Progesterone receptor modulators in breast cancer

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Abstract: Breast cancer has been treated successfully with selective estrogen receptor antagonists (SERMs) such as tamoxifen, receptor-depleting agents such as fulvestrant, and aromatase inhibitors such as anastrozole. Selective progesterone receptor modulators (SPRMs or PRMs) have not been studied as much and are currently under investigation for inhibition of mammary carcinogenesis in animal models and breast cancer prevention trials in women. They might follow tamoxifen and aromatase inhibitors in the adjuvant treatment of breast cancers with acquired resistance. These uses have not provoked ground-breaking progress or studies and PRMs do not have a high profile. Most in vitro and in vivo studies indicate that PRMs preferentially suppress cell proliferation and also induce apoptosis. In this review we summarized the data on the effects of PRMs and particularly of the antiprogestins RU-486 (mifepristone) and CDB-4124 (Progenta, Proellex or telapristone acetate) on breast cancer models. Both agents have been employed in preclinical and clinical studies for prevention and treatment of breast cancer. This author believes that PRMs should be investigated more intensely.

Key words: Progesterone receptor, PRMs, antiprogestins, mammary carcinogenesis, biomarkers

1. The case for progesterone receptor modulators (PRMs) for breast cancer

PRMs have shown benefit in women with uterine leiomyomas and endometriosis and those successes in clinical trials have led to the realization that the antiproliferative and pro-apoptotic actions seen may find use in other progesterone-sensitive tissues. As a prime developer of a lead PRM, we (Repros Therapeutics) soon realized that those young women who used a PRM for treatment of fibroids or endometriosis would face an extended treatment period of months, if not years. Those women needed assurance that the use of these hormonal agents was not going to increase their risk for breast cancer, particularly so in an era when seemingly innocuous treatments such as hormone replacement therapy (HRT) with progestins have been shown to increase the risks of breast cancer (Rossouw et al., 2002; Chlebowski et al., 2003; Rohan, et al., 2008). Any possibility that risks could be mitigated and thus prevention realized would be most welcome, to say the least, for women taking a new PRM. Indeed, this ‘new world’ of treatments would present a new way of looking at the problem. This paradigm requires giving up a purely estrogen-based or estrocentic view of breast cancer in favor of one that introduces other hormones as drivers. Progesterone appears to be one of those drivers but peptide growth factors including EGF and IGF-1 need to be considered alongside steroids (Dressing and Lange, 2009). We have always known that steroid hormone-responsive therapies have not been enough and the current evaluation of new disease usually requires the evaluation of Her2/neu status, nodal involvement, tumor stage and grade, some assessment of proliferation such as Ki67, and other markers with many patients opting for an analysis of tumor markers such as Oncotype DX. Luminal A is the term we now use to distinguish those tumors with the best chance for antihormone treatments, although the presence of Her2/neu (Luminal B) adds an additional layer of treatment for tumors that appear to be hormone-responsive. The coming age of individualized medicine for breast cancer would seem to require the use of multiple markers.

We are accustomed to the linkage of breast cancer with estrogen and its treatment with agents that oppose estrogen. This has been a tremendous medical advance for those women whose breast cancers present as estrogen receptor positive (ER+) status at biopsy and surgery. It is known that progesterone receptor presence (PR+) is estrogen-regulated and its function in normal and tumor cells requires estrogen and ER signaling (Chabbert-Buffet et al.,

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2. The introduction of PRMs

The relative lack of PRMs may be one reason that clinical progress has been relatively slow. The initial antiprogestin was RU-486 (mifepristone), which was synthesized in the early 1980s by Roussel-Uclaf and described by Philibert in meetings abstracts in 1981 (Philibert et al., 1981) and 1982 (Philibert et al., 1982) and in a monograph in 1984 (Philibert, 1984). In comparison, the synthesis of tamoxifen was in 1964 and its properties were described by 1966 (Harper and Walpole, 1966). The extensive antiglucocorticoid action of RU 486 was recognized early. Nevertheless, clinical uses were quickly shown by Baulieu's group (Herman et al., 1982). An initial indication was pregnancy termination and that use was constrained by ethical and political opposition that soon found its way into the mainstream media (Baulieu, 1994). This did much to delay research and development. An early worker characterized this as 'political chemistry' (Hogden, 1991). The search for new compounds was joined by the labs of C.E. Cook at Research Triangle Park and by the European firms Schering AG and Onganon. Noteworthy early compounds were RTI 3121-012 (CDB-2914), ZK 98 299, and ORG 31167, among others. The caution displayed by major American pharmaceutical companies in the 1990s over both the chemistry and the issue of pregnancy termination seemed to delay further invention on their part and we at Repros Therapeutics (then Zonagen, Inc.), as a small company, were able to move into the space later with less competition. Another complication was that the first compounds were also antiglucocorticoids and substantial work was done in synthesizing these mixed steroids and finding those with activity on one receptor or the other, i.e. dissociated binding and biological activity. By 1994, there were many potential PRMs. This entire story may be best appreciated through a supplement issue to Human Reproduction and a monograph (Beier and Spitz, 1994) published in 1994 based on a symposium held at Mohonk Mountain, New York, in the summer of 1992.

It was recognized early that antiglucocorticoid activity was unwanted in a potential drug to be used for antiprogesterational effects. As pointed out early by Horwitz (Horwitz, 1993), lower antiglucocorticoid activity can enhance the usefulness of any drug by allowing higher dosage. Thus, the early best candidates were judged against both activities. As an example, it has been shown that RU 486 was nearly a 10-fold better binder to the glucocorticoid receptor (hGR) than to the hPR and a 3–4-fold better binder to the hGR than dexamethasone (Kloosterboer et al., 1994). In contrast, he found that ZK 98 299 (onapristone or ZK 299) bound the hGR less avidly but with an approximately 5-fold selectivity for hGR over hPR. Nevertheless, both compounds went into clinical trials. In the case of ZK 299, phase II trials were terminated early due to liver toxicity found by liver function tests (Robertson et al., 1999). We at Repros Therapeutics have also found liver toxicity at high doses as well but believe that an optimal dosing regimen in terms of oral dose concentration as well as an appropriate schedule is required.

A spin-off of the Schering AG work on PRMs was the compound Asoprisnil, which was developed through TAP (Chen et al., 2006). Although its development appeared to be intended for fibroids and endometriosis, problems with uterine bleeding and endometrial changes interfered with its further development in a 2005 phase III study. It is unknown if there is to be further development for oncologic indications.

Another PRM compound developed early was CDB-2914 (Xu et al., 2005). A 2012 report in the New England Journal of Medicine (Donnez et al., 2012) highlighted a European study that showed efficacy in women with fibroids. This drug is approved for emergency contraception although it is not certain if it may be developed for breast cancer.

The compound CDB-4124 was synthesized at the Southwest Foundation for Biomedical Research under a
contract from the Contraceptive Research Branch, Center for Population Research, NICHD, and studied initially under a contract to Bioquals, Inc. Its chemical similarity to RU 486 is through its nor-19 steroidal backbone and 11b substitution. It differs at the C-21 position (Figure 1). Another compound from this first set was CDB-4059. These compounds and others were patented as composition-of-matter. In 1999, Repros Therapeutics acquired rights to at least 44 compounds that were 21-substituted. One of these became the lead compound, CDB-4124. We determined early that a mono-demethylated metabolite, CDB-4453, was always found along with the parent in animal and human serum. The metabolite was found to be active relative to the parent. Neither compound demonstrated significant antiglucocorticoid activity but showed high activity as an antiprogestin. The comparisons of these 2 compounds and RU 486 have been shown by Attardi et al. (2002, 2004).

3. PRMs in breast cancer trials

Early progesterone antagonists showed greater antitumor activity than tamoxifen or high-dose progesterone agonists and synergized with antiestrogens (Klijn et al., 2000). Clinical data are meager but at least 5 studies have used antiprogestins in women with breast cancer. One small clinical trial in women with metastatic breast cancer has shown that RU 486 has some efficacy against the disease (Mandelonde, 1987). Two other studies employed RU 486 (Romieu et al., 1987; Klijn et al., 1989) as a second-line adjuvant therapy. These first 3 trials demonstrated a complete or partial response of 12% and stable disease in 46% of 33 patients. Two more phase II trials used mifepristone (Perrault et al., 1996) or onapristone (Robertson et al., 1999) as first-line therapy. Perrault et al. (1996) reported values for partial response and stable disease similar to the 3 mentioned above. Interestingly, Robertson et al. reported a partial response in 56% of patients and stable disease in 11% but symptoms of adrenal insufficiency (Robertson et al., 1999). This was perhaps not totally unexpected; a rise in serum cortisol was seen when RU 486 was used to treat patients with meningiomas (Grunberg, 1994) and trials of onapristone in women have been closed down due to liver toxicity. Indeed, potential antiglucocorticoid effects counterindicate the long-term use of RU 486. In a study of women treated with RU 486 for endometriosis (Kettel et al., 1991), patients were anovulatory as expected (Liu et al., 1987) but serum estrogen levels remained consistent with midfollicular phase and there was evidence for hypercortisolemia and ACTH excess. Although RU 486 can serve as the paradigm for this class of antiprogestins, outcomes must be measured against possible antiglucocorticoid effects (Bertagna et al., 1984). In a study of 11 postmenopausal women with advanced breast cancer RU-486 induced a short-term clinical response in 1 patient and stable disease in 6 others (Bakker et al., 1990). The side effects of RU-486 in this study were mostly related to antiglucocorticoid properties of the drug and increased serum estradiol levels. Previous studies suggested that high dose RU-486 can elevate serum estradiol and progesterone levels, impacting endometrial cell proliferation (Kettel et al., 1991).

CDB-4124 and its metabolites appear to show less antiglucocorticoid activity compared to RU-486, suggesting an advantage in future clinical studies (Attardi et al., 2002, 2004). Despite the beneficial effects seen in women with uterine fibroids (Wiehle et al., 2008) and endometriosis (Spitz, 2009; Spitz et al., 2009), rare, idiosyncratic liver reactions at high doses (unpublished data) suggest that lower doses will be required for clinical studies.

Figure 1. Structures of RU 486 and CDB-4124.
4. PRMs in in vivo experimental models

Classical experiments have shown DMBA (dimethylbenz(a)-anthracene)-induced breast tumors are inhibited by ovariectomy and maintained by estrogen and progesterone (Huggins et al., 1962; Welsch, 1985). Progestins increase the incidence of spontaneous mammary tumors in dogs (Frank et al., 1979) and mice (Nagasawa et al., 1988). We found that progesterone can promote DMBA-induced mammary carcinogenesis in rats (Wiehle et al., 2007). Using progestin receptor knock-out (PRKO) mice, PR has been shown to be specifically important for DMBA-induced mammary carcinogenesis (Bakker et al., 1989). When RU-486 was used in DMBA-treated rats and in mice that spontaneously developed ER+ mammary tumors, a significant reduction in tumor incidence, multiplicity, and size was observed (Michna et al., 1989; Chatterton et al., 2002). In a separate study on the effects of RU-486 on DMBA-induced mammary tumors in rats, a reduction in tumor multiplicity was found in 90% of animals versus 75% of animals treated with tamoxifen (Bakker et al., 1989). The combination of both agents further increased their antitumor potential. The antiprogestins Org 31710 and Org 31806 (Kloosterboer et al., 1994) appear to be more potent than RU 486. In the case of ZK 299, its full agonism may contribute to its potency. The combination of tamoxifen and RU 486 is a potent suppresser of tumor growth analogous to chemical castration or ovariectomy (Bakker et al., 1987). The SERM 164,384 enhances the ability of onapristone to reduce tumors in the MXT mouse mammary tumor model (Nishino et al., 1994).

Interestingly, Dr Eva Lee of the University of California, Irvine, conducted studies demonstrating that progesterone provides growth signals for breast tumors in mouse models of spontaneously developing tumors carrying Brca-1 mutations (Poole et al., 2006). BRCA1, the protein product of the breast cancer susceptibility gene, is known to interact directly with ER and PR and modulates both ligand-dependent and ligand-independent transcriptional activities of ERα and PR. Mice in which both alleles of Brca-1 and p53 have been knocked out in the mammary tissue exhibited abnormal proliferation of mammary epithelial cells with increased branching and alveolar formation, and 100% develop breast tumors in a median of 6.6 months. Poole et al. (2006) found the PR levels were markedly elevated in the mutant breast cells due to failure of the E3 ligase activity of BRCA1 and subsequent proteosomal degradation of PR. Treatment of mice with mifepristone for 5 weeks completely blocked tumor formation. These groundbreaking animal studies, epidemiologic data, and observations of the clinical behavior of ER+ positive breast cancers including the observation that aromatase inhibitors have heterogeneous effects on circulating progestins argue for the investigation of combined estrogen and progesterone signaling blockade. Translational clinical studies to test the central hypothesis that combined ER and PR signaling blockade is safe and clinically effective in treating breast cancer have not been performed. A recent study has shown that the BRCA1 protein inhibits the binding of PR to certain progesterone response elements without an effect of the binding of ligand to PR (Katiyar et al., 2009).

Our results in the DMBA tumor model (Wiehle et al., 2007) agree with these trends and extend these data: PRMs CDB-4124 and CDB-4059 as well as RU-486 shrank established tumors and prevented the appearance of new tumors within the 28 day window of treatment (Table 1). Rats were given DMBA at 50 days of age. Animals were weighed and palpated for any sign of lesions or swellings. Possible tumors were measured in 2 dimensions. If suspect tumors grew to a size of 10 mm, the individual animal was randomized into 1 of 15 groups. We treated the animals for 28 days. Rats treated with vehicle alone had the original tumor used to activate their treatment regimen and an average of 1.67 more tumors, a total of 2.67 tumors per rat. The addition of progesterone increased the average number of tumors per rat to almost 5. CDB-4124 reduced tumor number. Since each animal was enrolled into treatment randomly based on finding 1 tumor of a given size, we conclude that treatment with antiprogestins at 3 of the 4 top dose levels reduced the number of tumors by suppressing the formation of new tumors during the 28-day treatment period. The tumor-inducing effects of progesterone overwhelmed the tumor suppressing effects of CDB-4124 if the dose of progesterone exceeded the dose of CDB-4124 by a factor of 5 or more. We measured tumors weekly during the trial. The data were corrected to exclude the nonmalignant tumor types. Tumors that increased in cross-sectional area by at least 33% were considered to be growing. Those that decreased by approximately 33% were considered to be regressing. Others were considered stable. Progesterone treatment resulted in aggressively growing tumors. The proportion of tumors regressing in the group given progesterone (8%) is statistically the same as that of the controls but the proportion of tumors growing was 10-fold higher (80%). On the other hand, RU 486 treatment led to no real difference in the proportion of tumors growing or regressing. CDB-4124 at 10 mg/kg body weight reduced the proportion of growing tumors but appeared to exert its prime effects by increasing the proportions of regressing tumors. At high doses, CDB-4124 might have some agonist activity with respect to growth, albeit not approaching that of progesterone.

We inferred that all 3 PRMs (RU 486, CDB-4124, and CDB-4059) belong to the same class of agents. However, at equal dose levels, CDB-4124 and CDB-4059 were more
effective than RU 486 in provoking tumor regression. The order of tumor-suppressing activity by these criteria was CDB-4059 ~ CDB-4124 > RU 486. When CDB-4124 is given alone at moderate concentrations or in excess of progesterone, its effects predominate on growth and regression. When progesterone is given alone or in excess over CDB-4124 its effects are strongly growth-enhancing. The effects of CDB-4124 are consistent with decreasing the size of DMBA-induced mammary tumors in the rat. CDB-4059 may be as effective. These effects are mimicked by effects on proliferation and apoptosis (see section below).

Christov et al. used a rat MNU model to show predicative value in breast cancer prevention/therapy for compounds that are both antiproliferative and proapoptotic (e.g. SERMs and AIs) and as compared to castration and an aromatase inhibitor (Christov et al., 2007). Tamoxifen had proven itself useful in this model (Christov et al., 2003). We treated nascent mammary tumors with CDB-

<table>
<thead>
<tr>
<th>Group</th>
<th>TX</th>
<th>Animals with tumors</th>
<th>Tumors</th>
<th>Tumor weight</th>
<th>Effects on weight</th>
</tr>
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<tbody>
<tr>
<td></td>
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<tr>
<td>1</td>
<td>No TX vehicle</td>
<td>12 / 13</td>
<td>92</td>
<td>32</td>
<td>4.7 g 1.91 G 0.80</td>
</tr>
<tr>
<td>2</td>
<td>No DMBA vehicle</td>
<td>0 / 10</td>
<td>0</td>
<td>0</td>
<td>0 g 0 G 0</td>
</tr>
<tr>
<td>3</td>
<td>RU486 10</td>
<td>13 / 14</td>
<td>93</td>
<td>27</td>
<td>2.47 g 1.19 G 0.16 P = 0.012</td>
</tr>
<tr>
<td>4</td>
<td>P4 10</td>
<td>10 / 10</td>
<td>100</td>
<td>49</td>
<td>7.34 g 1.5 G 0.5 P = 0.42</td>
</tr>
<tr>
<td>5</td>
<td>4124 20</td>
<td>13 / 13</td>
<td>100</td>
<td>20</td>
<td>4.71 g 3.4 G 0.125 P = 0.066</td>
</tr>
<tr>
<td>6</td>
<td>4124 10</td>
<td>10 / 11</td>
<td>91</td>
<td>18</td>
<td>0.48 g 0.26 G 0.075 P = 0.0003</td>
</tr>
<tr>
<td>7</td>
<td>4124 2</td>
<td>11 / 12</td>
<td>92</td>
<td>17</td>
<td>4.61 g 2.98 G 0.09 P = 0.012</td>
</tr>
<tr>
<td>8</td>
<td>4124 1</td>
<td>9 / 11</td>
<td>82</td>
<td>16</td>
<td>0.94 g 0.53 G 0.17 P = 0.0009</td>
</tr>
<tr>
<td>9</td>
<td>4124 0.1</td>
<td>12 / 12</td>
<td>100</td>
<td>29</td>
<td>5.1 g 2.26 G 0.77 P = 0.99</td>
</tr>
<tr>
<td>10</td>
<td>4124/P4 20/10</td>
<td>8 / 10</td>
<td>90</td>
<td>18</td>
<td>0.92 g 0.46 G 0.18 P = 0.005</td>
</tr>
<tr>
<td>11</td>
<td>4124/P4 10/10</td>
<td>10 / 12</td>
<td>83</td>
<td>14</td>
<td>3.82 g 2.73 G 0.185 P = 0.013</td>
</tr>
<tr>
<td>12</td>
<td>4124/P4 2/10</td>
<td>10 / 11</td>
<td>91</td>
<td>34</td>
<td>5.8 g 1.96 G 0.45 P = 0.19</td>
</tr>
<tr>
<td>13</td>
<td>4124/P4 1/10</td>
<td>14 / 15</td>
<td>93</td>
<td>45</td>
<td>4.28 g 1.19 G 0.49 P = 0.27</td>
</tr>
<tr>
<td>14</td>
<td>4124/P4 0.1/10</td>
<td>11 / 11</td>
<td>100</td>
<td>41</td>
<td>6.16 g 1.65 G 0.49 P = 0.66</td>
</tr>
<tr>
<td>15</td>
<td>4059 10</td>
<td>9 / 11</td>
<td>82</td>
<td>12</td>
<td>1.24 g 0.93 G 0.17 P = 0.10</td>
</tr>
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</table>
4124 in this breast tumor model of prevention (Wiehle et al., 2011). In our case, female Sprague Dawley rats at 50 days of age were intravenously injected with N-methyl-N-nitrosourea (MNU). Randomly selected animals were placed into 2 arms of the study (20 rats per arm) and received CDB-4124 at a high (30 mg) or low (3 mg) dose in the form of a subcutaneous pellet beginning 5 days after MNU. A third arm received a placebo pellet. The treatment was for 90 days. The occurrence of mammary tumors was monitored by palpation, starting 4 weeks after carcinogen administration. Paraffin tissue sections of tumors were stained for identification of tumor morphology as well as with antibodies for assessment of ER, PR, and proliferation- and apoptosis-related biomarkers. Blood samples were taken for the determination of estrogen and progesterone.

As shown in Table 2 and Figure 2, treatment with CDB-4124 resulted in dose-dependent reduction in mammary gland tumor size and multiplicity. CDB-4124 prevented the appearance and reduced the size of tumors in the 90-day period following carcinogen insult. The higher dose level of CDB-4124 was more effective. CDB-4124 treatment had no effect on the serum estrogen level in the animals compared to controls but the highest dose of CDB-4124 significantly reduced circulating progesterone by 45%. Thus, the effects were not due to estrogen suppression.

In the course of drug development, Repros Therapeutics conducted a 2-year carcinogenesis study (MPI1155-006). The mammary gland of animals treated for 2 years (the mean lifetime) with CDB-4124 showed fewer tumors and abnormalities than controls. However, placebo-treated animals developed spontaneously hyperplastic and premalignant mammary lesions, as well as cystic formations (Wiehle et al., 2011). This was essentially the opposite of the aim of this carcinogenicity study, which was to disclose overt tumor development across the animal organs and lifetime. We reviewed slides for mammary gland histology, proliferation (Ki-67 staining), and apoptosis markers from these tissues. Based on these data, CDB-4124, when given for 2 years to female Sprague Dawley rats, remodeled mammary morphology by: 1) decreasing lobular differentiation of mammary epithelial cells, as shown by a prevalence of single lobules; 2) stimulating cystic formations in the mammary gland; and 3) suppressing lobular proliferation. These results in the mammary gland are reminiscent of those seen in the mammary gland of PRKO mice (Bakker et al., 1989). CDB-4124 also decreased proliferative activity of mammary epithelial cells (Table 3), but did not significantly affect apoptosis.

5. Evidence for the cellular mode of action: uterine proliferation and apoptosis

Under investigation is whether progesterone antagonist may induce apoptosis by affecting pro- or antiapoptotic proteins. This topic has been covered with respect to CDB-4124 in a recent review (Christov and Wiehle, 2012) and will be mentioned below for breast tissue. At the same time, we believe much may be gained by looking at the effects of PRMs on other progesterone-sensitive tissues such as those in the female reproductive tract.

Asoprisnil, a PRM with mixed progesterone agonist/antagonist activities, was able to reduce uterine leiomyoma volume in a dose-dependent manner in the presence of follicular phase estrogen (Chen et al., 2006). Studies in vitro on cultured human uterine leiomyoma cells and matched normal myometria showed that asoprisnil decreased the PCNA-positive rate and PCNA protein expression in cultured leiomyoma cells. Asoprisnil increased the TUNEL-positive rate, cleaved caspase-3, and cleaved poly (adenosine 5’-diphosphate-ribose) polymerase expression and decreased Bcl-2 protein expression in cultured leiomyoma cells. These effects were dose- and time-dependent. However, in cultured myometrial cells, asoprisnil did not affect cell proliferation and apoptosis.

CDB-2914, another SPRM, was also shown to increase apoptosis and suppress cell proliferation in leiomyomas (Xu et al., 2005). In that study cultured human uterine leiomyoma cells were subcultured for 120 h and then stepped down to serum-free conditions for 12, 24, 48, and 96 h in the absence or presence of graded concentrations of CDB-2914 between $10^{-6}$ and $10^{-9}$ M. Compared with untreated control cultures, CDB-2914 decreased the number of viable cultured leiomyoma cells and the PCNA-positive rate in those cells and increased the TUNEL-positive (apoptotic) cells in a dose-dependent manner. Western blot analysis revealed that treatment

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Table 2. CDB-4124 inhibits the incidence, multiplicity, and weight of mammary tumors.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose-mg</th>
<th>Animals-no.</th>
<th>Incidence-%</th>
<th>Multiplicity</th>
<th>Burden-g</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>20</td>
<td>85</td>
<td>3.0</td>
<td>2.60</td>
</tr>
<tr>
<td>2</td>
<td>3.0</td>
<td>20</td>
<td>60</td>
<td>2.2</td>
<td>0.62*</td>
</tr>
<tr>
<td>3</td>
<td>30.0</td>
<td>20</td>
<td>35*</td>
<td>1.1*</td>
<td>0.26*</td>
</tr>
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</table>

* significant (P < 0.05).
with CDB-2914 significantly decreased PCNA and Bcl-2 and increased cleaved caspase-3 and cleaved PARP expression in a dose-dependent manner, suggesting the involvement of apoptosis in leiomyoma growth inhibition and disintegration.

The effects of CDB-4124 on cell proliferation and apoptosis were assessed in cultured human uterine leiomyoma smooth muscle (LSM) cells and control myometrial smooth muscle (MSM) cells in matched uterine cells (Luo et al., 2010). CDB4124 (10⁻⁸ – 10⁻⁶ M) or vehicle were added to the culture medium for 24, 48, or 72 h. CDB-4124 significantly decreased PCNA antigen, the number of viable LSM cells, and the antiapoptotic protein Bcl-2. CDB-4124 also increased apoptosis related biomarkers cleaved polyadenosine 5′-diphosphate-ribose polymerase and the tumor suppressor Krüppel-like transcription factor 11 in a dose- and time-dependent manner in LSM cells. In matched MSM cells, however, CDB-4124 did not affect cell proliferation or apoptosis, indicating that this PRM selectively targets leiomyoma but not normal uterine smooth muscle cells.

Not all cell systems and conditions show these effects (Roeder et al., 2011). In order to investigate the role of apoptosis in mediating the effects of PRMs on uterine leiomyomas, PR+ fibroid cells, known to be capable of apoptosis, were grown to 80% confluence in serum containing and treated for 48 h with 0, 10, 100, or 1000 nmol/L CDB-4124. However, no evidence for increased apoptosis or elevated caspase-3, determined by Western blotting, was found. The reasons for this lack of effect in this system were not identified. It was also unexpected in the face of clinical and in vitro data to the contrary. It is possible that the cells employed were resistant to PRMs, although they were PR+. It is also possible that the progesterone activation required growth factor action rather than classical action through the PR. The lack of a coactivator specific for growth in these tumors due to culturing remains possible although an unsatisfying answer and one difficult to answer. Subtle changes at the cellular level can have major effects. The phosphorylation and sumoylation of PR by intracellular mechanisms can impact PR action (Daniel and Lange, 2009). Fibroids are understood to be clonal in nature and thus may vary from one cell line to another. In any case, this lack of effect may emerge for some patients under treatment for fibroids with a PRM. We know not all fibroids respond the same way. This may justify thoroughly characterizing any subject on a PRM with a heterogeneous response, i.e. if a subject with multiple fibroids shows a different response for her individual fibroids.

PRMs may affect not only the growth and progression of leiomyomas but also suppress clinical symptoms of endometriosis. However, little is known on the morphological base of endometrial changes and the biomarkers mostly affected. Recent studies by a panel of pathologists have found that women treated with various PRMs given at different doses for different time intervals develop cystic glandular formations previously not known. They also reported apoptotic cells in cystic formations, suggesting antiprogestins may induce apoptosis in endothelial cells and thus suppress their proliferation (Mutter et al., 2008). In a recent study 58 premenopausal women with endometriosis or uterine leiomyomas were treated for 3 or 6 months with different doses of CDB-4124. Endometrial biopsies were taken and were independently examined by 3 pathologists (Ioffe et al., 2009). They confirmed previous observations for cystic formations among endometrial glands and reported lack of endometrial hyperplasia or carcinomas in treated patients. The condition of months-long PRM treatment did not provoke a condition similar to unopposed estrogen but rather to what they characterized as PAEC, PRM-

**Table 3.** Effect of various agents on proliferation in cynomolgous monkeys.

<table>
<thead>
<tr>
<th></th>
<th>Epithelium</th>
<th>Stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>TXT</td>
<td>BrdU-%</td>
<td>BrdU-%</td>
</tr>
<tr>
<td>Control</td>
<td>10.0 ± 2.5</td>
<td>2.6 ± 0.6</td>
</tr>
<tr>
<td>Lupron</td>
<td>3.1 ± 0.8*</td>
<td>2.2 ± 1.0</td>
</tr>
<tr>
<td>RU 486</td>
<td>12.6 ± 1.8</td>
<td>3.1 ± 1.0</td>
</tr>
<tr>
<td>CDB-4124</td>
<td>2.1 ± 2.2*</td>
<td>1.1 ± 0.25*</td>
</tr>
</tbody>
</table>

* P < 0.05, compared to controls (t-test).
associated endometrial changes, characterized by rare mitoses, atrophic/cystic structures, and apoptotic bodies.

We have compared the effect of RU486, CDB412, and Lupron (a GnRH agonist often used to treat human endometriosis) on the endometrium of cynomolgous monkeys (Macaca fascicularis). This was done to model endometriotic lesions of women and disclose effects of PRMs. The peritoneal cavity of each monkey received minced pieces of the monkey's own endometrium. Certain of these fragments attached and established themselves in the cavity as lesions resembling endometriosis in size and appearance. Groups of 7 monkeys were treated orally for 36 weeks with CDB-4124 at 3 different doses of RU-486 or with placebo. Another group received Lupron IM. Animals were inspected visually after 18 and 36 weeks of treatment and sacrificed at the end of the study. Monkeys receiving Lupron, RU486, and CDB-4124 clearly stopped cycling and had lowered levels of progesterone. Lupron decreased serum estrogen compared to control and pretreatment follicular phase levels but serum estrogen levels were not different in the monkeys receiving CDB-4124 and RU 486. Although showing little change in size, lesions in monkeys treated with CDB-4124 differed in appearance, occurring often as clear cysts. Three monkeys in each group were injected with BrdU within 24 h of sacrifice to assess tissue proliferation. Full thickness uterine sections were stained and examined microscopically for evidence of proliferation in terms of the % cells incorporating BrdU (Table 3). Lupron and CDB-4124 decreased proliferation in the epithelial cells of the endometrium and CDB-4124 also decreased stromal proliferation. RU-486 did not decrease proliferation in either cell type. RU-486 and CDB-4124 increased apoptosis in the epithelial cells of the endometrium and CDB-4124 also increased apoptosis in the breast (Table 4). Lupron did not alter apoptosis in either cell type. No treatment appeared to alter the stroma. This dichotomy between RU 486 and CDB-4124 in terms of proliferation may indicate a functional difference between these 2 antiprogestins.

6. Cellular mode of action: mammary proliferation and apoptosis

By employing MDA-MB-231 cells that are ER– and PR–, researchers transfected cells with PR-complementary DNA (Lin et al., 1999). They found that PR, in the absence of ER, affected cell morphology and cell differentiation biomarkers. Progesterone induced E-cadherin, cytokeratins, vimentin, and STAT 1, 3, 5a, and 5b expression, all cell differentiation biomarkers. The more interesting effect was the negative effect on growth. Follow on studies (Lin et al., 2003) indicated inhibition of the p42/44 MAPK pathway, an effect unlike previous work they cited where progestin activated MAPK. This suggested a set of pathways for regulation that included MAPK-cyclin D1. The authors thought the fact that these cells were ER– may have been a major contributor to the effects seen, an effect that implied cross-talk between ER and PR in a cellular system with converging signals.

The use of xenographs of breast cancer is a way of looking at human cancer cells in an animal model. Liang et al. (2007) used BT-474 and T47D cells without Matrigel as xenographs in nude mice. These cells will regress soon after inoculation but that regression is held up if progesterone or medroxy-progesterone acetate is given. The effects are sensitive to RU 486. Most interesting is the apparent dependency on VEGF.

When given by subcutaneous injection over a wide range of concentrations, CDB-4124 suppressed tumor growth of DMBA-induced mammary tumors in rats, dose-dependently as already described (Wiehle et al., 2007). Progesterone, on the other hand, stimulated DMBA-induced mammary carcinogenesis and tumor growth and this was associated with increased cell proliferation and decreased apoptosis, suggesting that PRMs may act as inhibitors of apoptosis. CDB-4124 significantly decreased cell proliferation in DMBA-induced mammary tumors, as evaluated by Ki-67 antibody reactivity (Table 5).

In order better to understand cellular mechanisms of CDB-4124-induced inhibition of mammary carcinogenesis, proliferation activity and apoptosis were examined in mammary tumors from the Sprague Dawley rats treated with N-methyl-N-nitrosourea (MNU) (Wiehle et al., 2011). As mentioned above, treatment increased latency, and, as shown in Table 2, decreased multiplicity (Figure 2), and tumor burden. Decreased size of mammary tumors was a consequence of decreased cell proliferation and induced apoptosis. Apoptosis was evaluated by TUNEL assay and cell proliferation by Ki-67 antibody that recognized the cells in all phases of the cell cycle. As shown in Table 6, CDB-4124 significantly suppressed Ki-67 positive cells in mammary tumors, from

Table 4. Effect of various agents on apoptosis in cynomolgous monkeys.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Uterus Epithelium</th>
<th>Uterus Stroma</th>
<th>Breast Epithelium</th>
<th>Breast Stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.2 ± 0.1</td>
<td>0.7 ± 0.2</td>
<td>0.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Lupron</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>1.4 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>RU 486</td>
<td>0.5 ± 0.1*</td>
<td>0.5 ± 0.1</td>
<td>1.2 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>CDB-4124</td>
<td>0.5 ± 0.2*</td>
<td>0.5 ± 0.1</td>
<td>2.6 ± 0.9*</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05, compared to controls (t-test).
30.5 ± 5.4% in the placebo group to 25.4 ± 14.4% in the low-dose and further to 10.3 ± 4.5% in the high-dose group of treated animals. Both doses of CDB-4124 induced apoptosis in mammary tumors, where 0.7 ± 0.4% apoptotic cells were detected in the control group vs. 1.4 ± 0.8% in the low-dose and 1.6 ± 0.8%, in the high-dose group. These data correlated with decreased proportion of PR+ tumor cells and with decreased serum progesterone. CDB-4124 did not affect serum estradiol. We examined PR expression in mammary tumors by immunohistochemistry. CDB-4124 decreased the proportion of PR+ cells from 48 ± 11% in placebo treated to 32 ± 12% in CDB-4124 (30 mg/kg) treated animals. Although trending lower, CDB-4124 did not significantly affect the proportion of ER+ cells in the samples.

Other work has shown the link between PRMs and apoptosis. CDB-4124 suppressed the growth of ER- and PR-expressing T47D cells and induced apoptosis (Wiehle et al., 2011). Cells were treated with 0, 0.1, 1.0, and 10.0 µM CDB-4124 for 3 days or 6 days, and cell number in triplicate was determined by cell counter. In addition, caspase 3 as a biomarker of apoptotic cell death was determined in cells grown on cover slips. CDB-4124 at 1.0 µM and 10.0 µM suppressed cell growth in a dose-dependent manner and the high dose induced cell death in both 3- and 6-days treated cells.

Women with leiomyoma treated with 50 mg of RU-486 every second day for 3 months underwent fine needle breast biopsies before initiation and after termination of treatment (Engman et al., 2008). A significant reduction in proliferating breast epithelial cells (Ki-67 positive) was observed in RU-486 treated vs. placebo treated patients, suggesting that antiprogestin treatment can prevent the development and progression of ER+ and PR+ breast cancer by inhibiting mammary epithelial cell proliferation. Apoptosis was not examined in these patients.

7. Humoral mode of action: serum steroids

One of the observations we made purposefully was the effect of CDB-4124 on serum steroid levels. A frequent finding was the presence of estrogen but a loss of progesterone along with cycling in animals and menstrual cycling in women. In an unpublished safety study (ZP-204), we determined that normal young women who were being assessed for evidence of liver toxicity provided little evidence for LH surges or serum progesterone over 10 weeks of treatment dependent on dose. There was some evidence of cycling at the lowest oral daily dose, 1 mg/day, but none at daily doses of 3 mg, 6 mg, 9 mg, or 12 mg. Serum progesterone in women at visit 12, i.e. in the last week of active dosing, as compared to the follow up (FU) month when they were no longer taking the drug, demonstrated a major difference (Figure 3). Even though these women could no longer be considered cycling by vaginal bleeding or LH surges, it could be argued that we “missed” the luteal phases at visit 12. This seems unlikely to be true for all groups and all individuals in the face of the FU subjects who demonstrated serum progesterone levels.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>% cells positive</th>
<th>Compared to controls (t-test)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>13.5 ± 7.8</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>RU 486 (10 mg/kg)</td>
<td>12.9 ± 7</td>
<td>P = 0.85</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>P4 (Progesterone) (10 mg/kg)</td>
<td>25.7 ± 5.8</td>
<td>P = 0.0007</td>
<td>9</td>
</tr>
<tr>
<td>6</td>
<td>CDB-4124 (10 mg/kg)</td>
<td>5.1 ± 4.2</td>
<td>P = 0.00</td>
<td>7</td>
</tr>
<tr>
<td>11</td>
<td>CDB-4124 + P4 (10 + 10)</td>
<td>15.5 ± 12.2</td>
<td>P = 0.66</td>
<td>8</td>
</tr>
</tbody>
</table>

ANOVA = 0.0001  [P4 > Control, RU486, CDB-4124 + P4 > CDB-4124].

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose mg/kg</th>
<th>Animals no.</th>
<th>Ki-67 LI%</th>
<th>P</th>
<th>Apoptosis LI-%</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>19</td>
<td>30.5 ± 7.1</td>
<td>0.7 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proellex</td>
<td>3.0</td>
<td>20</td>
<td>25.4 ± 14.4</td>
<td>1.4+0.8</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Proellex</td>
<td>30.0</td>
<td>20</td>
<td>10.3 ± 4.5</td>
<td>0.001</td>
<td>1.6 ± 0.8</td>
<td>0.01</td>
</tr>
</tbody>
</table>

The differences in the values are significant (P < 0.05) as compared to those of the control animals (Student-Fisher t-test).
typical of the luteal phase and with a mean progesterone level in excess of 3 ng/mL. Other studies showed similar effects at higher doses.

While we were not surprised at the effects at the higher doses, the efficacy at the lower doses was unexpected. This suggested a sensitivity of CDB-4124 towards provoking meaningful changes in the gonadotropin hormones that are responsible for cycling and for hormones, especially progesterone from the corpus lutea. This had been seen in early studies of RU 486 (Ortmann et al., 1994). The effects on cycling suggest a central effect on the hypothalamus/pituitary with major effects that result in acyclicity. The hypothalamus receives multiple inputs and the KNDy neurons of that tissue possess both ER and PR receptors, allowing them to respond to both up- and down-regulatory signals and control LH release by the pituitary by way of kisspeptins and/or GnRH (Skorupskaite et al., 2014).

We believe that the effects on serum progesterone constitute an additional source of effects on sensitive tissues. This drug-induced hormonal suppression broadly affects progesterone action. PRMs should be more effective when the level of ovarian progesterone is low. Thus, a PRM like CBD-4124 attenuates its own effects by reducing the competition from endogenous progesterone.

8. Conclusions

We would propose a second line adjuvant therapy for patients with hormone-dependent breast cancer for women who fail estrogen-based therapies. That could be composed of the use of a progesterone antagonist such as CDB-4124. This is supported by the DBMA results in rats and by the studies demonstrating effects on proliferation and apoptosis in many systems. CDB-54124 when used alone or in combination with other growth factor or cell-

signal-modifying agents could break out of the current dilemma where estrogen therapies fail because tumors have circumvented antiestrogen or estrogen depletion treatments. First line therapy for women with ER−/PR+ tumors should be considered as well, even though the number of women with such tumors is small. Window-of-opportunity trials such as currently underway at Northwestern under Seema Khan (NU 12B09, Pre-surgical Phase IIB Trial of Oral CDB-4124 vs. Placebo in Women with Stage I-III Primary Breast Cancer) can highlight possible efficacies while mitigating risk. Neoadjuvant use may find a place if strong antiproliferative effects can be documented.

We would propose a chronic prevention paradigm. The lesson taught here is from both the DMBA and the MNU studies in rats where potential tumors seem to be less likely to be detected by difference between groups. The effects seen on the mammary gland itself, on hormones, and proliferation/apoptosis are provocative and may be predictive. Taken together, these data indicate that CDB-4124, among others, can suppress the development of precancerous lesions and ER+ mammary tumors in rats, and may have implications for prevention and treatment of human breast cancer. Women treated with PRMs for benign gynecologic indications (fibroids and endometriosis) for relatively long periods are a set of individuals who may be monitored.

We would propose the inhibitory effect of CDB-4124 on mammary carcinogenesis to be a consequence, in part, of decreased progesterone action in the mammary gland. Perhaps this was shown already by the changes in ductal-lateral branching or stem cells, which is promoted by physiological levels of serum progestins. During pregnancy the high blood levels of progesterone stimulate ductal-lateral branching, leading to lobular development and milk production. This has been supported by recent data on BRCA1/p53-transgenic mice that have shown that RU-486 suppressed mammary carcinogenesis by inhibiting ductal-lateral branching and lobular differentiation of mammary epithelial cells (Poole et al., 2006). These data also suggest that patients with mutations in BRCA1 may benefit from antiprogestin therapy in breast cancer prevention and treatment studies. In a different study, PRKO mice have also shown distinctive mammary gland architecture with the presence of ducts, but lack of alveoli and lobules (Bakker et al., 1989). With the cessation of breast feeding and drop in the progesterone circulation level, apoptosis plays a critical role in disintegration of these structures. Castration of mice and rats similarly induces apoptosis and reduction of mammary lobular structures.

9. New Directions

Future studies should also address the role of CDB-4124 alone, or in combination with tamoxifen or other SERMs,
with agents like fulvestrant, and aromatase inhibitors on the modulation of ER and PR signaling. The potential involvement of specific coactivators and corepressors will need to be pursued (Katzenellenbogen et al., 1996) as well as the challenging proposals that PR can work at the same sites as ER or cross-talk with the ER or other nuclear receptors (Daniel et al., 2014; Lin et al., 2003; Vicent et al., 2006). The involvement of peptide growth factors such as EGF and IGF-1 needs to be addressed (Dressing and Lange, 2009). Interaction with the membrane PRs (Charles et al., 2010) that provoke early responses is entirely open to investigation. The development of resistance in ER+ breast carcinomas after treatment with tamoxifen may offer additional possibilities for clinical applications of progesterone receptor antagonists, alone or cooperatively with other SERMs. We know that CDB-4124 is compatible with the use of aromatase inhibitors from in vitro studies (Gupta et al., 2013). Any potential that progestins play a role in BRCA-1/-2 cancer is a high priority given the few choices faced by those women and the results that suggest the involvement of progesterone in that process.

The main challenges for clinical applications of PR modulators are their potential toxicity and their potential effects on the modulation of corticosteroids. The topical use of PRMs for breast cancer, an approach much like the vaginal suppository use for gynecologic disorders currently underway in clinical trials at Repros Therapeutics, is one way to avoid the first pass through the liver and metabolism and liver toxicity.

Development of novel PR modulators with high receptor binding affinity and low incidences of toxic effects is highly desirable for further study in the prevention and treatment of breast cancer. For completely new chemical entities to be brought out, agents should be evaluated with tools that will allow us to recognize their activity in breast tissues and breast cancer. The determination of their binding in cell-free extracts of PR+ tissues has always been a first step, as has the use of reporter assays that show potential activation of a convenient gene through a proximal promoter that is responsive to progestins. The classical way to differentiate new PRMs has been to test their antiprogestational activity in rabbit uterus. Given the nature of tissue-specificity inherent in PRMs, a method that demonstrates direct effects on breast tissue would be preferred. The xenographic model of Liang et al. would be favorable (Liang et al., 2007). The strong effects seen in virgin mammary glands of mice and rats suggest that the PRM could be disclosed in that living system as well. Clearly, the tissue specificity of PRMs and the discovery of specific progesterone-proliferative modulatory genes and their suppression by PRMs would be a considerable advance. This adds not only to the rationalization of progesterone's effects in the breast and breast tumors but also the increases the possibilities that markers of progestin and PRM action can be discerned.

One issue that is specific to the use of PRMs and presents a new situation is the realization that women taking a drug like CDB-4124 achieve a long-term state of low progesterone, a progestopenia. Given that a PRM usually has antagonist activity, perhaps the term aprostogenenic state is more appropriate. This is going to be the case for young women regardless of whether they are under treatment for benign gynecology or cancer. As pointed out, this lowering of progesterone contributes to drug efficiency but may have downsides. Pre- and postmenopausal females have neither high estrogen nor progesterone levels. The absolute level that characterizes partial from total lack of progesterone will be established fully as we utilize the more sensitive LC-mass spectroscopy for hormone analysis. As this becomes widespread, we will be able to delineate levels that are meaningfully low and sort out the low levels from the very low levels with reference to physiologic effects. At the same time, women on PRMs should retain tonic levels of estrogen in the absence of progesterone. While this might suggest the establishment of a state of unopposed estrogen, the direct effect of PRMs on the endometrium producing PAEC (Ioffe et al., 2009) avoids unopposed estrogenic stimulation.

The downsides to low estrogen for young women are considerable in terms of well-being and include vaginal dryness, hot flashes, and bone loss. The downsides to low progesterone are somewhat harder to identify. These women are amenorrheic but the condition is not unprecedented. Certain kinds of popular oral contraceptives allow ovulation and menstruation 3 to 4 times a year, a situation not unlike the use of CDB-4124 with off-drug holidays. Anovulation is the downside; these women are likely to be contracepted but without the estrogen that is in the usual oral formulation. This may not a problem for women who smoke and are in danger of clotting disorders: these individuals would welcome a new contraceptive that does not contain an estrogen. In a world where women seek oral contraception, another form is not a drawback unless lowering progesterone itself and blocking residual progesterone activities have negative effects. This should be an area of investigation to be followed.

In the last 10 years, it has become recognized that progesterones play a role in mammary proliferation and breast cancer presents a new challenge. Taken on the broadest possible terms, monthly cyclic progesterone and, in the modern world, exogenous progestins seem to pose a risk for breast cancer. Is breast cancer in older women the price women pay for being fertile when they were young? If that were the trade-off, widespread fertility trumps the marginal risk of dread disease after the child-bearing years, taking the long view of history with a nod to evolution. Certainly, given that Homo sapiens spent most of its history in small isolated groups where child-bearing and nurturing were existential, the deadly bargain makes a kind of perverse sense. However, it is not a good bargain for women in our times and perhaps there is an opportunity to cheat that fate, if that is indeed what we face.
References


