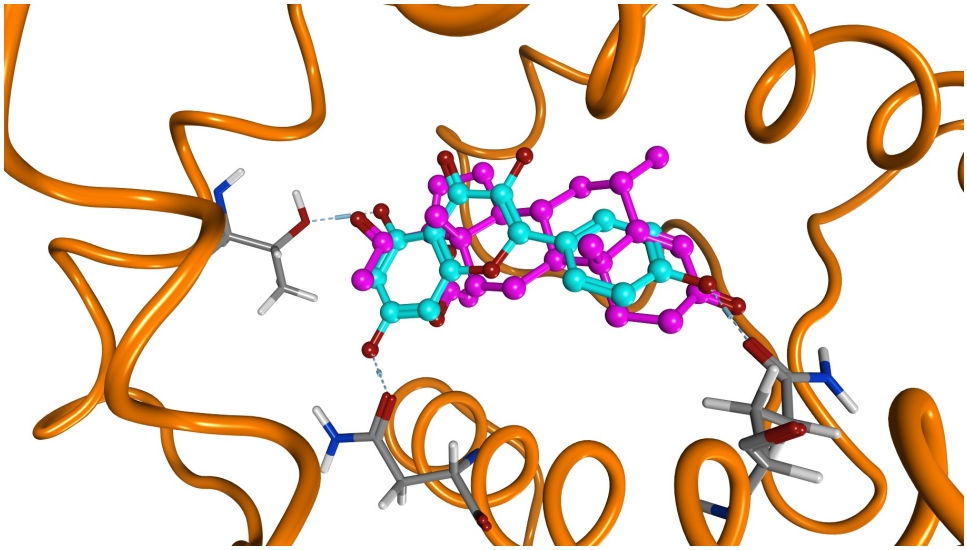

**Figure 15.** Kaempferol (cyan) and MPA (magenta) bound to the ligand binding domain of PR. The interactions of the aromatic rings of kaempferol and MPA are similar to $P_4$ in the ligand binding domain of the receptor. The 4'-hydroxyl group of kaempferol anchors to Gln725 analogous to the keto group of $P_4$ or MPA. Additionally, the 7-hydroxyl moiety on the phenyl ring forms a hydrogen bond with Asn719 and the 5-hydroxyl group interacts with Thr894. MPA gains more interactions through its ester linked arm that extends into the cavity formed by Leu715, Leu718 and Phe794.

| Ligand | Affinity (kcal/mol) |
|---|---|
| Kaempferol | -8.88 |
| $P_4$ | -11.76 |
| MPA | -14.36 |
| RU486 | -15.22 |

**TABLE VII**. KNOWN PROGESTERONE RECEPTOR LIGANDS USED IN THIS STUDY AND AFFINITY SCORES FOR THE BEST DOCKING POSES AFTER CO-MINIMIZATION IN THE BINDING SITE.

*Kaempferol does not increase uterine weight of OVX rats*

Since botanicals are mixtures and are often consumed as multi-botanical formulations, the ability of kaempferol to oppose genistein action in the uterus was investigated (161-163). Genistein is a phytoestrogen found in commonly used botanical supplements soy and red clover that activates ER and increases uterine weight and cell proliferation similar to $E_2$ (1, 2, 8, 138, 164). Based on a previous study, Sprague-Dawley rats (200 g) fed 375 μg genistein/g of food/day demonstrated significant uterine weight gain and proliferation (8). The dose of genistein and the length of treatment in this study were calculated based on the average amount of food consumed per day (15 g/animal/day), indicating that genistein at 5.625 mg/animal/day for 8 days should significantly induce uterine proliferation. Kaempferol's ability to block genistein-induced proliferation in OVX rats after 8 days of oral treatment was investigated **(Table VIII)**. As expected, the uterine wet weights of genistein-treated animals were significantly higher than control rats **(Table VIII)**. Oral administration of an equal dose of

kaempferol (5.625 mg/animal/day) did not significantly increase uterine wet weight compared to control group, indicating that kaempferol did not induce an estrogenic response in the uterus **(Table VIII)**, consistent with previous reports (165, 166)**.** Additive effects were not observed on uterine weight in animals co-treated with kaempferol and genistein.

| **Treatment (5.625mg/animal/day)** | **Uterine Weight (mg)** |
|---|---|
| Vehicle (control) | 63.3 ± 24 |
| Genistein | 90.0 ± 17 * |
| Kaempferol | 52.0 ± 25 |
| Genistein + Kaempferol | 95.7 ± 22 * |

**TABLE VIII.** GENISTEIN INDUCED UTERINE WET WEIGHT INCREASE IN OVX SPRAGUE-DAWLEY RATS. Animals were administered with vehicle control, genistein, kaempferol and geninstein + kaempferol for 8 days via oral gavage. Data are uterine wet weights (mg) 24 h after last treatment (n = 8 per group). Mean ± SEM (∗$p < 0.05$), significance against untreated control, as determined by one-way ANOVA test.

*Kaempferol inhibits uterine epithelial cell proliferation*

$P_4$ opposes ER-mediated proliferation in the uterine luminal epithelium, while also preparing the uterine stroma to respond to $E_2$ by inducing stromal proliferation (167-171). Therefore, the effect of kaempferol, genistein, and the combination on proliferation of rat uterine epithelial cells was investigated. Ki67 staining was utilized to quantify proliferation. Genistein significantly increased luminal epithelial proliferation as compared to control **(Figure 16A)**. These results are in agreement with previously conducted studies (1, 2, 8, 54, 164). Kaempferol alone did not increase luminal epithelial proliferation. Importantly, when given in combination with genistein, kaempferol decreased proliferation of the uterine luminal epithelial as compared to genistein alone **(Figure 16A-B)**. Due to minimal proliferation in the stroma, a semi-quantitative assessment method was used to investigate Ki67 expression. Co-administration of kaempferol and genistein stimulated proliferation of uterine stromal cells when compared to the individual treatments and vehicle control **(Figure 16C)**. These changes in luminal and stromal proliferation from co-administration of kaempferol and genistein are consistent with similar studies which assessed actions of $P_4$ in the presence of $E_2$ (169, 172).

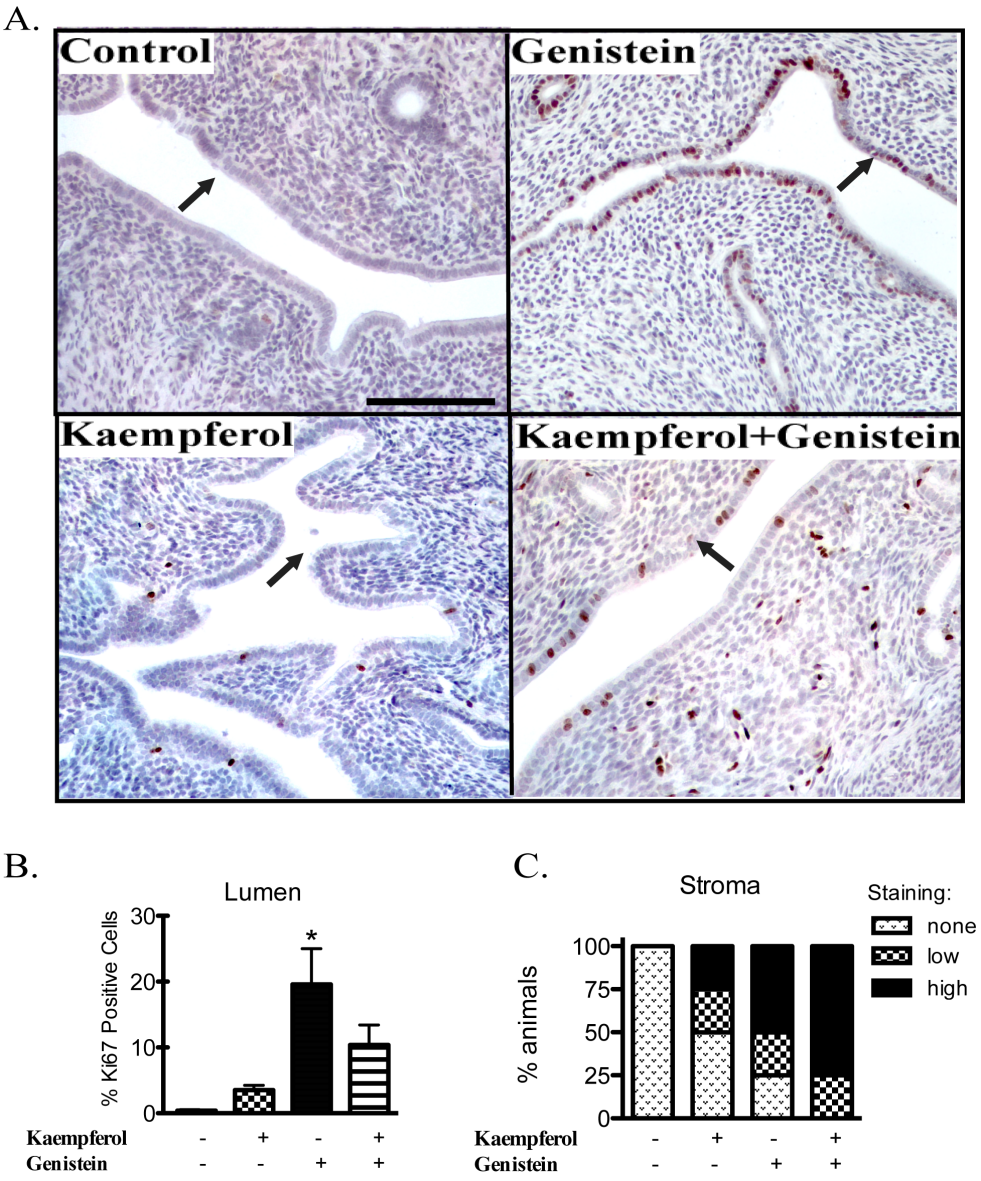


**Figure 16.** Cell proliferation in response to oral treatment with vehicle (control), genistein, kaempferol and genistein + kaempferol in OVX rat uteri. Representative sections of uterus immunostained for Ki67 (A). Results are represented as percentage of Ki67 positive cells in the luminal epithelium (B). In the stroma, Ki67 staining was categorized as low, medium or high, and shown as percentage of animals from each category (C). Data represented as mean ± SEM of Ki67 positive cells ($n$ = 4 per group). *$p < 0.05$, compared by one-way ANOVA test followed by Tukey's, Scale bar=100 μm.

*Kaempferol induces Areg mRNA expression and Hand2 protein levels in the uterus.*

The anti-proliferative action of $P_4$ in the uterine epithelial cells is mediated by Hand2 induction (54, 131). Immunohistochemistry and qPCR analyses were used to investigate if Hand2 induction correlated with the anti-proliferative effects of kaempferol. As predicted, an increase in Hand2 expression was observed in uterine stromal cells after kaempferol treatment **(Figure 17A)**. Hand2 protein expression was not induced by genistein, and a slight increase was observed in rats treated with both genistein and kaempferol **(Figure 17A)**. *Hand2* mRNA changes were not observed in any of the treated animals **(Figure 17B)**. The observed discrepancy between Hand2 protein and mRNA expression is likely based on the technique, as immunohistochemistry allows for analysis of specific uterine cell types (54, 173, 174), whereas the mRNA analyzed was a heterogeneous mixture of all uterine cell types.

Amphiregulin (Areg) is a secreted protein that is induced by $P_4$ in the uterus (44, 54, 175, 176). Kaempferol treatment significantly induced (5-fold) *Areg* mRNA compared to vehicle treated animals, suggesting that it can function to increase PR-regulated targets *in vivo* **(Figure 17C)**. Genistein blocked kaempferol-induced *Areg* expression, consistent with the antagonistic effects of $E_2$ on $P_4$-mediated induction of *Areg* (37, 172, 176, 177). Since Areg is a secreted protein, it was not investigated via immunohistochemistry (12, 37, 54, 176, 178) (54, 92, 175).

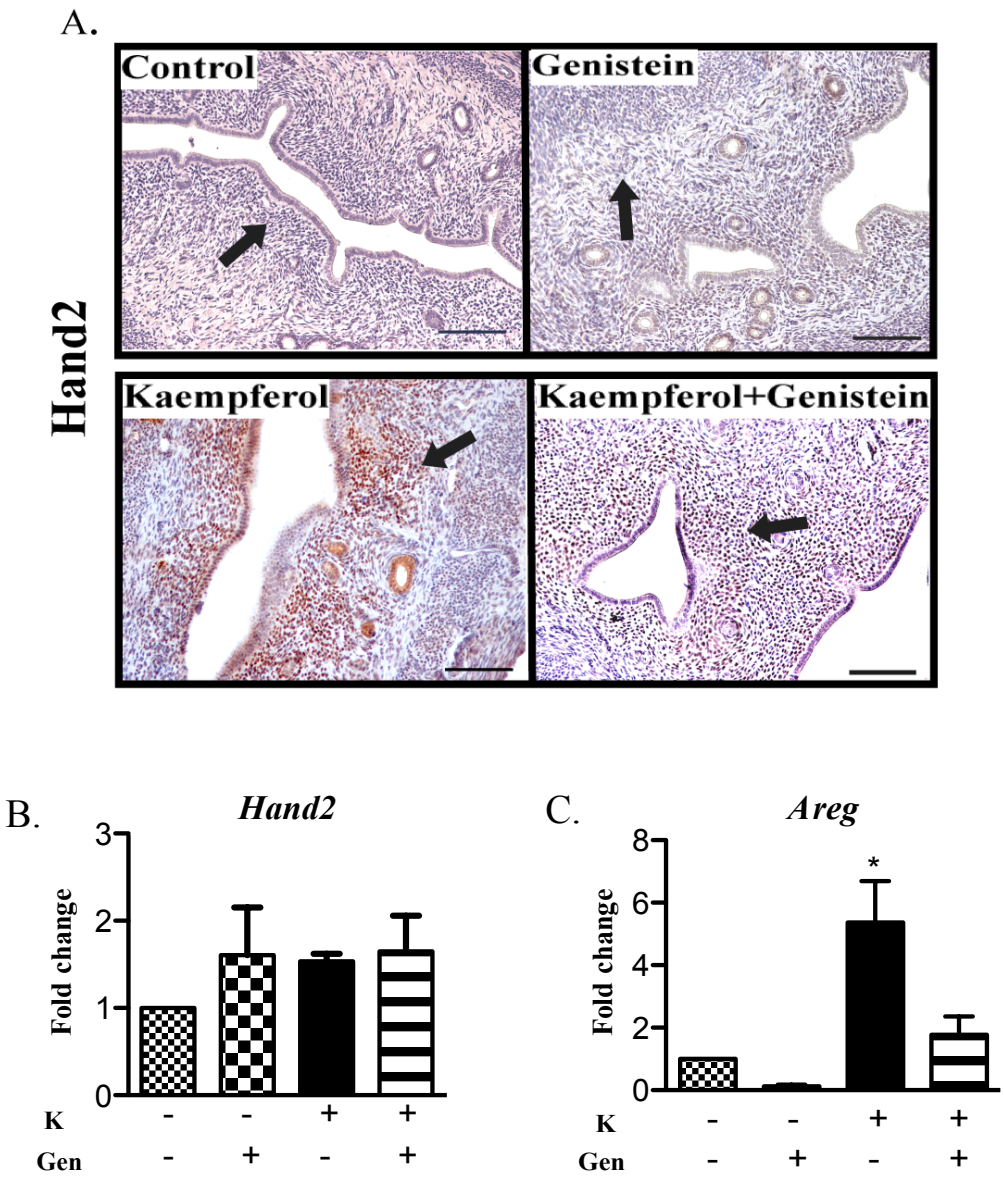


**Figure 17.** Immunohistochemistry and mRNA expression of $P_4$ targets (*Hand2* and *Areg*) in the rat uterus after treatment with vehicle control, genistein (Gen), kaempferol (K), and genistein+kaempferol for 8 days. Kaempferol induced expression of Hand2 protein (**A**) but not mRNA (**B**), and induced *Areg* mRNA (**C**) in the ovariectomized rat uterus. Arrows indicate the uterine stromal compartment. Bar =100 μm. qPCR analyses were performed using SYBR technologies. Results were normalized to *Rpl1* for Hand2 or *Gapdh* for Areg. Mean ± SEM (∗$p < 0.05$), compared by one-way ANOVA followed by Tukey's.

*Genistein and kaempferol treatment modulated uterine PR and ERα protein and mRNA expression*

Steroid receptor mRNA and protein levels can be influenced by several physiological factors, including exposure to $E_2$ and $P_4$ (92, 163, 179, 180). To investigate the effects of genistein and kaempferol on steroid receptor expression in the uterus (the myometrium, endometrial stroma, luminal and glandular epithelium), mRNA levels of ER and PRA were measured. To establish cell type specific PR and ER regulation of steroid receptor expression, protein levels were compared using immunohistochemistry.

ERα bound to $E_2$ triggers its proteasome-dependent protein degradation (54, 179). In order to study ERα regulation, first qPCR for the receptor was performed. *ERα* mRNA levels in whole uterus were not affected by genistein treatment, but was significantly induced by kaempferol and kaempferol combined with genistein **(Figure 18A)**. To investigate ERα protein expression in the different uterine cell types, immunohistochemical analyses were performed. As expected, genistein downregulated ERα protein expression **(Figure 18B)**. Interestingly, the induction of *ERα* mRNA in animals treated with kaempferol alone and in combination group (genistein and kaempferol) was significant and paralleled protein levels **(Figure 18A)**. The lack of uterine weight gain and ERα expression in kaempferol-treated rats confirmed that kaempferol did not function as an estrogenic compound.

PR is an ER-regulated target (1-4, 8, 9, 96, 181, 182). Genistein treatment significantly increased PR mRNA and protein expression, confirming that genistein acts as a phytoestrogen in rat uteri (3, 8, 9, 183). Interestingly, kaempferol and kaempferol combined with genistein also significantly upregulated PR mRNA **(Figure 18A)**. PR immunostaining in the vehicle-treated rat uteri was intense and localized to the nucleus throughout the luminal and glandular epithelial

cells, but exposure to genistein and kaempferol increased PR expression in the stroma **(Figure 18C)**. Kaempferol blocked genistein-induced proliferation and induced expression of PR target genes (*Areg mRNA* and *Hand2*), which is consistent with kaempferol functioning as a progestin. However, kaempferol acted without stimulating the degradation and loss of PR protein or mRNA expression, which typically occurs when PR binds a ligand **(Figure 18C).**

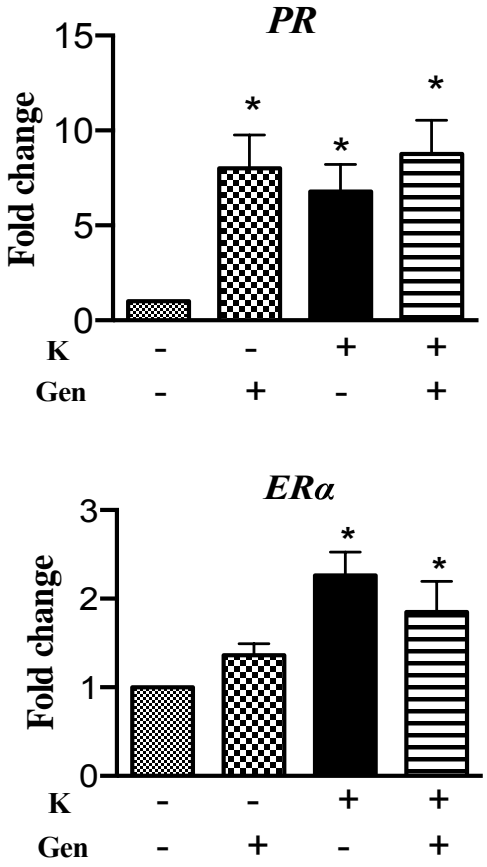


**Figure 18. (A)** Immunohistochemistry and mRNA expression of ERα and PR in the rat uterus after treatment with vehicle control, genistein (Gen), kaempferol (K), and genistein + kaempferol for 8 days. Kaempferol and genistein differentially regulate *PR* and *ERα* mRNA, protein expression, and localization in the uterus. qPCR analyses were performed using SYBR technologies. All results were normalized to *RPL1*. Mean ± SEM (∗$p < 0.05$) compared using one-way ANOVA followed by Tukey's.

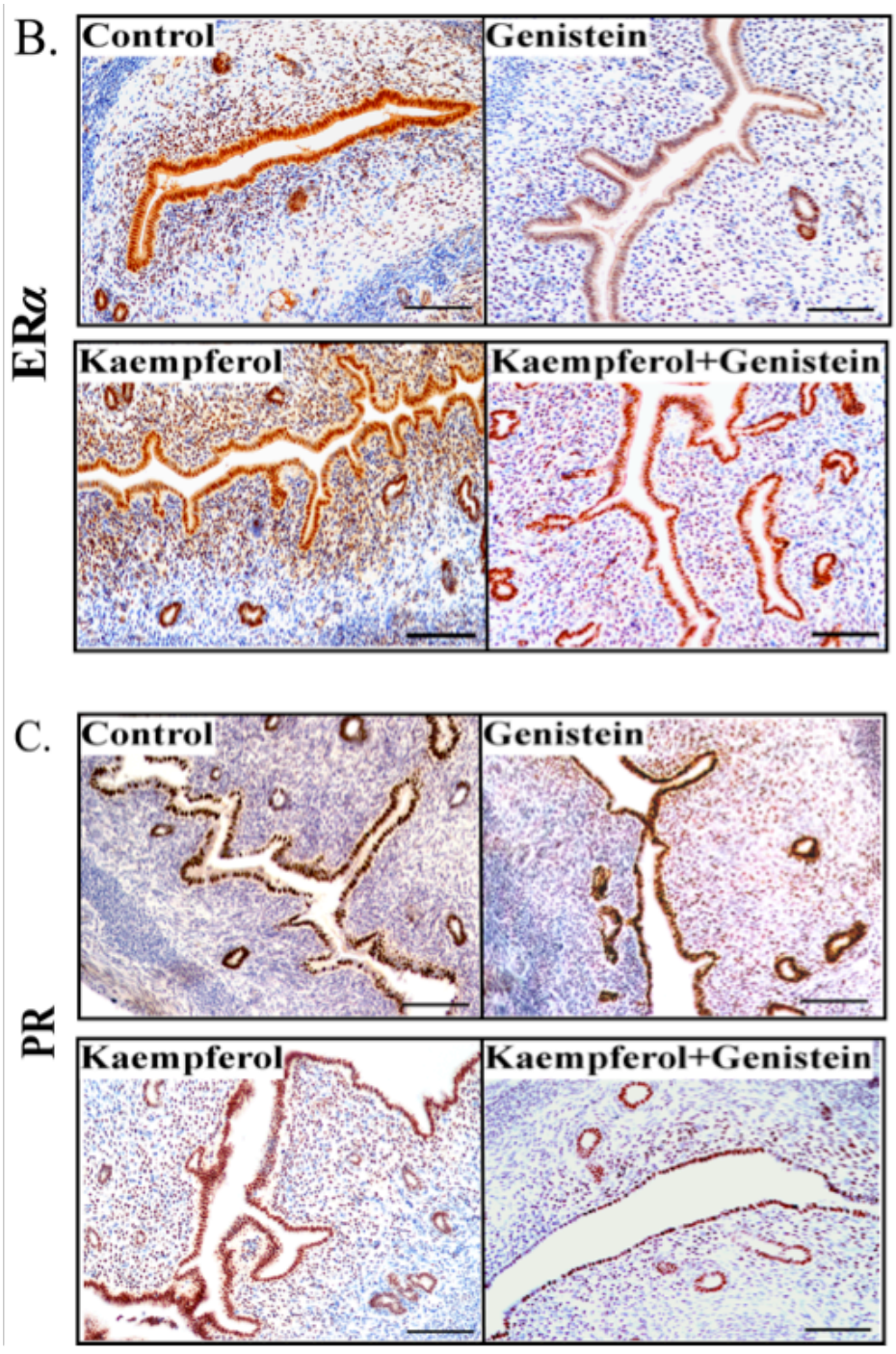


**Figure 18.** Uterine sections were stained for ERα (**B**) and PR (**C**) expression, n=8 per group. Bar =100 μm.

*Kaempferol, $E_2$ and genistein regulation of PR expression in human endometrial stromal cells (HESC).*

Upon $P_4$ binding, PR is targeted for proteasomal degradation, which could be partially responsible for the resistance to progestin therapy observed in endometriotic patients consuming progestins chronically (138). Increased PR expression is mediated by the interaction between ligand-occupied ER with ERE in the PR gene promoter (Classen-Linke et al., 2000; Petz et al., 2004). Since PR mRNA and protein was not reduced in the uteri of kaempferol-stimulated rats and instead actually increased, the regulation of the PR was investigated *in vitro*. PRA and PRB protein expression was analyzed in HESCs treated with MPA, $P_4$, kaempferol, $E_2$, and genistein **(Figure 19)**. As expected, after a 48-hr treatment with MPA, HESCs had reduced PR protein expression **(Figure 19A)**. HESCs exposed to $E_2$ or genistein had increased PR protein expression **(Figure 19B)**. The combination treatment of MPA and $E_2$ maintained PR expression at basal levels. Similar to the *in vivo* results in rat uteri, kaempferol increased PR protein expression **(Figure 19A-B).** When combined with genistein treatment, kaempferol decreased genistein-induced PR expression, similar to MPA when combined with $E_2$. PRE-luciferase induction by kaempferol in combination with genistein at higher concentrations was significantly greater compared to kaempferol alone, suggesting potential cooperation/cross-talk between phytoprogestins and phytoestrogens, which could lead to a more potent progestogenic effect in whole extracts (**Figure 20**). Similar trends were observed with positive control $E_2$ and MPA.

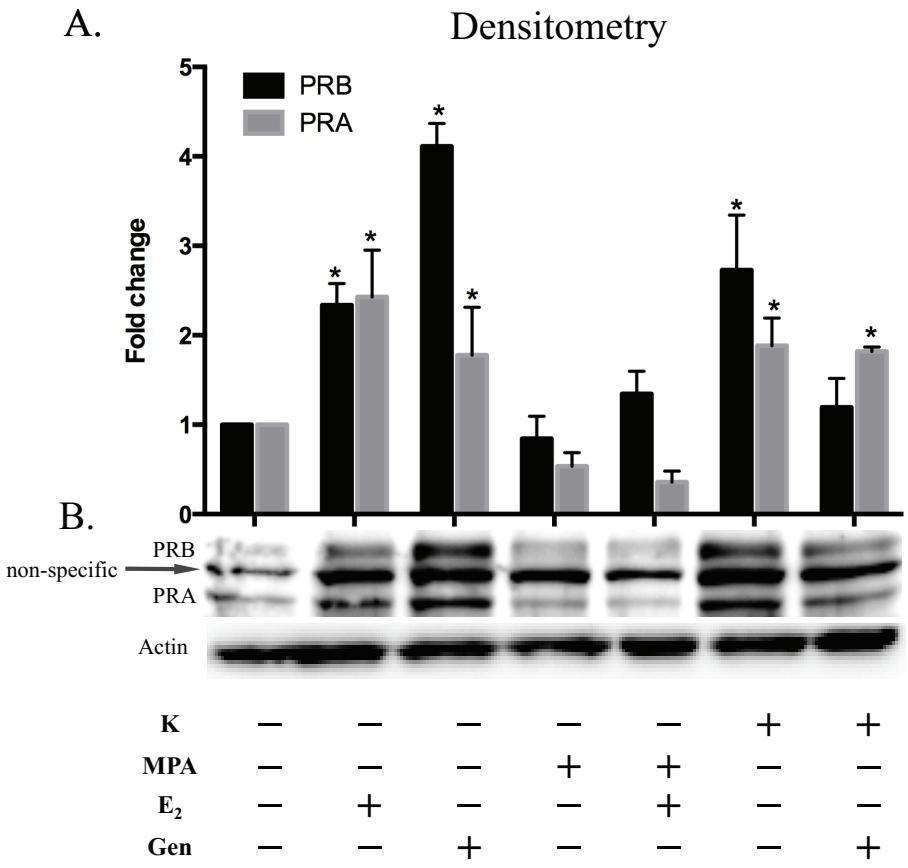


**Figure 19**. Regulation of PR protein expression in human endometrial stromal cells. Cells were incubated with pure compound for 48 hours. PRA and PRB were induced by $E_2$ (1 μM), genistein (5 μM) and kaempferol (20 μM) as determined by densitometry (**A**). PR fold change was analyzed using Image-J in triplicate experiments. * $p < 0.05$ indicates significant fold change of PR compared to basal DMSO. MPA (20 μM) did not induce PRA and PRB. Membranes were blotted for actin as a loading control (**B**). Image is a representative blot, experiment repeated in triplicate.

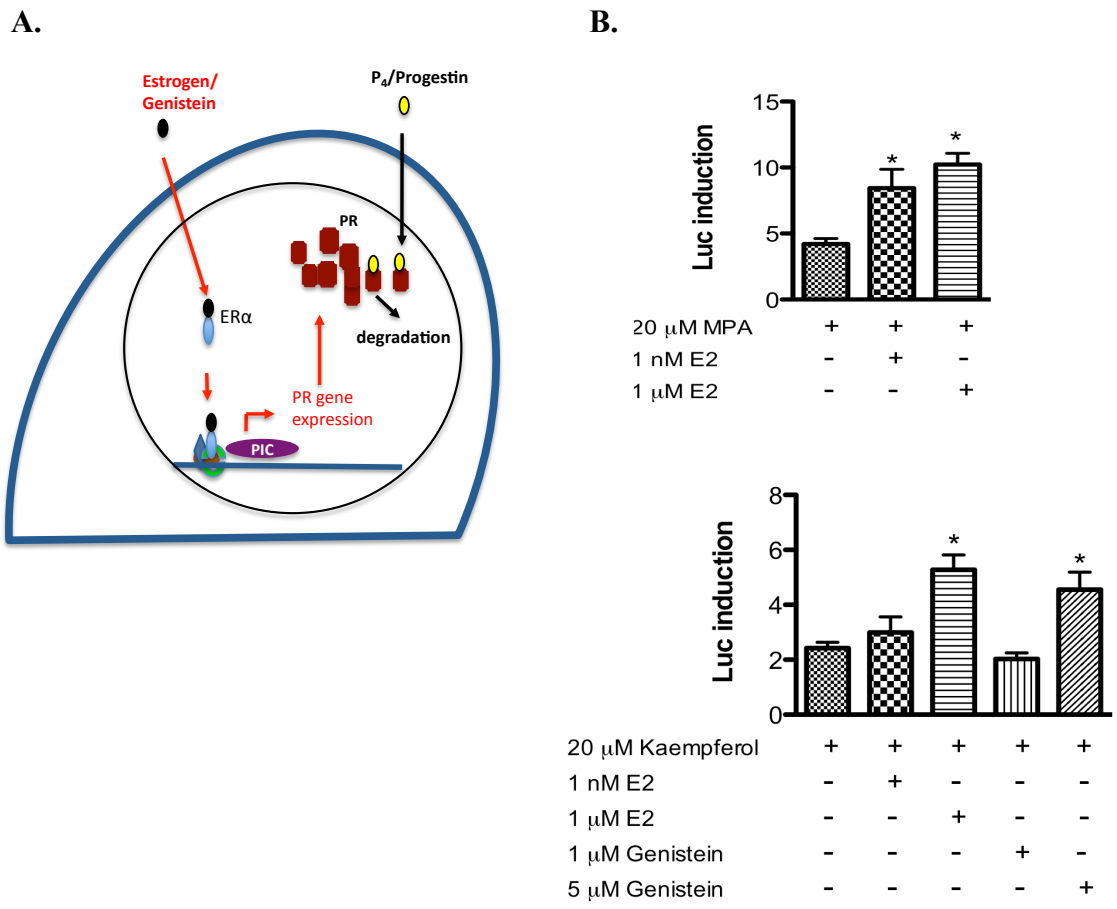


**Figure 20.** Investigation of estrogen and $P_4$/kaempferol crosstalk via PRE-luc in endometrial stromal cells. (**A**). Schematic showing the regulation of PR expression by both progestins and estrogens (**B**). MPA and $E_2$ co-administration in endometrial stromal cells resulted in enhanced transcription of the PRE-reporter gene compared to MPA alone. However only in the presence of higher $E_2$ and genistein concentrations were similar results observed with kaempferol treatments. * $p < 0.05$ indicates significant fold change of PR compared to basal DMSO.

*Kaempferol does not induce ERE-luciferase in HESCs.*

Enhanced PR protein expression in the uterine stroma from kaempferol treatment *in vivo* could be due to activation of ER, which in turn transcriptionally induces PR (182, 184). Although kaempferol was previously reported to function as an ER modulator in HeLa cells, rat primary osteoblasts and human breast cancer MCF-7 cells in a concentration range of 10-70 μM (12, 136, 185-187), the ability of kaempferol to activate the ER in HESCs has not been reported. Therefore, HESCs were treated with a vehicle control, genistein, kaempferol, and genistein combined with kaempferol and ER activation was monitored by ERE-luciferase transcription **(Figure 21)**. $E_2$ and genistein, but not kaempferol significantly induced ERE-luciferase expression in HESCs **(Figure 21)**. In agreement with PR protein expression **(Figure 21)**, genistein (5 μM) was as active as $E_2$ at inducing ERE-luciferase transcription **(Figure 21)**. The ER antagonist desmethylarzoxifen (DMA) (100 nM) significantly inhibited $E_2$-induced signaling suggesting that these activities are mediated through ER. Despite increased PR protein expression after kaempferol treatment, ERE transcription was not significantly activated **(Figure 21)**.

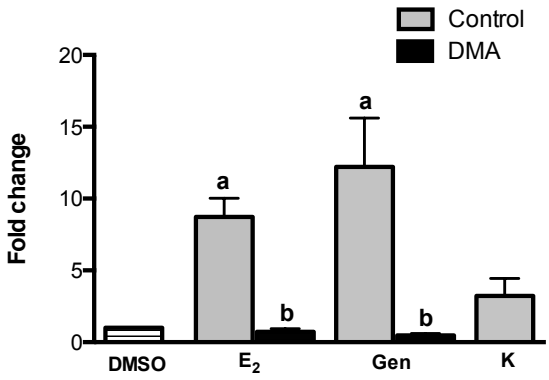


**Figure 21.** ERE-luciferase induction in human endometrial stromal cells. Endometrial stromal cells were transiently transfected with ERE-luciferase and ERα and treated with pure compounds $E_2$ (1 μM), genistein (5 μM), and kaempferol/K (20 μM) with and without the ER antagonist DMA (100 nM) for 48 hours. Data represent mean fold change ± SEM of relative light units normalized to β-gal in triplicate experiments. "**a**" indicates significant luciferase induction compared to basal DMSO; "**b**" indicates significant reduction of luciferase induction by DMA as determined by one-way ANOVA test.

*Kaempferol does not induce PSA-luciferase in MDA-MB-231 cells*

MPA is one of the most commonly used synthetic progestins (55, 116). Although MPA signals through PR, it also activates other nuclear receptors such as AR, thereby increasing side effects and the risk of breast cancer and cardiovascular disorders (116, 188, 189). Thus, it was important to evaluate if kaempferol also activates AR signaling. As PRE and ARE have similar consensus sequences, it is difficult to accurately determine androgen-specific activity when both PR and AR are expressed in a cell (180, 190). Therefore, in this experiment, MDA-MB-231 breast cancer cells, which express AR but not PR, were used. Prostate specific antigen (PSA) is an AR-regulated gene and its proximal promoter is highly responsive to androgens (191, 192). Thus, PSA-luciferase activity was measured in MDA-MB-231 cells to monitor AR activation. As expected, MPA activated PSA-luciferase in MDA-MB-231 cells, verifying that MPA stimulates AR-mediated transcription **(Figure 22)**. In the presence of RU486 (1 μM), an AR antagonist, MPA-induced AR signaling was completely inhibited, further demonstrating MPA activation of AR-mediated transcription **(Figure 22)**. Although there was a trend for increased activation with kaempferol, this was not statistically significant.

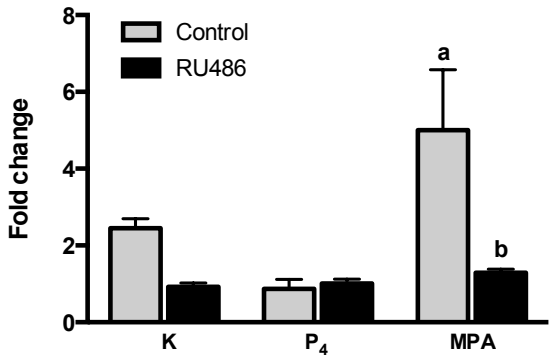


**Figure 22.** PSA-luciferase induction in MDA-MB-231 cells. MDA-MB-231 cells were transiently transfected with PSA-luciferase and treated with pure compounds kaempferol (20 μM), $P_4$ (1 μM), and MPA (1 μM) with and without AR antagonist RU486 (1 μM) for 48 hours. MPA activates PSA-luciferase and can be antagonized by RU486. Kaempferol and $P_4$ did not significantly activate AR signaling. Data represent mean fold change ± SEM of relative light units normalized to β-gal in triplicate experiments. "**a**" indicates significant luciferase induction compared to basal DMSO; "**b**" indicates significant reduction of luciferase induction by RU486 as determined by one-way ANOVA test.

## C. Discussion

Many studies have provided evidence that kaempferol may function as progestin, including (i) activation of PR signaling *in vitro* (9), (ii) antagonistic effects when a potent PR agonist is present (9), (iii) similar anti-inflammatory mechanisms when compared to $P_4$ (146, 157) and (iv) protection against ovarian cancer (9, 55, 86, 150-152, 157, 193). To date, there are no reports regarding the progestogenic effects of kaempferol *in vivo*. Therefore, this study investigated the effects of kaempferol on $P_4$ signaling in the uteri of OVX Sprague-Dawley rats and steroid receptor activation *in vitro*. In this study, the kaempferol treatment of cultured cells and animals were within the range used in previous studies (10-70 μM and 1-100 mg/kg, respectively) (8, 9, 157, 194, 195). These findings, together with previous data, collectively suggest that kaempferol may have the potential to provide progestogenic biological activity *in vivo,* particularly in the uterus.

Computational analysis demonstrated that kaempferol adopts binding poses, which closely mimic the binding conformation and the interactions commonly observed between the LBD of PR and established ligands. The hydrophobic and hydrogen bond interactions of kaempferol are highly analogous to those of the steroid scaffold of $P_4$. In addition to the interactions expected for the steroid-based backbone of MPA, it gains additional interactions with the binding site through its ester-linked appendage, which may be associated with its agonistic effects. Molecular modeling data are consistent with previous *in vitro* binding analysis performed with kaempferol and the PR ligand binding domain (9, 157). Future investigations using additional molecular modeling techniques to study the recruitment of coregulatory proteins are warranted to elucidate the molecular mechanisms of kaempferol as a selective progesterone receptor modulator.

One well-established function of $P_4$ is the inhibition of $E_2$-induced uterine cell proliferation (157, 169). As a result, progestins are used therapeutically to reduce the proliferation of $E_2$-dependent endometrial cancers and in endometriosis (146, 147, 169). Kaempferol reduced genistein-induced proliferation in luminal epithelial cells, while preparing the uterine stroma to respond to genistein, leading to stromal cell proliferation. Hand2 mediates the anti-proliferative effects of $P_4$ in the uterus (131, 150, 151). Although kaempferol treatment stimulated Hand2 protein expression in the uterine stroma, there was no change in *Hand2* mRNA expression. Hand2 mRNA and protein expression in kaempferol treated uteri likely do not correlate completely because Hand2 is expressed in a discrete area within the uterine stroma, easily detectable by immunohistochemistry, but constituting only a small portion of the total uterine mRNA. Previous *in vivo* studies have reported that $P_4$ treatment completely abolished $E_2$-induced proliferation in the uterine epithelium. (131, 151, 158, 168, 169). Although kaempferol significantly reduced genistein-mediated proliferation in the uterus, it did not completely eliminate proliferation. However, it is important to note that the differences between $P_4$ and kaempferol could be due to different routes of treatment administration (oral vs. subcutaneous), potency, and duration. $P_4$ is poorly orally bioavailable driving the administration of synthetic progestins, like MPA. Since genistein and kaempferol are biologically active in the uterus after oral administration, botanicals (or combination therapies) containing both estrogenic and progestogenic compounds might provide the desired benefits for mitigating menopausal symptoms while also preventing $E_2$-induced uterine hyperplasia. (86, 159, 160, 169).

Reduced PR protein following progestin administration occurs in $P_4$ responsive cell types, and may be used to study progestogenic action within a target tissue (9, 196, 197). Unexpectedly, the uteri of kaempferol-treated rats maintained expression of PR *in vivo* and in HESCs. This

finding was especially intriguing because PR induction is linked to estrogenic signaling (131, 182, 184), which was not observed with kaempferol treatment, *in vitro* or *in vivo* as demonstrated by no change in ERE-luciferase expression and a lack of increased uterine weight. While kaempferol has been described as a partial ER agonist in human breast cancer cells and cervical cells (13, 185, 186), multiple studies investigating the estrogenic actions of kaempferol *in vivo* detected no uterotrophic estrogenic effects (12, 165, 194), which is further corroborated in this study. Progestin therapy resistance occurs in some populations of patients with endometrial cancer and endometriosis due to reduced or loss of PR protein expression after prolonged treatment (12, 198, 199). Endometrial cancer is the most common gynecological malignancy in the United States and the fifth most common cancer among women in the world (55, 63, 138). The anti-proliferative effects of kaempferol, without simultaneously downregulating PR expression raises an interesting possibility that a novel therapeutic approach could be attempted using kaempferol as an alternative for longer-term management of endometriotic symptoms (193, 199-205).

The mechanism of progestin action is complex and may exert effects other than those traditionally expected from progestogenic activity (138, 206). Progestins currently available for prescription, such as MPA, interact with other steroidal receptors, including the AR, mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) (156, 182, 184, 206). It has been proposed that AR, GR and MR activity enhance the proliferation of mammary epithelial cells, increasing breast cancer risk (12, 41, 136, 185-187). The androgenic nature of MPA has been suggested to be one mechanism through which it may give rise to blot clots, heart attacks and hypertension (12, 136, 207, 208). Therefore, an ideal progestin would be devoid of non-specific GR, MR, and AR activity (55, 116, 178). Contrary to MPA, kaempferol did not

significantly activate AR *in vitro*. However, further studies are needed to confirm kaempferol's action on GR and MR.

Taken together, the results from this study demonstrate that kaempferol functions as a progestin *in vivo* to mediate anti-proliferative effects of genistein in the uterus and modulate steroid receptor expression, without activating AR and ER signaling. The notion that phytoprogestins can be identified provides a new type of endocrine modulator, which could interact with and change endocrine signaling. Endocrine disruption is a critical issue as women are already consuming botanical-based therapies for a variety of conditions, such as infertility, menopause, and premenstrual symptoms (116, 154, 155, 188, 189). Identification and characterization of progesterone-like molecules from natural sources might allow for informed decisions regarding their use as part of a complicated multi-botanical formulations or as an alternative to current progestin therapies.

# V. IDENTIFICATION AND BIOLOGICAL CHARACTERIZATION OF PROGESTINS FROM HOPS (*HUMULUS LUPULUS*)

## A. Introduction

Strobiles of *Humulus lupulus* L., Cannabanaceae, more commonly known as hops, have been cultivated for more than 1000 years, primarily in Eurasia (209). The reputation of hops as nature's most coveted beer favoring agent is indisputable. As a result, beer connoisseurs and brewers around the world have invested heavily in hops cultivation in the pursuit of the perfect beer. The use of hops to support women's health started in 1973. German women would take baths with brewery sludge containing 30% hops for its medicinal properties on the gynecologic system (210). Furthermore, evidence supports the traditional use of hops in the U.S, Iran, Romania and France for the improvement of women's reproductive health (209). Mainstream use of hops among menopausal women stems from the growing evidence supporting that hops possesses estrogenic activity in humans (211-216). The use of botanical extracts for the potential alleviation of menopausal symptoms contributes to an estimated 13.7 billion dollars spent annually (217).

Botanical dietary supplements are consumed sometimes instead of conventional medicine for menopause-related symptoms for a variety of reasons including the perception that they are 'safer' as they are typically not associated with uterine cancer (4, 99). The toxicity of hops extracts in cell-based assays have limited its investigation *in vitro*, but an initial bioassay screen suggested that natural progestins can be identified from hops extracts (9). Furthermore, hops has been a focus of the UIC Botanical Center. The goal of this chapter was to discuss the biology of

potent progesterone ($P_4$)-like compounds in hops and the experimental evidence to support this hypothesis.

Botanicals are complex mixtures of many bioactive constituents. Consequently, a challenge with screening natural products is the presence of spectroscopic properties, toxic compounds and fatty acids that interfere with receptor binding. This is exemplified by the toxicity observed in hops. Xanthohumol is a major prenylated flavonoid found in hops and has been explored for its chemopreventive properties (102). In the human body, xanthohumol is metabolized into many flavonoid derivatives. Although not toxic in the body, higher concentrations of xanthohumol may be cytotoxic in cell-based assays. Since 20 µg/mL of supercritical $CO_2$ extract was toxic in T47D breast cancer epithelial cells, several chromatographic steps were proposed to remove the masking toxicity and to facilitate further investigation. Once the bioactive, non-toxic fraction is generated, standard chromatographic techniques (semi-preparative HPLC) will be used in future analyses by our collaborator, Dr. Brian T. Murphy, to isolate the metabolite(s) and complete structures will be determined using various one- and two-dimensional NMR experiments ($^1H$, $^{13}C$, 1D-selective TOCSY, COSY , HSQC, HMBC, HSQMBC, NOE, etc) and high-resolution MS analysis.

**B. Hops Fractionation and Extraction**

Hops plant material was not available at UIC Botanical Center at the time of these experiments due to depletion in alternative experiments being conducted at UIC, thus a new source was acquired. Previously, hops was extracted using supercritical $CO_2$ to allow for more selective separation and to produce an extract free of bitter acids (218). On the negative side, this extraction method has polarity limitations because $CO_2$ is non-polar and has restricted dissolving

power, hence, it might eliminate more polar compounds (219). In the present study, *Humulus lupulus* hops pellets were acquired from Bell's Brewery and were extracted using different liquid solvent systems. The present extraction technique will allow for the inclusion of the bitter acids in addition to other small molecules present in hops to better evaluate potential PR modulators. 100 g of pellets were ground up, extracted with a mixture of methanol and chloroform at room temperature, and shaken at 220 rpm over the weekend (~60 hours), while exposed continuously to visible light. Following filtration, the initial crude extract was dried to completion *in vacuo* with low heat (10-37°C). Hops extract was redissolved in 50% $H_2O$ and 50% hexane, followed by successive hexane partitioning to remove fatty acids (3 x 500mL). The $H_2O$ layer was partitioned against ethyl acetate (6 x 500mL). Using the T47D PRE-luciferase assay to guide the fractionation process, strong progestogenic activity was traced to the non-toxic $H_2O$ fraction. The $H_2O$ extract at 60 μg/mL induced a 41.7 fold change of PRE-luciferase transcription (**Figure 23A**) and 74 fold change of ZBTB16 expression over basal (**Figure 24**). Progestogenic activities were attenuated in the presence of 1 μM PR antagonist RU486, suggesting PR mediated induction of the reporter and endogenous genes (**Figure 24**). The active $H_2O$ layer was further partitioned with butanol (3 x 500mL), in which progestogenic activity was found in the butanol layer. The butanol extract at 60 μg/mL induced a 45.7 fold PRE-luc induction and 64 fold induction of ZBTB16 expression over basal (**Figure 23A** and **24**). To identify the location of metabolite(s) that exhibit potent induction of the PRE construct, the butanol fraction was chromatographed using flash column chromatography by Sephadex gel-filtration, reversed phase C18 (**Figure 23B**) and phenyl-hexyl semi-preparative HPLC (**Figure 24C**).

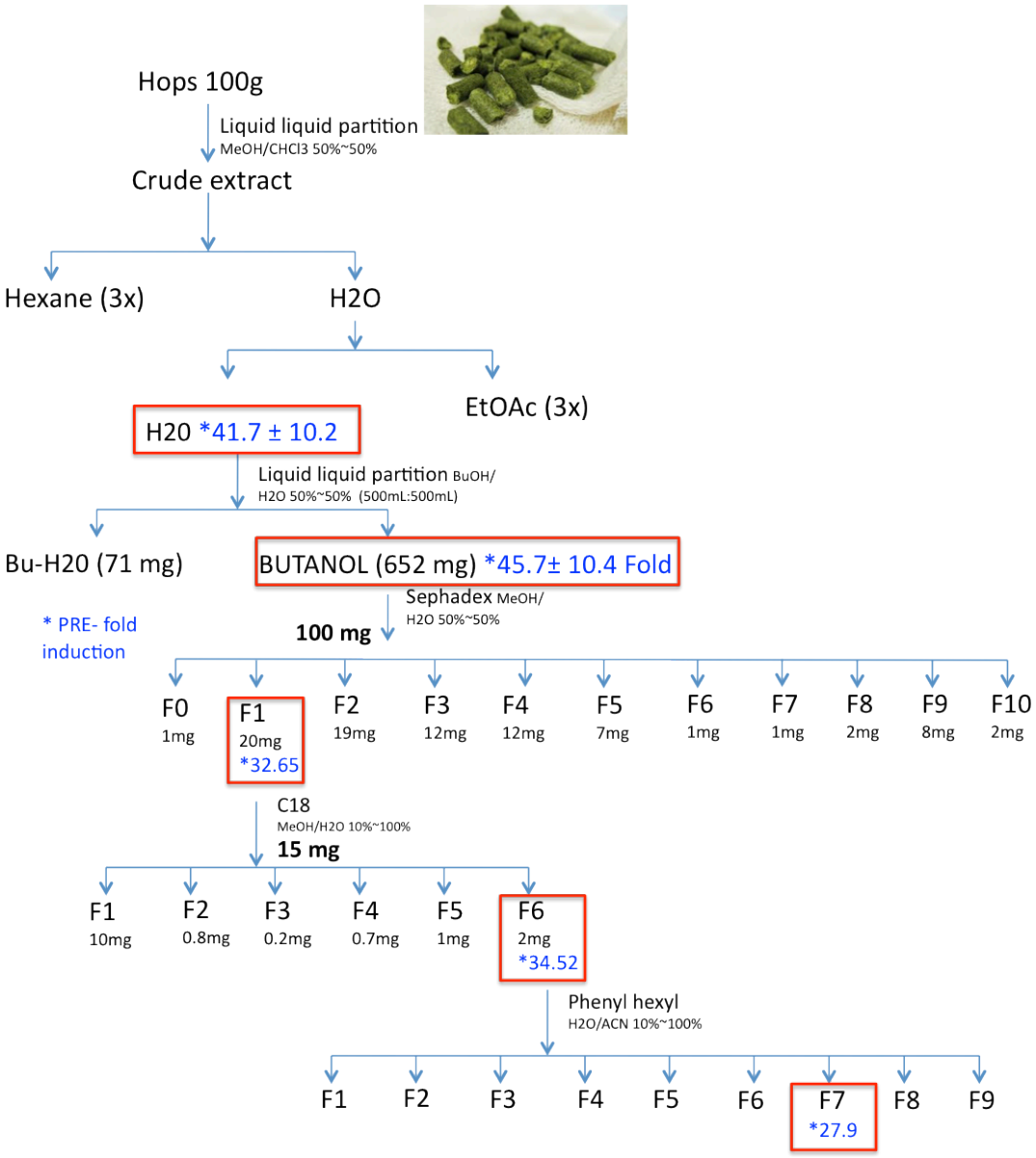


**Figure 23 A.** Hops extraction scheme. Progestogenic activity identified in polar hops fraction.

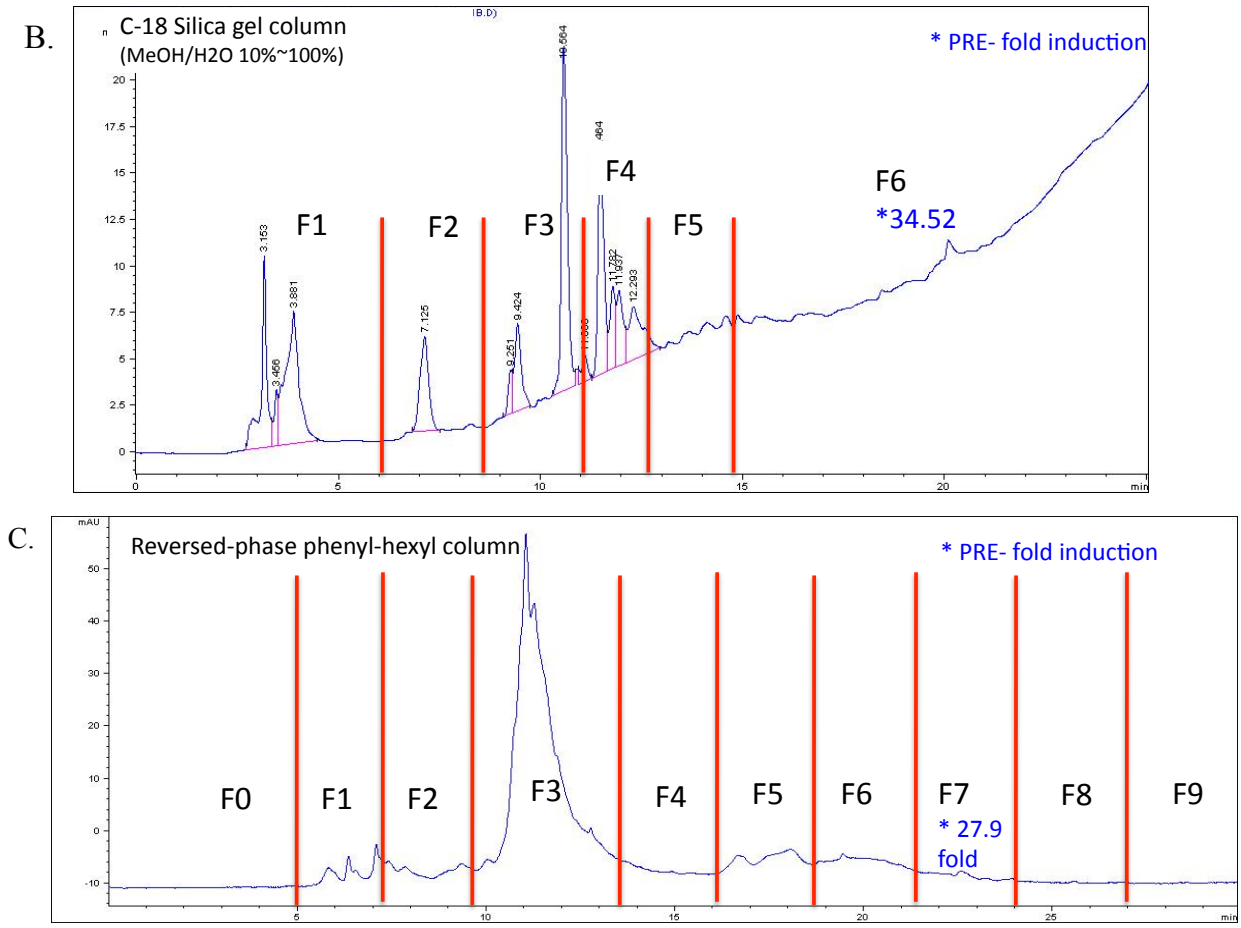


**Figure 23. B.** Chromatogram of C18 reverse phase column of active F1 fraction, **C.** reverse-phase phenyl–hexyl semi-prep column of F6 fraction extracted from C18 column. Resulting separated yielded F7 with 27.9 fold PRE-luc induction.

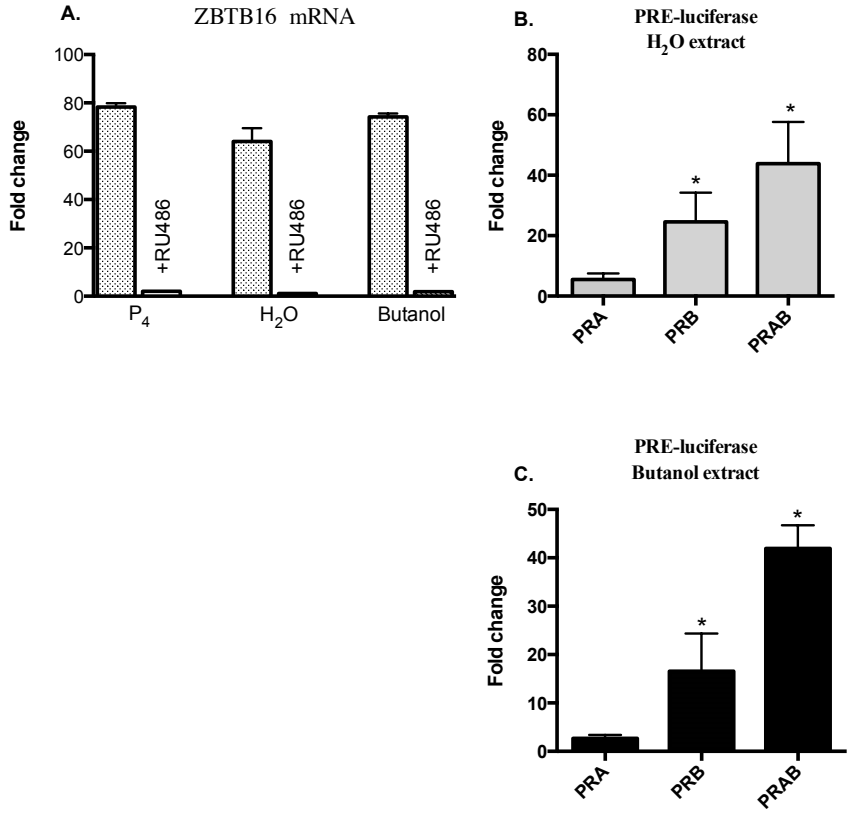


**Figure 24.** Progestogenic activities of hops $H_2O$ and butanol extracts. (**A**) ZBTB16 mRNA expression in breast cancer epithelial cells and PRE-luciferase fold induction in endometrial epithelial cells in the presence of (**B**) 60 μg/mL $H_2O$ and (**C**) 60 μg/mL butanol extracts. 6.29 μg/mL $P_4$ gave a 13.97 fold change in PRAB endometrial epithelial cells (data not shown). *$p<0.05$, significant differences from the control DMSO value were determined by t-test.

## C. Identification of Progestins from Hops.

The $H_2O$ extract displayed extremely high activity in the PRE-luciferase assay, as the $H_2O$ and butanol fraction induced a >40 fold PRE-luc expression, while the positive control $P_4$ used at 1 μM was ~50 fold in T47D breast epithelial cells (**Figure 23A**). Additionally, these activities were comparable to $P_4$ in the activation of endogenous $P_4$ regulated gene promoter (ZBTB16), further suggeting the presence of highly active natural progestins in hops (**Figure 24**). The detection of PR ligands in the $H_2O$ and butanol extracts was unexpected because known progestogenic molecules have moderate polarity and would be partitioned into the ethyl acetate layer. As a result, the partition of the active extracts between successions of increasing polar solvents were completed in hopes of revealing a potentially new structural class of progestin.

Since the uterus is a major target of $P_4$ action additional studies were conducted to evaluate $P_4$ activities of active extracts in a human endometrial cell line. Due to low PR levels in the endometrial stromal cells (HESCs), $E_2$ incorporation is required to study PRE-luc expression. Therefore, to investigate $P_4$ activity of hops independent of $E_2$, human endometrial epithelial cell lines (Ishikawa) from the Blok laboratory were used (**Figure 24 B, C**). Ishikawa cells do not endogenously express PR, so the stable incorporation of PRA or PRB allowed the evaluation of PR isoform specific activity. Furthermore, there is a growing body of evidence demonstrating the role of PR isoforms in mammary and uterine development, changes in pregnancy, and alterations in cancers (220, 221). For example, in humans, nearly all PR expressing cells have relative levels of both isoforms indicating that heterodimers are responsible for transcription of most $P_4$ regulated genes (222). On the contrary, alterations in PRA:PRB expression ratios were observed in the progression to malignancies (11). Significant increases in predominantly one PR isoform were reported in malignant breast and endometrial tissues (11, 223, 224). In breast cancer tissues,

PRA levels predominate in ductal carcinomas in situ and in invasive tumors (225), whereas in endometrial cancers, the loss of one PR isoform is a determinant of higher histological grade and poor prognosis (224). The loss of coordinated PRA:PRB expression may underlie the onset of these gynecologic pathogenesis. Therefore, the specificity of PR binding must be determined in the human diseases where coexpression is the norm and a disruption in balance of PR expression is seen in malignancy. Phytoprogestins identified in plant extracts may provide new molecules with PR specific activity that could be tested *in vitro*.

To determine if our hops extract displayed any receptor specificity, 60 μg/mL hops $H_2O$ and butanol extracts were tested in Ishikawa cell lines expressing PRA, PRB, or both (**Figure 24**). Interestingly, both extracts demonstrated preferential signaling through PRB (**Figure 24**). To further confirm these results, PR isoform specific gene regulation (PRA, amphiregulin; PRB, betaintegrin4) could be measured using qPCR analysis. The chromatographic separation using sephadex generated 10 subfractions with F1 as the most active fraction (**Figure 23A**). To reduce the complexity of F1, separation over a reversed phase C18 column was employed (**Figure 23B**). F6 displayed the highest progestogenic activity (**Figure 23B**), and further separation with reversed phase phenyl-hexyl semi-preparative HPLC yielded F7 with a 28-fold activity (**Figure 23C**). Unfortunately, due to low final yield, NMR analysis was not conducted. Based on HPLC chromatogram, molecules in F7 were weakly UV active (**Figure 23C**). Ideally, at this stage, semi-preparative HPLC together with evaporative light scattering detection (ELSD) would be the optimal technique to purify the metabolite(s) since ELSD relies on the physical abundance of a molecule in the mixture rather than its spectroscopic properties. However, it is important to consider the possibility that poor UV activity is due to low abundance of the active molecule(s). This highlights the importance of structural elucidation efforts as that will assist with the

procedure optimization process for the extraction of this particular class of molecule(s). Isolating the molecule(s) responsible for the observed bioactivity of F7 will require a large-scale extraction with an optimized procedure to provide enough material to pinpoint and characterize the active compounds. Once a procedure is established, different sources of hops could be tested to determine if these progestogenic effects are conserved between different varieties.

## D. Discussion

In this chapter, biological activity consistent with a progesterone receptor agonist was confirmed in cell-based assays with hops extracts made from pellets. These molecules activated a reporter gene in two major $P_4$-responsive cell-lines (breast and uterus), were blocked by receptor antagonists, activated endogenous PR regulated genes, and were extracted by polar solvents. It is perhaps coincidental, but nonetheless important to note that hops pellets, and not a supercritical $CO_2$ spent extract exhibited the most active progestogenic activity to date. After assembling the evidence from existing literature (218, 219) (polarity limitations in supercritical $CO_2$ extraction method) and the results in this study (progestogenic polar water layer), it is possible that previous supercritical $CO_2$ extracts may not be the ideal starting material for investigating natural progestins in hops. Therefore, future extraction of hops pellets with the above methodology (**Figure 23A**) is needed to identify novel PR ligands and to gain a better understanding of the progestogenic actions of hops. Considering that the phytoprogestin has poor UV absorbance and is found in the previously uninvestigated polar $H_2O$ and butanol layers (**Figure 23A-C**), the molecules responsible for the bioactivity may belong to a new structural class of progestins. However, it is also important to consider the possibility that this lack of observable UV absorbance may be due to low abundance of the active molecule(s). This is an important question,

as it will shed light on whether hops contains natural progestins and if these biological effects are translated through human consumption. Sadly, due to dwindling resources we were unable to move forward with this vital research.

In rodent models, PR differs in expression patterns depending on the cell-type within the reproductive system. Often, a predominant expression of one isoform is observed (226) . For instance, PRA is the predominant isoform in murine uteri and mice lacking PRA lost $P_4$ mediated inhibition of uterine hyperplasia (11, 226). These findings suggest that PRA signaling is necessary in the mouse uterus for protective biological activity against estrogens (227). During development and pregnancy, PRB is more highly expressed In the rat mammary gland (228). Also, proliferating cells in the rat mammary gland consistently expressed PRB perhaps indicating that mammary cellular proliferation in the rat is controlled preferentially by PRB (143, 228). These animal models in part suggest that PRA induction is protective in the uterus while PRB induction might increase breast proliferation. Thus, molecules that are PRA selective may be safer for menopausal women. Since $H_2O$ and butanol extracts demonstrated preferential signaling through PRB, the exposure to hops may have an adverse effect on the endocrine system and may pose a health concern. However, since PR expression and function vary between, species, tissues and cell types, it is important to practice caution when extrapolating results between species (11).

A potential problematic situation with the current use of botanical dietary supplements is the formulation and standardization of extracts to contain only plant-derived estrogens, as this may lead to adverse uterine events similar to taking estrogen monotherapy. In the case of hops, its estrogenic activity has been traced to 8-PN; the most potent phytoestrogen to date with undesirable uterotrophic effects. Since hops pellets contained highly active phytoprogestins, this

may be the most appropriate starting material for the future production of an ideal commercial hops extract containing phytoestrogens to help with menopausal symptoms and phytoprogestins to confer uterine protection. In any case, detailed preparative guidelines have been developed and described in this chapter for the generation of a progestogenic crude extract, which may provide a useful resource for future chemical analysis and formulation of hops.

# VI. CONCLUSIONS AND FUTURE DIRECTIONS

## A. Summary of Results

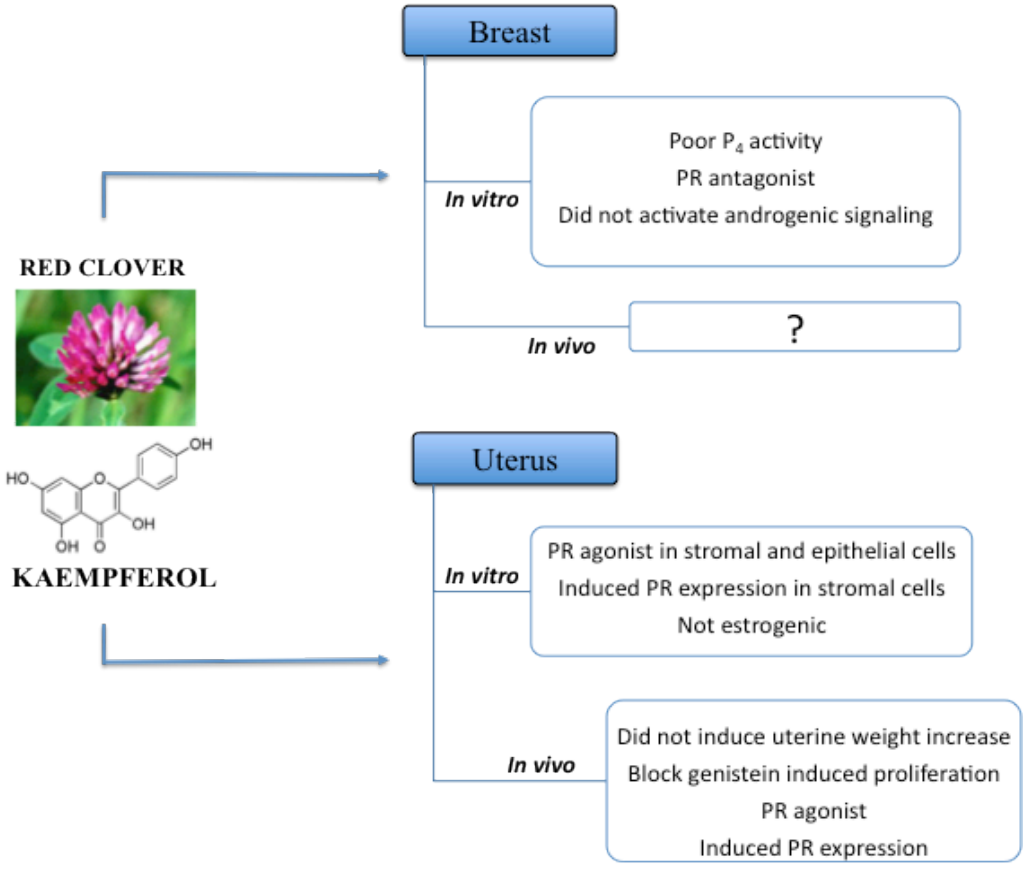


**Figure 25**. Summary of kaempferol steroidal activity *in vitro* and *in vivo*. Kaempferol demonstrated diverse actions and functioned as a novel progestin.

A sizeable number of women are self-prescribing botanical alternatives for a variety of endocrine related health issues, such as infertility, menstrual disorders, menopause, pre-menstrual symptoms and endometriosis (92, 229). The general dissatisfaction of HRT, the perceived safety, and the widespread availability of botanical supplements have significantly fueled botanical use among menopausal women (92, 93, 99). Although substantial research efforts have been focused on the hormonal formulation of phytoestrogens for HRT, compounds modulating other steroid receptors remain largely underexplored. This oversight is alarming because environmental estrogens can have a profound effect on a woman's health, making the identification of progestin substances from botanicals of great significance. The primary goal of this study was to expand our understanding into botanicals' mechanisms of action in the context of progesterone-like bioactivity in botanicals that are commonly consumed for women's health. Moreover, the identification of novel, natural progestins with selective PR signaling will provide an avenue to generate new, safer therapies potentially impacting women's health in terms of menopause, contraception, uterine disorders, reproductive support and ovarian cancer.

The investigation on the progestogenic activity of red clover and hops extracts indicated that novel PR modulators were present and could be identified through a series of chemical and biological techniques that have been established through the work of this dissertation. Kaempferol, identified in red clover, showed progesterone-like activity *in vitro* and *in vivo* (**Figure 28**). Unexpectedly, kaempferol demonstrated multi-functional steroidal effects suggesting that it is not a strict progestin, but rather an eccentric PR modulator. Unlike conventional progestins, kaempferol has tissue specific progestogenic effects, induced PR expression without activating ER or AR, and blocked genistein induced uterine proliferation. These intriguing findings warrant further investigation especially since kaempferol is universally

present in plant matter and our food supply. Additional studies will clarify the promise and peril of kaempferol to facilitate how to best standardize the active compounds to produce a potentially safer therapeutic. The implication of these results toward future experiments is discussed.

## B. Future Directions

### 1. *New botanicals, needle in the haystack?*

In pharmacognosy, much information has been generated on the pharmacological/biochemical profile of extracts *in vitro*, *in vivo*, and in human studies. Gathering data is only the beginning in the drug discovery process. The challenge lies in the extraction and processing of information from large databases to answer biology questions. Fortunately, a relational database of all natural products, NAPRALERT, founded by Dr. Norman R. Farnsworth, is a powerful tool that can be exploited to facilitate this process in the future. More specifically, botanicals can be prioritized based on ethnomedicinal use and scientific data to simplify the screening and discovery of novel phytoprogestins. As a search strategy to identify additional botanicals for new sources of phytoprogestins, a spreadsheet with prioritized taxa, filtered for ethnomedical usage and similarities in terms of chemistry to kaempferol was generated (**Table IX**). Additional refinement such as subtracting those with "bad criteria" including toxicity, estrogenic and androgenic signaling will narrow down the number of plant species with potential selective activity for further evaluation. Once the botanical(s) of interest are carefully selected, a virtual-based *in silico* molecular docking screening of available chemical libraries of isolated compounds from the botanical(s) against the PR will be completed. If the positive hits are commercially available, they will be subjected to bioassay testing for PR binding and progestogenic activity. In the case that the botanical composition is unavailable,

identification of the bioactive constituents from these botanicals could be attempted. Licorice, yam, damiana, and ginger are great candidates for further screening as they all have documented use indicating that they are likely to contain progestins or androgens (3, 9).

| | |
|---|---|
| PROGESTAGENIC EFFECT | x |
| ANTIPROGESTERONE EFFECT | |
| RECEPTOR BINDING(PROGESTIN) DECREASED | |
| PROGESTERONE RECEPTOR BINDING INHIBITION | |
| PROGESTERONE SYNTHESIS INHIBITION | |
| PROGESTERONE RECEPTOR AGONIST ACTIVITY | XXX |
| PROGESTERONE SECRETION STIMULATION | |
| PROGESTERONE SECRETION INHIBITION | |
| PROGESTERONE RECEPTOR BINDING ACTIVITY | XX |
| PROGESTERONE RECEPTOR STIMULATION | XXX |
| PROGESTERONE 11-ALPHA-HYDROXYLASE INHIBITION | |
| | |
| ESTROGEN BINDING SITE-TYPE II BINDING EFFECT | |
| ESTROUS CYCLE DISRUPTION EFFECT | |
| ESTROGENIC EFFECT | |
| ANTIESTROGENIC EFFECT | |
| ESTRONE SULFATASE INHIBITION | |
| ESTROGEN METABOLISM STIMULATION | |
| RECEPTOR BINDING(ESTROGEN) DECREASED | |
| ESTROGEN SYNTHESIS STIMULATION | |
| ESTROGEN RECEPTOR BINDING EFFECT | |
| ESTROGEN BINDING INHIBITION | |
| ESTROGEN RECEPTOR-ALPHA TRANSCRIPTION INHIBITION | |
| ESTROGEN RECEPTOR(ALPHA) BINDING EFFECT | |
| ESTROGEN RECEPTOR(BETA) BINDING EFFECT | |
| ESTROGEN RECEPTOR(ALPHA) STIMULATION | |
| ESTROGEN RECEPTOR(ALPHA) INHIBITION | |
| ESTROGEN RECEPTOR LEVEL INCREASE | |
| ESTROGEN RECEPTOR TRANSCRIPTION STIMULATION | |
| ESTROGEN RECEPTOR(BETA) EXPRESSION INHIBITION | |
| ESTROGEN SYNTHETASE INHIBITION | |
| ESTROGEN RECEPTOR MODULATION | |
| | |
| 5-ALPHA-ANDROSTANE-3-ALPHA-17-BETA-DIOL FORMATION | |
| ANDROSTENEDIONE 6-BETA-HYDROXYLASE INHIBITION | |
| ANDROGENIC EFFECT | |
| ANTIANDROGENIC EFFECT | |
| RECEPTOR BINDING(ANDROGEN) DECREASED | |
| ANDROGENIC RECEPTOR BINDING ACTIVITY | |
| ANDROGEN RECEPTOR INHIBITION | |

**Table IX.** Example of pharmacological activities available for sorting on NAPRALERT. ‘X’ symbolizes priority and desired criteria.

2. *Development of a high throughput assay*

Previously, procedures designed to lower the chemical complexity of each plant extract to narrow down the active compounds within a simpler mixture were described. Within the simpler bioactive fraction, there could still be a complex assortment of chemicals. Biochromatograms could be used to pinpoint the exact location of the active constituents within the mixture. This method uses HPLC to separate the contents of the active fraction into a 96-well microplate. Each well will represent a time point (**Figure 29**); for example, a solvent system of 1 mL/min will effectively separate a 20 mL bioactive fraction over 20 minutes and will occupy 20 wells in the microplate. The collected fractions in each well be dried to completion and reconstituted in DMSO. Qualitative screening of the occupied wells for progestogenic activity using a PRE-luciferase assay might reveal the exact elution time of the active target metabolite. Unfortunately a bottleneck in this process is in the screening of large number of samples very rapidly for biological activity in different cell lines.

Currently, it takes 5 days to run a PRE-luc bioassay; plate, transfect, starve, treat, lyse. Furthermore, the current bioassay system utilizes a 24-well plate and can only accommodate 12 duplicate samples/time points, which severely limits the throughput of this assay. Not only is this process time consuming, but it is also cost ineffective, especially when screening large numbers of samples. An approach to overcome this bottleneck is to develop stable PRE driven luciferase-based reporter system in breast and endometrial cell lines. In the long run, this method would reduce reagent cost and time considerably as it would eliminate the need for transient transfection methods and controls (B-gal). Once fully validated, these ready to screen cell lines can be seeded directly into a 96-well cell culture plate and subsequently treated with the collected fractions (**Figure 29**). Given that more samples can be screened with each assay, one

could also collect factions at a shorter time point, e.g 30 seconds rather than a minute, which allows for the "fine tuning" of the screening process. Reporter gene expression/florescence readout in each well will correspond directly to the collected fractions. This approach provides an efficient and simplified "high-throughput" screening for progestogenic molecules within an active fraction.

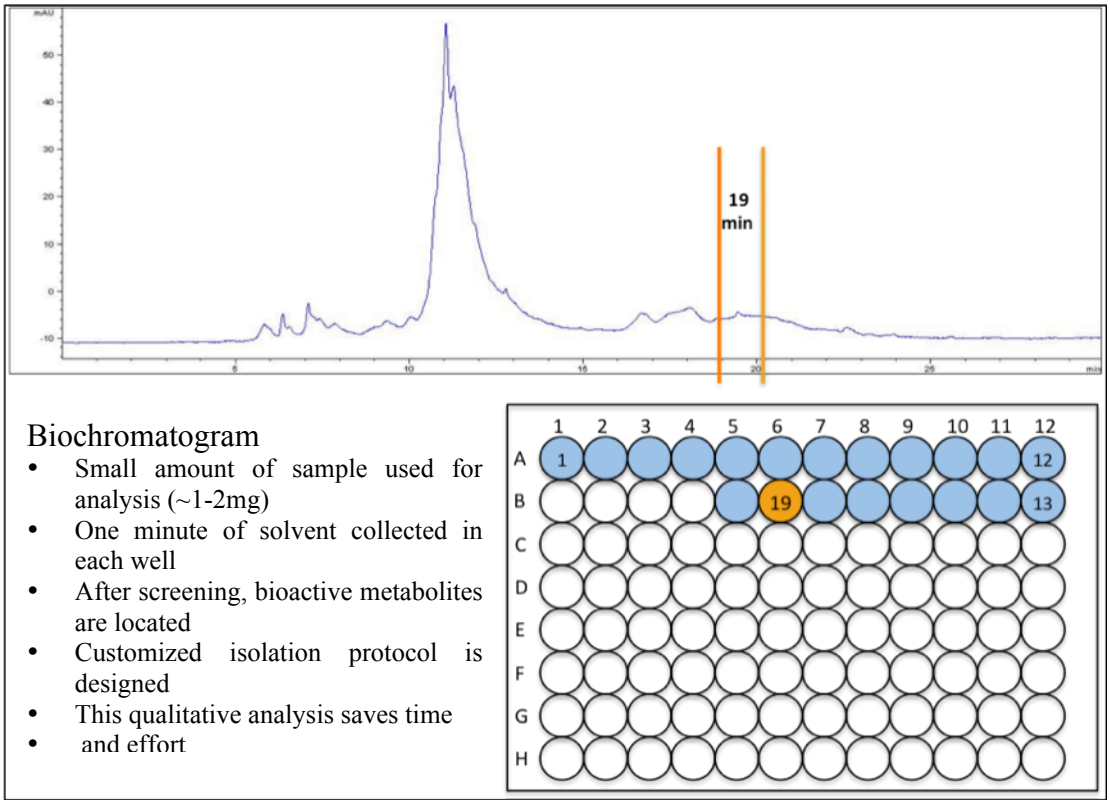


**Figure 26.** Identification of bioactive compounds using biochromatogram. Adapted from grant application, Botanical Dietary Supplements Pilot Project.

3. *Phytoprogestins-phytoestrogen signaling interactions in vivo*

Most of the completed studies of kaempferol were focused on its signaling in the uterus. Although kaempferol demonstrated marginal PR signaling in cell-based models of breast cancer (9), the question remains whether kaempferol-mediated progestogenic effects occur in the breast *in vivo. In vivo* evaluation is more biologically relevant and predictive, especially since kaempferol is subjected to complex pharmacokinetics and rapid metabolism (189, 230). Numerous clinical studies have supported that the addition of the progestin component with estrogen in HRT, not estrogen monotherapy, led to an increase in breast cancer events in postmenopausal women (44, 45, 231). In other words, these clinical data suggest that the specific type of progestins and route of administration modulates the adverse effects in the breast (117). More specifically, this breast cancer risk highlights the importance of better understanding whether phytoestrogens and phytoprogestins can affect breast proliferation and alter the risk for cancer. As a quantitative readout of PR activity, rat mammary gland end bud development, proliferation and specific gene induction from combined phytoestrogens and phytoprogestins will be investigated. To assess end bud formation, three random fields at 20X per gland can be captured microscopically to count the number of buds. The other inguinal mammary gland is then harvested for mRNA to evaluate the expression of serine protease inhibitor Kazal type 3 (Spink 3), defensinß1 (Defß1) and g- protein coupled receptor 105 (GPR105). Spink3 and Defß1 expression is predicted to increase from progestins alone and in combination with estrogen, but not with estrogen alone (232). Estrogenic compounds alone in the absence of progestins should enhance GPR105, and the combination will have the lowest expression level (232).

Evaluating the effects of phytoprogestins and phytoestrogens *in vivo* will also be dependent on the route of delivery. Generally, the investigation of progestogenic compounds *in*

*vivo* is administered subcutaneously (s.c) or via intra-peritoneal (i.p), on the basis that $P_4$ itself has poor oral bioavailability. i.p and s.c routes are artificial exposure routes that are useful to circumvent extensive gastro-intestinal first-pass effects subsequently producing the highest bioavailability of the substances and faster onset of action (233). Although these methods are acceptable routes of administration, they poorly reflect human exposure when investigating the role of progestins in orally administered hormone therapy and in botanical dietary supplements. In the completed *in vivo* study of kaempferol, oral gavage was selected to better resemble the route of administrations to humans. On the negative side, the overall efficacy of kaempferol in this study might have been compromised due to extensive metabolism by gut and hepatic microsomes, leading to lowered systemic absorption and weaker endpoint progestogenic signaling *in vivo.* Therefore careful attention to detail is necessary when comparing future studies to published endpoint steroidal effects (gene and protein expression profiles), as they may vary between different routes of administration. If oral administration is not sensitive enough to evaluate the inducibility of target genes such as Spink3 and GPR105, then it may be worthwhile to investigate other delivery options (s.c or i.p), which may contribute to experimental refinement.

4. *Physiological relevance and tissue distribution*

One major drawback of new potential phytoprogestins as a novel PR modulator is that they may be subjected to complex pharmacokinetic events, therefore may have poor localization and distribution in its target tissues (189, 230). To investigate these discrepancies, an important future work would be to determine uterine and breast tissue distribution of kaempferol and its metabolites using MALDI imaging following oral administration (234). MALDI imaging mass

spectrometry allows the detection, tracking and visualization of multiple analytes such as small molecule drugs, metabolites, proteins, peptides, lipids and polymer at the molecular level of specific tissues (234, 235). Rat and breast tissue sections will be introduced in the MALDI instrument, UV pulsed laser then scans over a selected area while collecting mass spectra at every time point (234, 235). Next, the image data is process with a software and MALDI-image converter, generating "analyte- specific images based on selected masses" (235). The distribution and localization of kaempferol and its conjugates throughout the tissue sections can be visualized from the generated images. Furthermore, these images may also shed light on the cell-type specific (epithelial and stromal) uptake and distribution within the different compartments of the uterus and breast. Since kaempferol was progestogenic in rat uteri, it is expected that a fair concentration of kaempferol or its metabolites should accumulate throughout this tissue.

5. *Alternative receptor targets*

*PGRMC1*

The chemical structures of phytoprogestins and progesterone might confer different mechanisms of action. Therefore, the most effective use of phytoprogestins as chemopreventive agents ultimately relies on understanding the mechanism of action at the molecular, cellular, tissue and organ levels as well as in the animal. The PR-ligand complex binds PRE on DNA directly and recruits various coregulator proteins that could activate or suppress PR dependent transcription. In fact, depending on the PR ligand, phytoprogestins could differentially affect transcription of PR target genes through selective recruitment of cofactors giving rise to different gene expression in different tissues (13, 81, 163). The activation of nuclear PR is critical for blocking estrogen-induced uterine proliferation (10, 14, 236); however, more recent studies have

shown that some actions of progesterone are mediated through non-nuclear PR mechanisms, for instance through PGRMC1 and PGRMC2 signaling (26-30, 32). The expression of PGRMC 1 is highly tissue specific and is expressed in reproductive tissues, neural tissues and the gastrointestinal tract (26-30, 32). The complex progesterone signaling network controlling cell growth and proliferation is often deregulated in cancer cells, which could be used as a therapeutic target. The signaling of PGRMC1 has been shown to suppress apoptosis and promote cell growth and survival in multiple types of cancer (30, 237, 238). The elucidation of phytoprogestin mechanisms of action in other cell types (for example, ovarian cancer) and phytoprogestins-PGRMC1 associated ligand-binding function is critical to provide a new way of treating different diseases. Multiple studies have documented that flavonoids can function as PR antagonists in a breast cancer cell model (6, 9, 113). To date, there are no studies investigating their actions on PGRMC1. If phytoprogestins are able to antagonize PGRMC1, that could be used as a therapeutic approach to target PGRMC1 cancer associate phenotypes. Furthermore, novel ligands are useful tools as drug and molecular probes to elucidate PR mechanisms, as the diversity of the molecules will result in unique protein conformations that alter coregulatory proteins and gene transcription.

*Glucocorticoid, mineralocorticoid receptor*

Glucocorticoid receptors (GR) belong to the nuclear receptor family and regulate various fundamental cellular events. More specifically, GR regulates inflammatory and immune responses, and the metabolism of sugars, fats and proteins to generate energy (81). Additionally, GR can also regulate physiological functions of the reproductive system (47-49, 239, 240). GR plays a complex role in various aspects of breast cancer biology (239, 240). PR and GR share

overlapping sequence and structural similarities (240). In fact, PR-GR crosstalk is required to mediate a set of unique processes in breast cancer cells such as proliferation, migration and adhesion (240). $P_4$ binds to GR with relatively low affinity and mediate partial GR agonistic effects (240). Unlike $P_4$, synthetic progestins such as MPA and norethindrone displayed a stronger affinity for GR and are thought to exert negative effects on breast cancer risk when used in HRT (241). Clinical observations from the WHI trial demonstrate that women taking combined estrogen/progesterone as compared to estrogen alone had a higher risk of developing breast cancer (44, 45, 242). Ideal progestins will bind more specifically to the PR and display little or no GR modulation. A future goal would be to compare the binding affinity and specificity of extracts and compounds that bound the PR for binding to the GR.

Mineralocorticoid receptor (MR) is mainly known for its role in modulating water electrolyte homeostasis and blood pressure. The consistency of results from clinical studies provides greater support for the beneficial effects of MR antagonist on the cardiovascular system. $P_4$ binds to MR and has potent anti-mineralocorticoid properties *in vitro* and was able to antagonize aldosterone *in vivo,* conferring cardiovascular protection. Clinical progestins on the other hand, have a wide range of mineralocorticoid properties. MPA demonstrated greater binding to MR compared to norethindrone, but did not exhibit anti-mineralocorticoid activity in rat models. To investigate if phytoprogestins can function as an ideal progestin, it is therapeutically significant to investigate whether they can interact with MR, compete with aldosterone for MR binding, and if they can mediate anti-MR properties similar to $P_4$, in hopes to recapitulate the beneficial effects on blood pressure and cardiovascular function.

6. *Anti-progestins*

Preliminary data suggested that anti-progestins are present in dogwood and black cohosh. PR antagonism is advantages in breast cancer models to block proliferation in cells with tumorigenic potential, but harmful in the uterus as $P_4$ protects against $E_2$ driven growth (113). Therefore, an important future direction would be to identify and characterize these molecules in relation to tissue specific antagonistic effects. Particularly, the discovery of molecules that are antagonistic in the breast but agonistic in the uterus would be clinically interesting as they may be excellent selective progesterone receptor modulators. On the negative side, the exposure to botanicals containing anti-progestins may be disadvantages to women trying to conceive and potentially dangerous to pregnant women. Additional work is warranted to extract, fractionate and characterize dogwood and black cohosh to identify potential anti-progestins as part of the safety profile of botanicals as modulators of the endocrine system.

7. *Standardization, friend or foe?*

Botanical dietary supplements are not manufactured under carefully monitored conditions (243). Without official standardization, formulations can vary widely from batch to batch, resulting in variation in the final product. Some of the factors that determine the compositional profile of a botanical extracts include: the source and quality of the original plant material, where/how it was harvested, extraction techniques, solvent systems and formulation methods (243). Therefore production standardization of herbal medicine is a useful procedure to ensure batch-to-batch consistency and reproducibility (243). Considering the sizable number of menopausal women who take dietary supplements and the widespread availability of these products (such as black cohosh, red clover, soy products among others), this topic is immensely

important. The challenge comes when trying to commercially reproduce a particular formulation to recapitulate laboratory preparations to achieve the expected results (243). Clinical trials and experimental findings are therefore only relevant to the specific herbal preparations (dosage, dose form and administration route) used in the research and it unlikely reflective of other extracts of the same botanical (243). Given the poor regulation and the vast inconsistency in commercially available herbal supplements, a pressing problem in this field is in establishing experiments and model systems that are reasonably predictive of human utility and outcomes, especially when transitioning between different formulations (243).

With the help of sophisticated laboratory techniques, many of these products have been standardized to the desired active constituents (phytoestrogens) for their pharmacological benefits on menopausal symptoms (243). Although standardization is a step in the right direction to reduce variation and improve reproducibility, there are safety concerns to this quality assurance practice. For example, red clover and hops crude extract demonstrated protective effects against phytoestrogen-driven uterine hyperplasia *in vivo* (4). Since red clover extract activated PR, it is likely that natural progestogenic compounds are present to block the adverse estrogenic signaling in the uterus (9). Therefore, if the concentration of phytoestrogens is altered for standardization purposes, and the other non-active constituents are removed, the final product many not be representative of the 'safer' original red clover and hops crude extract. More specifically, standardization to merely include plant-derived estrogens in the absence of progestins might increase the risk of developing endometrial cancer similar to estrogen alone. Once a phytoprogestin is identified from red clover and hops, it is favorable to methodologically standardize the extracts to contain both the beneficial effects of phytoestrogens and the protective effects of phytoprogestins. Ideally, the entire manufacturing process from the

acquisition of the plant material, processing, extraction, storage, formulation, to the packaging of the finished product should be executed under a regulated and systemic protocol. Unfortunately, there is no legal system to enforce this procedure in manufacturing practices due to a lack of official FDA standards and guidelines. All in all, there is an urgent need for stronger rules to ensure the safety of dietary supplements.

8. *Synergistic interactions*

Unlike most pharmaceutical drugs, the therapeutic actions of botanical medicines stem from the highly complex mixtures and interactions of different bioactive constituents within the extract (243). When it comes to botanical extracts, it is imperceptive to think in terms of specific quantities of a single active constituent. For instance, in the case of plant progestins, it is likely that the final progestogenic effects are dependent on the composition of the rest of the extract. Although kaempferol was identified as the molecule with the most potent PR signaling activity in red clover, its low abundance and potency profile does not account for the overall activity seen in the crude extract. There could be multiple explanations to this observation. First, other components of the extract, even with seemingly unrelated physiological activities may mediate "crosstalk" with kaempferol, potentially leading to additive or synergistic effects on PR signaling. Therefore, testing purified kaempferol alone may not accurately represent the overall progestogenic actions of red clover. Further complicating this issue is the background matrix that may affect multiple processes including bioavailability, solubility, stability, metabolism and uptake (243). As a rule of thumb, activity of the active compound should increase through successive fractionation because of its increasing concentration. In the event that PR activity is lost, metabolites from surrounding fractions will be isolated and bioactivity analyzed using

recombination approach to assess synergism. To put it simply, fully characterizing red clover in hopes to tease apart the key constituents underlying the safety profile of the entire botanical extract may be time consuming and costly. A second possibility is that kaempferol is not the most active progestin and there could be other undiscovered PR modulators that are more potent in red clover.

### C. Pitfalls

Americans spend 25 billion dollars a year on dietary supplements and these numbers are steadily increasing (244-246). Regrettably, despite their widespread use, there is very limited scientific backing and surveillance in terms of the safety and efficacy of these supplements that many rely on (243). Unlike pharmaceutical drugs, botanical dietary supplements are not subjected to the rigorous, strict laboratory testing and clinical trials used by the FDA. The lack of federal regulation gave the supplements industry "free range", through which, anyone with the means to manufacture, market and distribute their products with herbal ingredients can do so. This loophole, together with the general dissatisfaction with conventional hormone replacement therapy fueled the rapid expansion in the development and sale of botanical dietary supplements for menopausal symptoms.

## D. Final Remarks

The comprehensive framework outlined in this thesis provides a promising avenue for the identification of potentially better and safer compounds capable of activating PR signaling from botanical extracts used for women's health. More importantly, women are already consuming many of these botanical extracts and should be aware of their potential PR modulating activities. Therefore, identification and characterization is important to understanding the actual benefits and hidden risks of these botanicals that many women find appealing. Given the lack of oversight and poor regulation of the dietary supplement industry, supplementation with purified flavonoids, such as kaempferol, should be taken with caution as their use may exert effects on the endocrine system.

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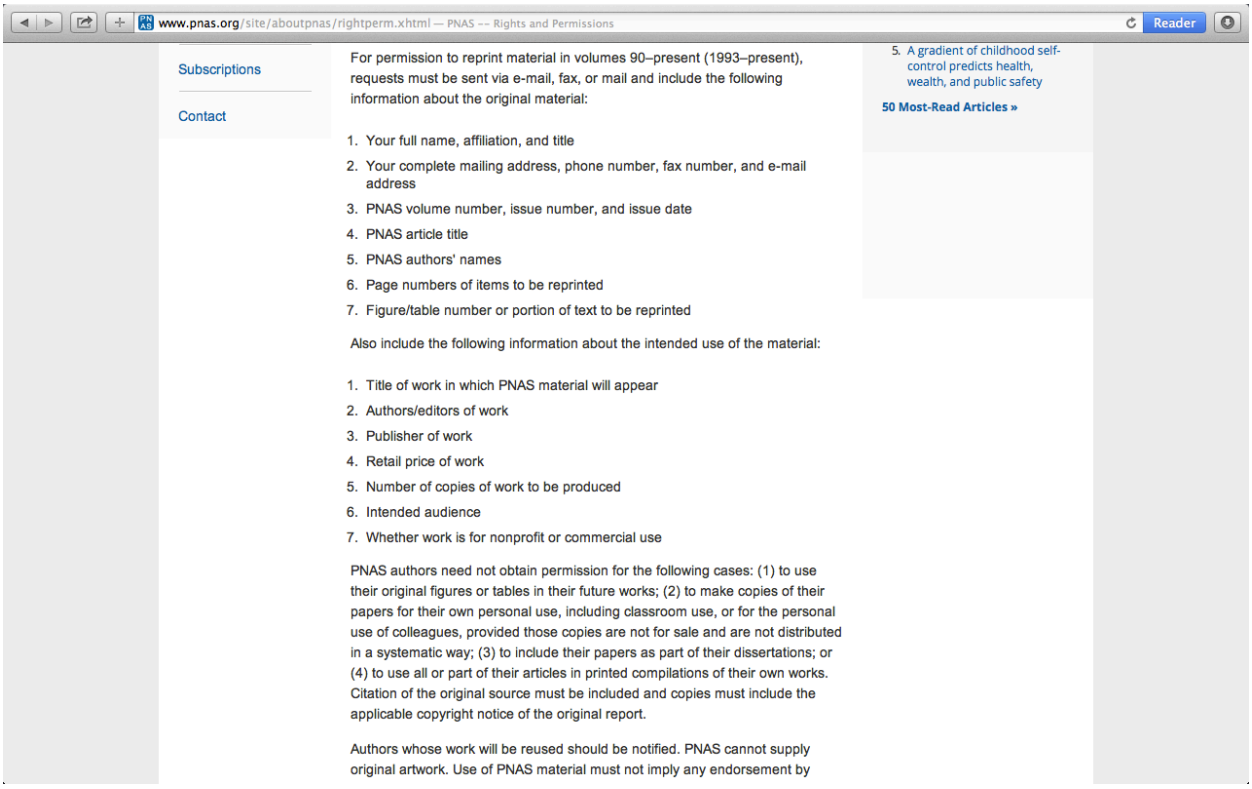

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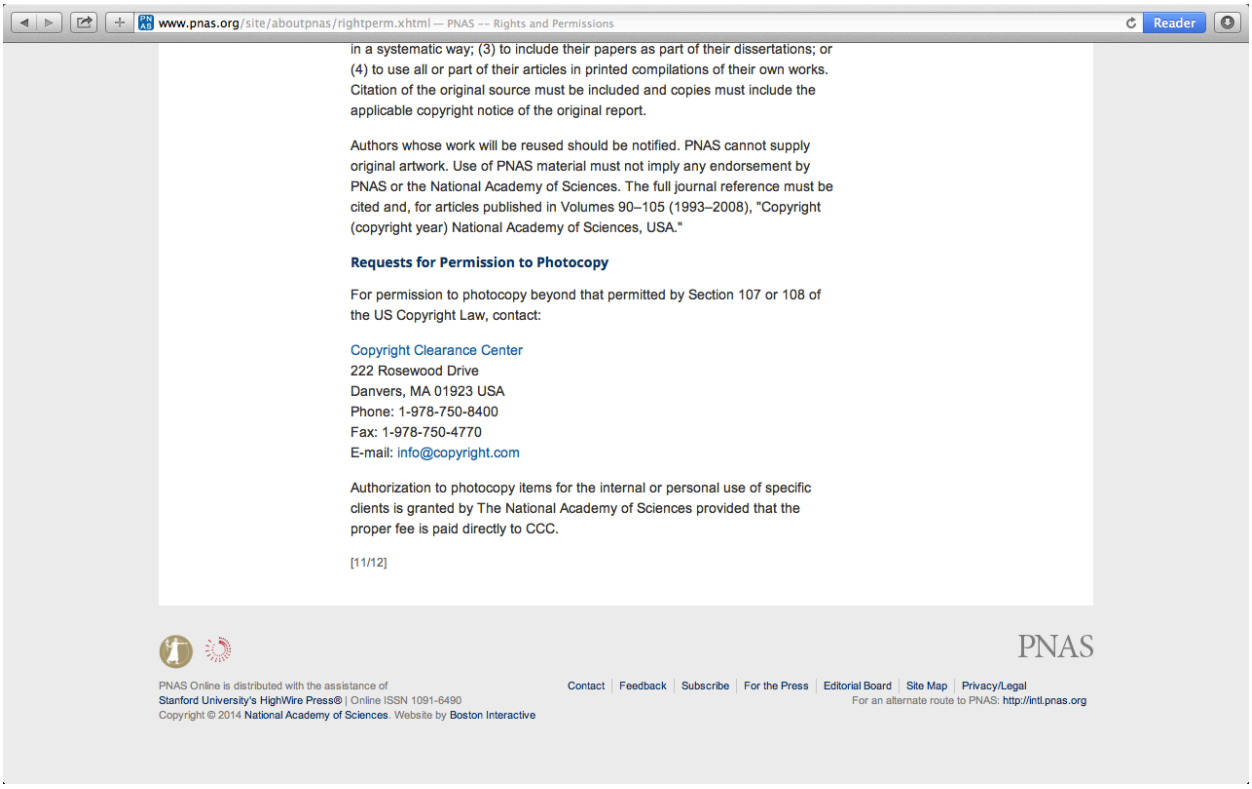

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# IX. VITA

## EDUCATION

**University of Illinois at Chicago, Chicago IL**

Ph.D in Medicinal Chemistry
Field of study: Cellular and molecular cancer endocrinology, nuclear hormone receptor biology 2009-2014

**Dissertation:** "Identification and biological characterization of progesterone receptor modulators from botanicals *in vitro* and *in vivo* for women's health."

**Beloit College, Beloit, WI** 2005-2009

B.S in Biochemistry
GPA: 3.89/4.0

## RESEARCH EXPERIENCE

Graduate Research Assistant, University of Illinois at Chicago 2009–2014

Mentor: Dr. Joanna Burdette
Responsible for complex projects requiring assay development methods, expertise in mammalian cell culture and animal models

- Biological characterization of phytoprogestins in breast cancer epithelial cells and endometrial cell lines using transient transfection, gene induction experiments and western blot analysis
- Evaluate the actions of kaempferol in animal models using qPCR, histological analysis and imaging techniques
- Identification of non-steroidal progestins from botanicals for the improvement of women's health via liquid and solid phase extraction followed by structural elucidation experiments.
- Familiar with the development of mouse cancer models of high-grade serous ovarian carcinoma using tissue specific driven Cre crossed with transgenic *Pten*, *Kras* and *p53* mice. Assisted with histological analysis and genotyping

## HONORS AND AWARDS

Glaxo Smith Kline, Bronze Level Recognition Award
Glaxo Smith Kline, Sharing Science Symposium Best Science Award
Dean's Scholar UIC Medicinal Chemistry Department Nominee
W.E van Doran Scholar Award
Graduate Student Council Travel Fellowship Award, University of Illinois at Chicago
Society for the Study of Reproduction, Larry Ewing Memorial Trainee Travel Award
UIC University Graduate Fellowship
Ann M. Verville Scholar's Award
Willian J. Trautman Award In Physical Chemistry
Phi Beta Kappa
American Chemical Society Undergraduate Award in Analytical Chemistry
Midwest Society of Cosmetic Chemist Annual Scholarship Award Recipient
Beloit College World Affairs Scholar

**MEETINGS AND PRESENTATIONS**

Glaxo Smith Kline internal meeting project presentation (oral)
Glaxo Smith Kline Sharing Science Symposium Presentation (poster)
University of Minnesota, Illinois, Kansas, Iowa Medicinal Chemistry annual meeting (oral)
National Center for Complementary and Alternative Medicine (NCCAM) annual lecture. NIH, MD (poster)
UIC Reproductive Endocrinology Meeting (oral)
3rd and 4th Annual Illinois Symposium on Reproductive Sciences. Chicago, IL (poster)
The Society for the Study of Reproductive Sciences. Penn State, PA (poster)
UIC College of Pharmacy Research Day (poster)
American Chemical Society National Meeting. Salt Lake City, UT (poster)

**PUBLICATIONS AND PAPERS**

**May Fern Toh, Burdette, J.E.** (2010) Botanical Mechanisms of Action. Fitoterapia 82(1):67-70 2010

**May Fern Toh, Johann Sohn, Ping Yao, Shao-Nong Chen, Judy Bolton and Joanna Burdette.** Biological Characterization of Non-Steroidal Progestins from Botanicals for Women's Health. *Steroids* 2012 Jun;77(7):765-73, 2012

**May Fern Toh, Emma Mendonca, Sharon L. Eddie, Michael Endsley, Daniel Lantvit, Leen J. Blok, Pavel A. Petukhov, and Joanna E. Burdette.** Kaempferol Exhibits *In Vivo* Progestogenic Effe*cts* in an Ovariectomized Rat Model. *Nutrition and Cancer: An international Journal (under review),* 2013