CDB-4124, a Progesterone Receptor Modulator, Inhibits Mammary Carcinogenesis by Suppressing Cell Proliferation and Inducing Apoptosis*†

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ABSTRACT

CDB-4124 (Proellex or telapristone acetate) is a modulator of progesterone receptor (PR) signaling, which is currently employed in preclinical studies for prevention and treatment of breast cancer and has been used in clinical studies for treatment of uterine fibroids and endometriosis. Here we provide evidence for its action on steroid hormone signaling, cell cycle regulated genes and \textit{in vivo} on mammary carcinogenesis. When CDB-4124 is given to rats at 200 mg/kg for 24 months, it prevents the development of spontaneous mammary hyperplastic and premalignant lesions. Also, CDB-4124 given as subcutaneous pellets at two different doses suppressed, dose-dependently, N-methyl-N-nitrosourea (MNU)-induced mammary carcinogenesis. The high dose (30 mg, over 84 days) increased tumor latency from $66\pm24$ days to $87\pm20$ days ($p<0.02$), decreased incidence from 85% to 35% ($p<0.001$), and reduced multiplicity, from 3.0 to 1.1 tumors/animal ($p<0.001$). Tumor burden decreased from 2.6 g/animal to 0.26 g/animal ($p<0.01$). CDB-4124 inhibited cell proliferation and induced apoptosis in MNU-induced mammary tumors, which correlated with a decreased proportion of PR+ tumor cells and with decreased serum progesterone. CDB-4124 did not affect serum estradiol.

In a mechanistic study employing T47D cells we found that CDB-4124 suppressed G1/0-S transition by inhibiting cdk2 and cdk4 expression, which correlated with inhibition of estrogen receptor (ER) expression. Taken together, these data indicate that CDB-4124 can suppress the development of pre-cancerous lesions and carcinogen-induced ER+ mammary tumors in rats, and may have implications for prevention and treatment of human breast cancer.
Introduction

In clinical trials with hormone replacement therapy (HRT) it was found that postmenopausal women treated with estrogen and progesterone developed higher incidence of breast cancer as compared to placebo-treated women suggesting that the progesterone might be responsible for the carcinogenic effect of HRT (1, 2). In addition to an increase in breast cancer, HRT also increased benign breast proliferative lesions, further supporting the hypothesis that the combination of estradiol and progesterone may promote mammary carcinogenesis (3). It is generally accepted that PR is an estrogen-regulated gene and its synthesis in normal and tumor cells requires estrogen and ER (4, 5). As a result of endocrine therapy with tamoxifen, ER and PR in breast cancer cells may decrease, but complete loss of receptor does not occur (6-8). In animal experiments, progestins increase the incidence of spontaneous mammary tumors in dogs (9) and mice (10) and promote dimethylbenzanthracene (DMBA)-induced mammary carcinogenesis in rats (11, 12). Using progestin receptor knock-out mice (PRKO) mice, the PR has been shown to be specifically important for DMBA-carcinogenicity (13), indicating a sensitivity that would not seem to require the ER. When the well known antiprogestin, RU-486 (mifepristone) 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 (14, 15). 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 (16). The combination of both agents further increased their antitumor
potential. Antitumor effect of RU-486 has been associated with reduced mitotic activity and increased apoptosis (17-20).

In a study of eleven postmenopausal women with advanced breast cancer RU-486 induced a short-term clinical response in one patient and stable disease in six others (21). The side effects of RU-486 in this study were mostly related to anti-glucocorticoid properties of the drug and increased serum estradiol levels. Previous studies have shown that RU-486 at high doses can elevate serum estradiol and progesterone levels, impacting endometrial cell proliferation (22). Data from several clinical trials in patients with advanced breast cancer treated with RU-486 or onapristone, another antiprogestin, have shown a favorable response in 10-12% of the patients and stable disease in 42-46% of the patients (23). The combination of PR antagonists (mifepristone, ORG 31710, onapristone) with antiestrogens (tamoxifen, raloxifene, ICI 164384), or with aromatase inhibitors (atemestane) showed greater antitumor efficacy than when given alone, suggesting potential clinical efficacy in patients resistant to long-term therapy with tamoxifen (23). At the present time, clinical investigation of combined therapy with antiestrogens is lacking.

In an attempt to decrease the undesirable side effects of RU-486, newer classes of antiprogestins have been developed that have similar binding affinities to PR and a decreased potential of raising estradiol and glucocorticoid levels. We have previously reported that a new antiprogestin, CDB-4124 (17\(\alpha\)-acetoxy, 21-methoxy-11\(\beta\) (4-N,N'-dimethylamino)-19 norpregna-4,9-diene,3,20-dione), given by subcutaneous injection at 20, 10, 1 or 0.1 mg/kg suppressed the growth of DMBA-induced mammary tumors in rats in a dose-dependent fashion at the three highest doses (12). Both RU-486 and
CDB-4124 have exhibited comparable binding affinities to rabbit uterine PR and human breast PRA and PRB, suggesting similar effects on receptor signaling. However, CDB-4124 and its metabolites appear to show less antiguercocortcoid activity compared to RU-486, suggesting a clinical advantage (24, 25). Despite the beneficial effects seen in women with uterine fibroids (26) and endometriosis (27, 28), rare, idiosyncratic liver reactions at high doses (unpublished data) suggest that lower doses will be required for the treatment of conditions considered to be non-life-threatening.

Here, we present the results of three studies with CDB-4124; (i) a long-term carcinogenicity study of approximately 24 months, (ii) an 84-day, animal, mammary cancer prevention study, and (iii) a short-term mechanistic study in human T47D cells. In summary, we found evidence that CDB-4124 apparently suppressed the development of benign, hyperplastic, and pre-malignant lesions when compared to those spontaneously appearing in the control group. In the cancer prevention study, there was a reduction in carcinogen-induced mammary tumors; with decreased cell proliferation and increased apoptosis. When T47D cells which express both ER and PR receptors were employed, CDB-4124 inhibited cell proliferation and this was associated with inhibition of cdk2, cdk4, and cell cycle progression, as well as with down regulation of ER and PR signaling. Taken together these data indicate that CDB-4124 has strong inhibitory effect on mammary carcinogenesis and on the growth of T47D ER⁺ and PR⁺ breast cancer cells.
MATERIAL AND METHODS

Animal Models:

Long Term Carcinogenicity:

The long-term, 24 month (life-time) study was performed on female, Sprague-Dawley (Hsp: (SD/BR) rats. Rats at the age of 50 days were randomized in control (placebo) and CDB-4124-treated groups, with 60 animals per group at the beginning of experiment. N-Methyl-N-Nitrosourea (Ash Stevens Inc., Detroit, MI) was dissolved in sterile acidified saline (pH 5.0), and injected intraperitoneally (50 mg/kg body wt.), in 50 day old rats. Control animals at 50 days of age received intraperitoneal (ip), sterile saline. CDB-4124 (Repros Therapeutics Inc., The Woodlands, TX) was mixed with a vehicle comprised of 74.1% (w/w) Gelucire 44/14 (USP, NF lauroyl macroglycerides/lauroyl polyoxylglycerides, from Gattefosse, USA) and 25.9% w/w polyethylene glycol (PEG 400) and given by gavage at three doses; 20, 70 and 200 mg/kg/day (0.5 ml solution/animal, 7 days/week). Treated animals were followed for a period of approximately 24 months. The animals were asphyxiated with CO2. Tissue samples were taken from abdominal mammary glands at the time of animal’s sacrifice. The samples were fixed in 10% formalin (pH-7.2) overnight, and embedded in paraffin.

Mammary Cancer Prevention:

In the cancer prevention study, rats at the age of 50 days were injected with carcinogen to initiate mammary carcinogenesis and 6 days later CDB-4124 pellets were implanted subcutaneously. The occurrence of mammary tumors and their growth was monitored.
twice a week. MNU was prepared as described above. CDB-4124 was formulated in pellets (Innovative Research of America, Sarasota, FL) of two doses: 30.0 mg/pellet and 3.0 mg/pellet. Placebo pellets for control animals were also prepared. Pellets, designed to release CDB-4124 over a 90 day period, were subcutaneously implanted in the back interscapular region of the rats.

Animals were sacrificed by CO₂ asphyxiation 90 days after carcinogen administration, and 84 days after subcutaneous implantation of progesterone pellets. In addition to tumor latency and volume, incidence, multiplicity, and tumor burden were also determined at the end of experiment. Mammary tumors were separated from the surrounding tissues, their mass was determined on an analytical balance, and they were fixed in formalin. Tissue sections (3-4 microns) were prepared and stained with hematoxylin and eosin (H&E) or used for ICH to identify proliferating and apoptotic cells, as well as ER and PR expression. Proliferating cells in mammary tissues and tumors were determined by using Ki-67 monoclonal antibody (Neo Markers, Fremont, CA) and ABC kit (Vector, CA). The slides were counter-stained by hematoxylin for identification of tissue morphology. More than 1X10³ mammary epithelial cells (MEC) among lobular structures or cells from tumor periphery were evaluated for Ki-67 labeling. Cells in apoptosis were identified by TUNEL assay, as described in a recent publication (26). Slides were counter-stained with methyl-green for identification of tumor and tissue morphology. ERα and PRA in mammary tumors were evaluated by ICH using mouse monoclonal antibodies, Ab-14 (1:100) for ERα and Ab-4 (1:50) for PRA (NeoMarkers). ABC kit and Diaminobenzidine (DAB) were used to detect ER and PR protein. The slides were counterstained with hematoxylin.
**Estradiol and progesterone serum levels:** Blood samples were collected at the time of animal sacrifice. Serum was isolated and sent to Endocrinology Research Laboratory at Cornell University Animal Health Diagnostic Center for estradiol and progesterone detection by radioimmunoassay.

**Statistical analysis:** Comparisons of tumor incidence curves for treated and control animals were made using life table analysis and the log-rank test. Tumor multiplicity data were compared using Armitage test for trend in proportion. Body and tumor mass data were compared by the two-tail Fisher t-test using ANOVA.

**Short-Term Mechanistic Study in Human T47D Cells:**

**T47D cell growth:** T47D cells (The American Cell Type Collection, Manassas, VA), which express both ER and PR were cultured in six-well plates at 10,000 cells/well using MEM supplemented with 100 µM penicillin, 100 µg/ml streptomycin, 10% FBS, 200 µM L-Glutamine, and 100µM MEM non-essential amino acid. Cells were treated for 3 or 6 days with 0.0, 0.1, 1.0 or 10.0 µM CDB-4124 in dimethyl sulfoxide (DMSO). Cell growth was compared to equal treatment with a placebo/DMSO control. The cell culture medium was refreshed every 48 hrs with placebo or CDB-4124. At the end, cells were treated with 0.05% trypsin, washed in PBS, and quantitated using a cell counter.

**Cell cycle analysis:** Cells were grown in 6 well-plates and treated for 3 days or 6 days with 0.1, 1.0 or 10 µM CDB-4124 or with placebo (DMSO). At the end of treatment, the cell culture medium was discarded; cells were washed by PBS, treated with trypsin, washed in PBS again, and fixed in 70% ethanol. For cell cycle analysis, cells were incubated with 0.1% RNase, stained by propidium iodide, and DNA content evaluated.
by FACS (BD Biosciences, San Jose, CA). The percentage of cells in G1/0, S, and G2M phases was calculated by using a multicycle program. At least 1X10^4 cells per time point were analyzed.

**Western blotting:** ERα, PRA, PRB, cyclin D1, cdk2, cdk4, and cdk6 expression in control and CDB-4124 treated cells was determined by Western blot. Cells were treated for 3 or 6 days with placebo or 1.0 μM CDB-4124. Cell lysates were prepared with RIPA buffer (10mM NaF, 137 mM NaCl, 1mM NaVO4, 10 mM EDTA, 1% NP-40, 1 mM DTT and protease inhibitors, Sigma Inc., MO) and the total protein was isolated. For ERα and PRA, corresponding antibodies, as indicated above were used. For PRB expression a rabbit polyclonal antibody (Cat#: 3178, Cell Signaling) was employed. For cyclin D1, cdk2, cdk4 and cdk6 expression, sc-246 mouse, sc-163 (goat), sc-260 (rabbit) and sc-177 (rabbit) antibodies (Santa Cruz Biotech. Inc., CA) respectively were used.

**Results**

**Long-term carcinogenicity study**

**Survival of animals:** In the course of development of CDB-4124 by Repros Therapeutics Inc., a life-time (i.e., 2-year) carcinogenicity study was completed in rats at MPI of Matawan, MI. Treated animals were followed for approximately 24 months. Similar numbers of animals survived in CDB-4124-treated vs. placebo-treated group, indicating little added toxicity of the agent when used over the natural life span of the animal. Since both the control and CDB-4124 treated animals were gavaged for approximately 24 months, tissue damage and inflammatory responses in the mouth
cavity as well as the development of lung inflammation and abscesses were common in all groups. By comparison, differences in overall body weights of placebo-treated vs. CDB-4124-treated animals were unremarkable. In the high dose group (200 mg/kg) there was a tendency toward body weight decrease (12.5%), but the difference with respect to placebo-treated animals was insignificant. The average range of body weights in the 20 mg/kg/day and 70 mg/kg/day female groups fell within range of the high dose and the controls with no statistical difference between the groups (Table 1).

Microscopic histological examination of the internal organs (liver, spleen, heart, lung, intestine, brain, kidneys) of the animals in the long-term experiment did not reveal substantial differences, in terms of tumors between the CDB-4124 and control animal groups. As a result, the internal organs of the low dose group were evaluated via gross observation.

**CDB-4124 decreased fibroadenomas and lobular hyperplasia:** Mammary gland morphology was assessed by comparing hematoxylin/eosin-stained tissue sections of the abdominal glands from both the placebo and high dose CDB-4124 treated animals (Figures 1A-F). Higher incidences of fibroadenomas (p<0.014) and hyperplastic lesions with atypia (p<0.003) were found in the control as compared to the CDB-4124 treated groups. In 11 animals from the placebo group, the lobular hyperplasia occupied a large area of mammary gland parenchyma with accumulation of secreted transparent material (Figure 1C). In one animal a tumor nodule with characteristics of follicular adenoma was also detected (Figure 1D). In the animals treated with CDB-4124, an increase in cystic formations (Cys) was also found (Figure 1B and Table 2). In both placebo and CDB-4124 treated animals calcifications among mammary gland parenchyma were also
observed. As shown in Table 2, even small doses of CDB-4124 (20, 70 mg/kg) decreased fibroadenoma and lobular hyperplasia development. CDB-4124 also reduced ductal lateral branching that leads to reduction in lobular structures in mammary gland (Figures 1E and 1F). These data indicate that CDB-4124 administered for approximately 24 months inhibits spontaneous mammary carcinogenesis by reducing lobular hyperplasia (benign and atypical) and development of benign tumors.

**CDB-4124 suppresses cell proliferation and induces apoptosis:** In order to understand better the potential cellular mechanisms of CDB-4124 induced alterations in mammary gland architecture, proliferation activity, and apoptosis of mammary epithelial cells in lobular structure of control and treated with CBD-4124 animals were examined. As shown in Table 3, CDB-4124 significantly decreased the percentage of Ki-67 positive cells, from 16.5±5.4% in placebo to 9.1±3.4% (p<0.001) in CDB-4124-treated animals. However, CDB-4124 was not as effective in inducing apoptosis, as evidenced by the values of apoptotic cells in placebo 1.0±0.7% vs. 1.2±0.7% (p<0.2) in CDB-4124-treated animals.

**CDB-4124 inhibits MNU-induced mammary carcinogenesis:** Pellets containing CDB-4124 at 0, 3.0 or 30.0 mg were subcutaneously implanted 6 days after carcinogen administration and the animals were sacrificed 84 days later. Analysis of animal body weight at the end of experiment did not show significant differences between the control and CDB-4124-treated animals (Table 1). All mammary glands were palpated starting 4 weeks after carcinogen administration to determine the latency of mammary tumors. The first palpable tumor was detected in the placebo group 49 days after carcinogen administration vs. 55 days in the low dose and 59 days in the high dose group CDB-
4124 groups (Table 4). The average tumor latency for the control group was 66±24 days vs. 74±21 days for the low dose and 87±20 days (p<0.02) for the high-dose CDB-4124 groups. CDB-4124 also suppressed the incidence and multiplicity of mammary tumors in a dose-dependent manner (Table 4, Figure 2). Tumor incidence was 85% in placebo treated animals and progressively decreased to 60% in the low-dose group and further to 35% in high-dose group (p<0.001). CDB-4124 also decreased tumor multiplicity; from 3.0 tumors/rat in the control group, to 2.2 and 1.1 tumors/rat in the low- and high dose (p<0.001) treated animals respectively (Table 4). Tumor weight (burden) at the end of experiment was also reduced; from 2.16 g in placebo group to 0.62 g (p<0.025) in the low dose group, and to further to 0.26 g (p<0.01) in high dose group. Histological examination of mammary tumors of CDB-4124 treated animals revealed an increase in intercellular spaces between tumor cells and the development of cystic formations.

**CDB-4124 inhibited cell proliferation and induced apoptosis in mammary tumors:**

To determine the potential cellular mechanism of CDB-4124 induced inhibition of mammary carcinogenesis, proliferating (Ki-67 positive) and apoptotic cells were evaluated in NMU tumors of control and CDB-4124 treated animals. CDB-4124 significantly suppressed Ki-67 positive cells in mammary tumors, from 30.5±5.4% in the placebo treated group to 25.4±14.4% in low-dose group, and further to 10.3±4.5% (p<0.001) in the high-dose group of treated animals (Table 3). Both doses of CDB-4124 also induced apoptosis in mammary tumors, where 0.7±0.4% apoptotic cells were detected in the control group vs. 1.4±0.8%, in low-dose (p<0.05) and 1.6±0.8%, in the
high-dose group (p<0.01). These data indicate that CDB-4124 suppresses mammary carcinogenesis and tumor growth by inhibiting cell proliferation and inducing apoptosis. **CDB-4124 decreased serum progesterone, but had no effect on estradiol:** To determine if the inhibitory effect of CDB-4124 on mammary carcinogenesis is associated with alterations in serum progesterone and/or estradiol, blood samples were collected from the abdominal vein at the time of animal sacrifice. The values of both hormones in the serum of the control, low dose (3 μM), and high dose (30 μM) treated animals are summarized in Table 5. The data show that CDB-4124 progressively decreased the serum progesterone level from 14.6±9.5 ng/ml in the placebo treated group to 12.5±7.9 ng/ml in the low-dose group, and further to 7.9±4.4 ng/ml (p<0.05) in high-dose group. CDB-4124 had little effect on serum estradiol at both dose levels. **CDB-4124 suppressed PR expression in mammary tumors:** Since antiprogestins have been shown to modulate PR signaling, we assumed that this might affect both ER and PR expression in mammary tumors. To test this hypothesis, we evaluated both receptors by ICH in parallel sections of tumor tissue. As shown in Table 5, 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 (p<0.01). Although trending lower, CDB-4124 did not significantly affect the proportion of ER+ cells in the samples. **CDB-4124 suppressed the growth of T47D cells:** In an attempt to model the effects of CDB-4124 on cell proliferation and apoptosis in mammary tumors *in vivo, in vitro* studies with T47D cells, which express both ER and PR were performed. 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 (Figure 3). CDB-4124 at 0.1 μM did not affect
cell growth after either 3 days or 6 days of treatment, whereas doses of 1.0 µM and 10.0 µM suppressed cell growth in a dose dependent manner. After 3 days of treatment with CDB-4124, the cell number decreased from 138.4±4.2 x 10^3 cells/ml in the placebo treated culture to 112.5±10.5 x 10^3 cells/ml at 1.0 µM CDB-4124 and to 68.2±6.7 x 10^3 cells/ml at 10.0 µM CDB-4124. After 6 days of treatment, the difference in cell number further decreased from, 560±32.6 x 10^3 cells/ml in placebo treated, to 464±24.3 x 10^3 cells/ml in 1.0 µM and further to 48.6±6.5 x 10^3 cells/mL in cells treated with 10.0 µM CDB-4124.

**CDB-4124 inhibits G1/S cell cycle progression:** We also examined if CDB-4124 differentially affected various cell cycle phases. T47D cells were treated for 3 or 6 days with 1.0 µM CDB-4124, a dose that suppressed cell growth, as shown above. As shown in Table 6, CDB-4124 decreased the percentage of cells in S-phase from 12.2±1.8% in 3-day placebo treated to 8.5±1.6% in CDB-4124-treated cells, and from 14.1±1.7% in 6 day placebo treated cells to 9.3±2.3% in CDB-4124 treated cells (p<0.01). In the 6-day CDB-4124 treated group, a significant increase in G1/0 cells was also observed (p<0.05). These data suggest that CDB-4124 may inhibit the transition of cells from G1 to S-phase of the cell cycle.

**CDB-4124 decreased ERα, but had no effect on PRA and PRB expression:** To support the data showing the effect of CDB-4124 on ER and PR expression in mammary tumors, T47D cells were treated for 3 or 6 days with 1.0 µM CDB-4124 and PRA, PRB and ERα expression was determined by Western blot (Figure 4A). Both PRA and PRB were differentially expressed at both time points, but CDB-4124 at
1.0 µM failed to affect the expression of either progesterone receptor. Conversely, CDB-4124 decreased ERα expression in both, 3 day and 6 day treated cells.

**CDB-4124 inhibits cdk2 and cdk4 but not cyclin D1 and CDK6 expression in T47D cells:** To corroborate the findings from the cell cycle analysis, cells treated for 3 or 6 days with 1.0 µM CDB-4124 or placebo were lysed, the total protein was isolated, and the expression levels of cyclin D1, cdk2, cdk4, and cdk6 were determined by Western blot (Figure 4B). Analysis of the blot implied that CDB-4124 did not affect cyclin D1 or cdk6 expression, but decreased cdk2 and cdk4 expression in both the 3 day and 6 day treated cells.

**Discussion/Conclusions**

The results from this study indicate that CDB-4124 is an efficacious inhibitor of benign, hyperplastic, pre-malignant, and spontaneous tumors when the agent is provided chronically; and an inhibitor of MNU-induced mammary carcinogenesis in rats, associated with inhibition cell proliferation and induction of apoptosis in mammary epithelial and tumor cells when treated for up to 90 days. The inhibitory effect of CDB-4124 on mammary carcinogenesis is apparently a consequence of decreased progesterone circulation level but not of serum estradiol, which remained unchanged in treated animals. In T47D cells, which express both ER and PR and are established tumor cell models for investigating PR modulation by antiprogestins, CDB-4124 suppressed cell growth and this correlated with inhibition of cdk2 and cdk4 expression and consequently with G1/0-S cell cycle block. These alterations in cell cycle
progression appear to be associated with a down-regulation of ERα expression, and a decrease in the population of PR+ cells in MNU-induced tumors.

The proliferation and apoptotic markers were investigated in those NMU-induced tumors remaining after the treatment period. We did not sacrifice animals at more than one time period. Those tumors investigated could represent resistant tumors except that incidence, size, and multiplicity all decrease with treatment in concert and the results appear to be dose-dependent without evidence of an evident change in growth rate. It would be interesting to observe the effects of CDB-412 on tumors that become estrogen-resistant as discussed below. We also cannot rule out that the tumors investigated at the end of the study were under the influence of lower doses of agent. We believed that the CDB-4124-releasing pellets would provide agent in a steady manner but we did not measure the serum levels of CDB-4124 throughout the study. In a previously study using DBMA-induced rat mammary tumors, we did observe that established breast tumors responded to CDB-4124 with changes in proliferation and apoptosis markers concomitantly with decreases in size and number.

The long-term (24 month) carcinogenicity study revealed that the addition of increasing doses of CDB-4124 in rats did not trigger pathological alterations in parenchymal organs when examined at the end of experiment. However, histological examination of mammary gland in CDB-4124-treated rats revealed dose-dependent inhibition of lobular hyperplasia and fibroadenomas that was associated with the development of cystic formations in mammary gland parenchyma. The inhibitory effect of CDB-4124 on mammary carcinogenesis appears to be a consequence of decreased ductal-lateral branching. In support of this hypothesis are recent data on BRCA1/p53-
transgenic mice indicating that RU-486, another antiprogestin, suppressed mammary carcinogenesis by inhibiting ductal-lateral branching and lobular differentiation of mammary epithelial cells (29). Those 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 (13). The apparent relationship between mammary gland architecture and cyst formation that may have ductal or lobular origin is apparently a consequence of decreased functional activity of MECs as result of CDB-4124 treatment. This has also been observed in the endometrium of women treated for uterine fibroids and endometriosis with the same agent (30).

The inhibitory effect of CDB-4124 on cell proliferation we see in both, long and short (3 month) term treatment correlates well with recent data from a clinical trial with mifepristone (RU-486). Women with leiomyoma treated with 50 mg RU-486 every second days for 3 months underwent fine needle breast biopsies before initiation and after termination of treatment (5). A significant reduction in proliferating breast epithelial cells (Ki-67 positive) were 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. In previous studies using the same carcinogenesis model, tamoxifen at 1.0 mg/kg body weight had similar effects on tumor multiplicity and burden (31, 32).

We may speculate that a combination of low dose tamoxifen and low doses of CDB-4124 could synergistically inhibit breast cancer development and progression. As
it has been demonstrated that tamoxifen suppresses the growth of only about 50% of ER+ breast carcinoma (2, 3), antiprogestins alone or in combination with tamoxifen may improve the clinical outcome (29). Moreover, in the course of breast cancer treatment with tamoxifen about 50% of ER+ tumors develop resistance to treatment, suggesting that PR inhibitors may have utility and improve prognosis.

Our *in vitro* studies on T47D cells revealed that CDB-4124 induced dose-dependent inhibition of cell growth and this was associated with inhibition of cell proliferation (S-phase fraction) and induction of apoptosis. The decrease in S-phase fraction coincided with an increase in G1/O cells, suggesting a G1/S cell cycle block. This observation was supported by a reduction in cdk2 and cdk4 expression as determined by western blot. CDB-4124 did not appear to affect cyclin D1 and cdk6 expression, suggesting that antiprogestins may modulate specific cell cycle targets as part of their antiproliferative action. Since, p27^Kip1^ and p21^Kip1/Waf1^ are inhibitors of cdk2 and cdk4 (19, 20) respectively, their expression was also evaluated, but no significant changes were found (data not shown). Additional studies need to be conducted by employing adequate cell systems and modulators of cdks in order to determine their selective response to antiprogestins. Since, CDB-4124 in addition to inhibiting cell proliferation also induced apoptosis in mammary tumors, we assessed Bcl-2, Bax, caspase 3, and cleaved caspase-3 expression in control and treated with CDB-4124 T47D cells. However, no significant changes in the above biomarkers of apoptosis were found in CDB-4124 treated cells vs. placebo treated cells (data not shown), suggesting differential molecular mechanisms in mammary epithelial *in vitro* and *in vivo*. 
We also found that CDB-4124 inhibits ERα, but not PRA and PRB expression in T47D cells which contradicts the results from MNU-induced mammary tumors where the same antiprogestin preferentially suppressed PR+ cells, as determined by ICH. Since ERα has been shown to regulate PR expression (33), we expected that CDB-4124-induced down-regulation of ERα in T47D cells would lead to down-regulation of PRs well. In fact, in MNU-induced mammary tumors CDB-4124 appears also to decrease ER+ cells, from 58±14% in placebo-treated to 49±21% in CDB-4124 treated animals, although the difference between groups was not statistically significant (Table 5). We may speculate that small decreases in ERα expression may trigger more significant decreases in PR expression, as has been previously reported (6, 33). However, various factors may affect ER and PR expression, such as the dose and duration of CDB-4124 treatment. We may speculate that a dose of 1.0 µM over 3 days and/or 6 days of treatment are efficacious for ER modulation but not for the analysis of PR expression. Doses of compound realized in vitro may be substantially higher than those seen in vivo, as well.

By employing T47D cells which express both ER and PR, we sought to confirm the observations from the in vivo studies using the MNU carcinogenesis model and to generate mechanistic information on the effect of CDB-4124. The in vivo results supported the in vitro data with regard to proliferation inhibition. It has been noted that there is conflicting, almost paradoxical data on progestin and antiprogestin effects in tissues and cell lines (34). Importantly here, the effects of progesterone agonists in T47D cells have been seen as clearly antiproliferative (35, 36). The data reporting the stimulatory effects of progesterone on breast cancer development in post-menopausal...
women (1-3, 37, 38) and the luteal effects of progesterone in the breast (39) are two cases that point to progesterone as a bad player with respect to proliferation. Our own work that reported on the effects of progesterone and antiprogestins on DMBA induced tumors (12), in conjunction with that given here further supports of the view that progesterone can stimulate proliferation. In the present case, we have used an antiprogestin in T47D cells at a relatively high culture concentration (1-10 μM). The observed activity of CDB-4124 could be as a progesterone agonist in that setting. We have seen earlier in Wiehle et al (12) that CDB-4124 given to animals with breast tumors appeared to have agonist-like effects at the highest dose and antagonist activity at lower doses. It is interesting that the NMU model used here provides constant but relatively low amounts of the antiprogestin (3 or 30 mg over 90 days) and retains antagonism towards cell growth. While we cannot exclude that some of the effects of CDB-4124 are mediated through protein kinases and other growth factors (34, 40), the observations from this study support the more limited view that antiprogestins suppress proliferation in normal mammary gland and nascent mammary tumors regardless of other factors.

Future studies should also address questions regarding the role of CDB-4124 alone, or in combination with tamoxifen or other SERMs, on the modulation of ER and PR signaling, and/or on potential involvement of co-activators and co-repressors. The development of therapy 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. However, the main challenge for clinical applications of PR modulators is their potential toxicity, as well as
their potential effects on the modulation of corticosteroids, as previous clinical studies have shown. Therefore, 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.
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MNU-Induced Mammary Cancers
Effect of CDB-4124 on Tumor Multiplicity

- Gp 1 MNU Vehicle
- Gp 2 MNU 3mg CBD-4124
- Gp 3 MNU 30mg CBD-4124

Days Post MNU

Tumors per Rat

35 42 49 56 62 69 76 80 84 Autopsy
Effect of CDB-4124 Concentration on Cell Count

![Graph showing the effect of CDB-4124 concentration on cell count over 3 and 6 days.](image-url)
**Table 1: Effects of CDB-4142 on Animal Body Weight at the End of Experiment**

<table>
<thead>
<tr>
<th>Study</th>
<th>treatment</th>
<th>Dose (months)</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>Mean Body Weight (g)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>gavage</td>
<td></td>
<td>study end</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toxicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>23.5</td>
<td>0</td>
<td>16</td>
<td>489</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>23.5</td>
<td>20</td>
<td>16</td>
<td>461</td>
<td>ns</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>23.5</td>
<td>70</td>
<td>22</td>
<td>476</td>
<td>ns</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>23.5</td>
<td>200</td>
<td>22</td>
<td>420*</td>
<td>0.1</td>
</tr>
<tr>
<td>Prevention</td>
<td></td>
<td></td>
<td></td>
<td>examined</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>3</td>
<td>0</td>
<td>20</td>
<td>253</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>3</td>
<td>3</td>
<td>20</td>
<td>247</td>
<td>ns</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>3</td>
<td>30</td>
<td>20</td>
<td>245</td>
<td>ns</td>
</tr>
</tbody>
</table>

In toxicity study the animals' weight was monitored for 23.5 months whereas in cancer prevention study animals were sacrificed 3 months after ip injection of animals with MNU.

*In the long-term experiment the high dose of CDB-4124 (200 mg/kg) slightly decreased the body weight but the difference with control group was not significant (p<0.1).*
**Table 2:** Development of Fibroadenomas, Lobular Hyperplasia, Atypical Lobular Hyperplasia and Cystic Formations in Mammary Gland of animals followed for 23.5 months

<table>
<thead>
<tr>
<th>Lesions (number)</th>
<th>CDB-4124 Dose in mg/kg body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>N at Study End</td>
<td>16</td>
</tr>
<tr>
<td>Fibroadenomas</td>
<td>11**</td>
</tr>
<tr>
<td>Lobular hyperplasia</td>
<td>40**</td>
</tr>
<tr>
<td>Atypical lobular hyperplasia</td>
<td>11**</td>
</tr>
<tr>
<td>Cystic structures</td>
<td>3**</td>
</tr>
</tbody>
</table>

The above lesions were identified by pathologist on formalin fixed, paraffin embedded tissue sections stained by hematoxylin-eosin (H&E).

**significant difference in the values between CDB-4124- and placebo-treated animals (p<0.05)**
Table 3: Effects of CDB-4124 on Cell Proliferation and Apoptosis

<table>
<thead>
<tr>
<th></th>
<th>CDB-4124 (mg/kg)</th>
<th>N</th>
<th>Ki-67 (%)</th>
<th>p</th>
<th>Apoptosis (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammary Lobules</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>0</td>
<td>16</td>
<td>16.5 ± 5.4</td>
<td></td>
<td>1.0 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>CDB-4124</td>
<td>200</td>
<td>22</td>
<td>9.1 ± 3.4</td>
<td>0.001</td>
<td>1.2 ± 0.7</td>
<td>ns</td>
</tr>
<tr>
<td>NMU Tumors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>0</td>
<td>19</td>
<td>30.5 ± 7.1</td>
<td></td>
<td>0.7 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>CDB-4124</td>
<td>3</td>
<td>20</td>
<td>25.4 ± 14.4</td>
<td>ns</td>
<td>1.4 ± 0.8</td>
<td>0.05*</td>
</tr>
<tr>
<td>CDB-4124</td>
<td>30</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>

Cell proliferation was determined by Ki-67 antibody and ABC kit. Cells in apoptosis were identified by TUNEL assay, as recommended by ApopTag kit (see material and methods). At least 1000 lobular cells were examined in toxicity study and the values compared with those of placebo0-treated animals. In mammary tumors peripheral tumor areas free of necroses were examined for Ki-67 and apoptotic cells. The differences in the values are significant (p<0.05) as compared to those of the control animals (Student-Fisher t-test).
Table 4: Tumor Latency, Incidence, Multiplicity, and Burden in CDB-4124 Treated Mammary Tumors

<table>
<thead>
<tr>
<th>Dose (mg/pellet)</th>
<th>N</th>
<th>First tumor (days)</th>
<th>Latency (days)</th>
<th>Incidence (%)</th>
<th>Multiplicity</th>
<th>Burden (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
<td>49</td>
<td>66.4±23.5*</td>
<td>85**</td>
<td>3.0**</td>
<td>2.16 ± 4.40*</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>55</td>
<td>73.5±21.1</td>
<td>60</td>
<td>2.2</td>
<td>0.62 ± 1.87*</td>
</tr>
<tr>
<td>30</td>
<td>20</td>
<td>59</td>
<td>87.1±19.5*</td>
<td>35**</td>
<td>1.1**</td>
<td>0.26 ± 0.33*</td>
</tr>
</tbody>
</table>

*significant (p<0.05, Student-Fisher t-test)  ** significant (p < 0.001, X²)
Table 5: Effects of CDB-4124 on Serum* Estradiol, Serum Progesterone**, and Hormone Receptors in MNU-Induced Mammary Tumors††

<table>
<thead>
<tr>
<th>Dose (µM)</th>
<th>Progesterone (ng/mL)</th>
<th>Estradiol (pg/mL)</th>
<th>% PR +</th>
<th>% ER +</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.6 ± 9.5</td>
<td>36.1 ± 5.1</td>
<td>48 ± 11 (n=13)</td>
<td>58 ± 14 (n=13)</td>
</tr>
<tr>
<td>3</td>
<td>12.5 ± 7.9§</td>
<td>38.3 ± 6.3§</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>30</td>
<td>7.9 ± 4.4†</td>
<td>34.7 ± 7.6§</td>
<td>33 ± 12 (n=14)†</td>
<td>49 ± 21 (n=14)§</td>
</tr>
</tbody>
</table>

*Blood was collected at the time of animals’ sacrifice and the serum was isolated

**As determined by radioimmunoassay

††At least 1X10^3 cells from the periphery of the tumor were examined

† p<0.01
‡ p<0.05
§ not significant
ND=not determined
Table 6: Effects of CDB-4124 on Cell Cycle Progression of T47D Cells

<table>
<thead>
<tr>
<th>Phase</th>
<th>3-days</th>
<th></th>
<th>6-days</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CDB-4124</td>
<td>Control</td>
<td>CDB-4124</td>
</tr>
<tr>
<td>G1/0 (%)</td>
<td>80.4 ± 3.2</td>
<td>85.2 ± 3.8</td>
<td>75.3 ± 2.5*</td>
<td>81.9 ± 3.2*</td>
</tr>
<tr>
<td>S (%)</td>
<td>12.2 ± 1.8†</td>
<td>8.5 ± 1.7†</td>
<td>14.1 ± 1.7†</td>
<td>9.3 ± 2.3†</td>
</tr>
<tr>
<td>G2M (%)</td>
<td>7.5 ± 2.1</td>
<td>5.2 ± 1.2</td>
<td>10.6 ± 1.4</td>
<td>8.8 ± 2.0</td>
</tr>
</tbody>
</table>

*significant increase compared to control
†significant decrease compared to control
Cancer Prevention Research

CDB-4124, a Progesterone Receptor Modulator, Inhibits Mammary Carcinogenesis by Supressing Cell Proliferation and Inducing Apoptosis

Ronald D Wiehle, Daniel D Lantvit, Tohru Yamada, et al.


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