

## Research Article

**CDB-4124, a Progesterone Receptor Modulator, Inhibits Mammary Carcinogenesis by Suppressing Cell Proliferation and Inducing Apoptosis**Ronald Wiehle<sup>1</sup>, Daniel Lantvit<sup>2</sup>, Tohru Yamada<sup>2</sup>, and Konstantin Christov<sup>2</sup>**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 *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  $66 \pm 24$  days to  $87 \pm 20$  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<sup>+</sup> 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 G<sub>1</sub>/G<sub>0</sub>-S transition by inhibiting CDK2 and CDK4 expressions, which correlated with inhibition of estrogen receptor (ER) expression. Taken together, these data indicate that CDB-4124 can suppress the development of precancerous lesions and carcinogen-induced ER<sup>+</sup> mammary tumors in rats, and may have implications for prevention and treatment of human breast cancer. *Cancer Prev Res*; 4(3); 414–24. ©2011 AACR.

**Introduction**

In clinical trials with hormone replacement therapy (HRT) it was found that postmenopausal women treated with estrogen and progestin developed higher incidence of breast cancer than 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 progesterone receptor (PR) is an estrogen-regulated gene and its synthesis in normal and tumor cells requires estrogen and estrogen receptor (ER; refs. 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 knockout 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<sup>+</sup> 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 anti-tumor potential. Antitumor effect of RU-486 has been associated with reduced mitotic activity and increased apoptosis (17–20).

In a study of 11 postmenopausal women with advanced breast cancer, RU-486 induced a short-term clinical

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response in 1 patient and stable disease in 6 others (21). 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 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% to 12% of the patients and stable disease in 42% to 46% of the patients (23). The combination of PR antagonists (mifepristone, ORG 31710, onapristone) with antiestrogens (tamoxifen, raloxifene, ICI 164384) or with aromatase inhibitors (atamestane) showed greater antitumor efficacy than when given alone, suggesting potential clinical efficacy in patients resistant to long-term therapy with tamoxifen (23).

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 3 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 antiglucocorticoid activity compared with 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 not considered to be life threatening.

Here, we present the results of 3 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 premalignant lesions when compared with 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 downregulation 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<sup>+</sup> and PR<sup>+</sup> breast cancer cells.

## Materials 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. CDB-4124 (Repros Therapeutics Inc.) was mixed with a vehicle comprised of 74.1% (w/w) Gelucire 44/14 (USP, NF lauroyl macrogol-21 glycerides/lauroyl polyoxyglycerides; Gattefosse) and 25.9% w/w polyethylene glycol (PEG 400), and given by gavage at 3 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 CO<sub>2</sub>. 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 a carcinogen (*N*-methyl-*N*-nitrosourea (MNU; Ash Stevens Inc.) to initiate mammary carcinogenesis, and 6 days later CDB-4124 pellets were implanted subcutaneously. The MNU was dissolved in sterile acidified saline (pH 5.0), and injected intraperitoneally (i.p.; 50 mg/kg body weight), in 50-day-old rats. Control animals at 50 days of age received i.p. sterile saline. The occurrence of mammary tumors and their growth was monitored twice a week. CDB-4124 was formulated in pellets (Innovative Research of America) of 2 doses: 30.0 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 CO<sub>2</sub> 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 immunohistochemistry (IHC) to identify proliferating and apoptotic cells, as well as ER and PR expressions. Proliferating cells in mammary tissues and tumors were determined by using Ki-67 monoclonal antibody (Neo Markers) and ABC kit (Vector). The slides were counterstained by hematoxylin for identification of tissue morphology. More than  $1 \times 10^3$  mammary epithelial cells (MEC) among lobular structures or cells from tumor periphery were evaluated for Ki-67 labeling. Cells in apoptosis were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, as described in a recent publication (26). Slides were counterstained with methyl green for

identification of tumor and tissue morphology. ER $\alpha$  and PRA in mammary tumors were evaluated by IHC using mouse monoclonal antibodies, Ab-14 (1:100) for ER $\alpha$ , and Ab-4 (1:50) for PRA (NeoMarkers). ABC kit and diaminobenzidine 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 2-tailed Fisher *t* test using ANOVA.

### Short-term mechanistic study in human T47D cells

**T47D cell growth.** T47D cells (The American Cell Type Collection), which express both ER and PR, were cultured in 6-well plates at 10,000 cells/well using MEM supplemented with 100  $\mu$ mol/L penicillin, 100  $\mu$ g/mL streptomycin, 10% FBS, 200  $\mu$ mol/L L-glutamine, and 100  $\mu$ mol/L MEM non-essential amino acid. Cells were treated for 3 or 6 days with 0.0, 0.1, 1.0, or 10.0  $\mu$ mol/L CDB-4124 in dimethyl sulfoxide (DMSO). Cell growth was compared with equal treatment with a placebo/DMSO control. The cell culture medium was refreshed every 48 hours 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  $\mu$ mol/L 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). The percentage of cells in G<sub>1</sub>/G<sub>0</sub>, S, and G<sub>2</sub>M phases was calculated by using a multicycle program. At least  $1 \times 10^4$  cells per time point were analyzed.

**Western blotting.** ER $\alpha$ , PRA, PRB, cyclin D1, CDK2, CDK4, and CDK6 expressions 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  $\mu$ mol/L CDB-4124. Cell lysates were prepared with RIPA buffer (10 mmol/L NaF, 137 mmol/L NaCl, 1 mmol/L NaVO<sub>4</sub>, 10 mmol/L EDTA, 1% NP-40, 1 mmol/L DTT, and protease inhibitors; Sigma Inc.) and the total protein was isolated. For ER $\alpha$  and PRA, corresponding antibodies, as indicated earlier in the text, were used. For PRB expression, a rabbit polyclonal antibody (category no. 3178, Cell Signaling) was employed. For cyclin D1, CDK2, CDK4, and CDK6 expressions, sc-246 (mouse), sc-163 (goat), sc-260 (rabbit), and sc-177 (rabbit) antibodies (Santa Cruz Biotech. Inc.), 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, Mattawan, MI. Treated animals were followed for approximately 24 months. Similar numbers of animals survived in CDB-4124-treated group versus placebo-treated group, indicating little added toxicity of the agent when used over the natural life span of the animal. Because 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 versus 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 histologic 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 H&E-stained tissue sections of the abdominal glands from both the placebo and high-dose CDB-4124-treated animals (Fig. 1A-F). Higher incidences of fibroadenomas ( $P < 0.014$ ) and hyperplastic lesions with atypia ( $P < 0.003$ ) were found in the control as compared with 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 (Fig. 1C). In one animal, a tumor nodule with characteristics of follicular adenoma was also detected (Fig. 1D). In the animals treated with CDB-4124, an increase in cystic formations was also found (Fig. 1B; Table 2). In both placebo-treated 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 and 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 (Figs. 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.

**Table 1.** Effects of CDB-4142 on animal body weight at the end of experiment

Study	Treatment (months)	Dose (mg/kg)	n	Mean body weight (g)	P
Toxicity		Gavage	study end		
1	23.5	0	16	489	
2	23.5	20	16	461	ns
3	23.5	70	22	476	ns
4	23.5	200	22	420 <sup>a</sup>	0.1
Prevention		Pellets	Examined		
1	3	0	20	253	
2	3	3	20	247	ns
3	3	30	20	245	ns

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

<sup>a</sup>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 ( $P < 0.1$ ).

Abbreviation: ns, not significant.

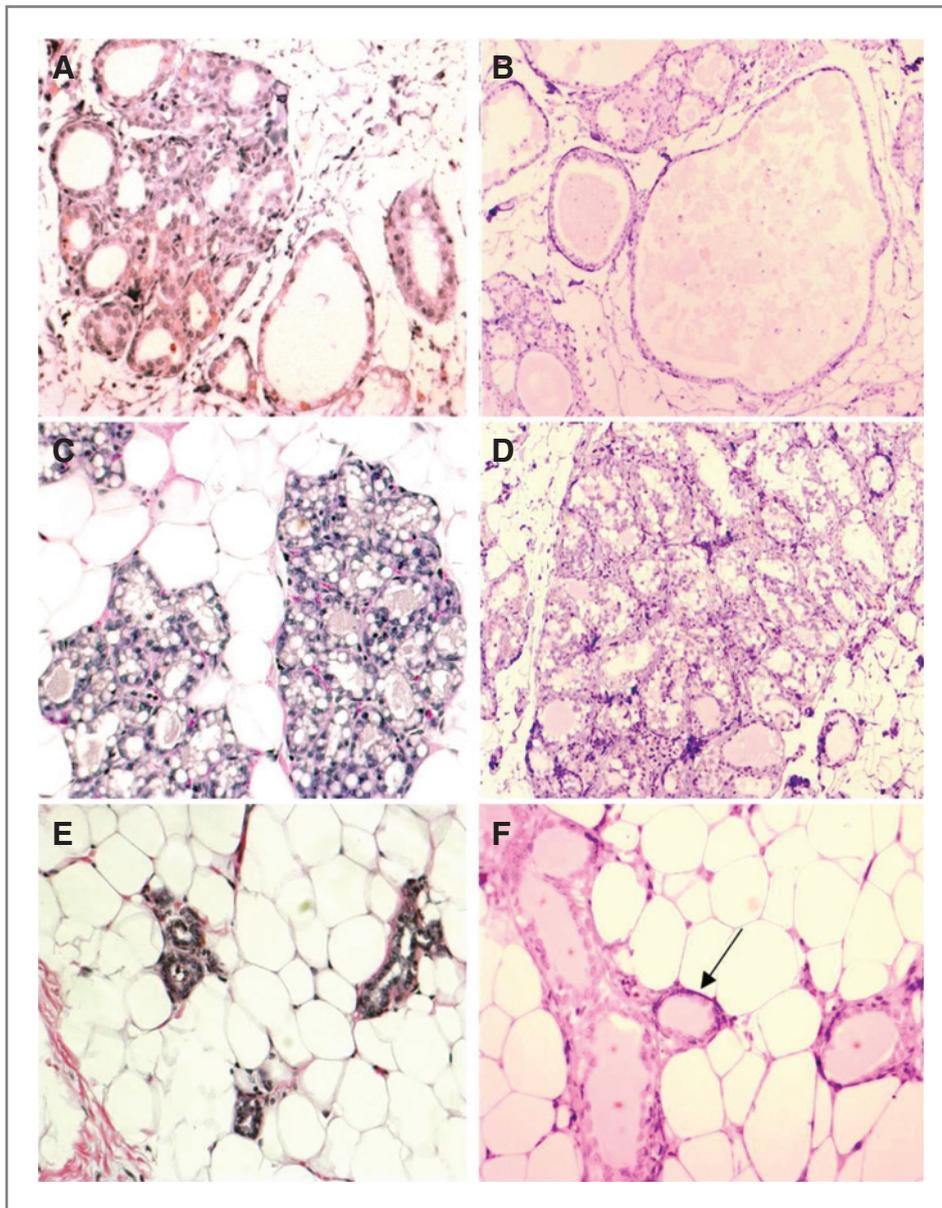
**CDB-4124 suppresses cell proliferation and induces apoptosis.** "To understand better the potential cellular mechanisms of CDB-4124-induced alterations in mammary gland architecture, proliferation activity, and apoptosis of MECs of control and treated with CDB-4124 animals were examined." As shown in Table 3, CDB-4124 significantly decreased the percentage of Ki-67-positive cells, from  $16.5 \pm 5.4\%$  in placebo to  $9.1 \pm 3.4\%$  ( $P < 0.001$ ) 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  $1.0 \pm 0.7\%$  versus  $1.2 \pm 0.7\%$  ( $P < 0.2$ ) 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 the 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 versus 55 days in the low-dose and 59 days in the high-dose CDB-4124 groups (Table 4). The average tumor latency for the control group was  $66 \pm 24$  days versus  $74 \pm 21$  days for the low-dose and  $87 \pm 20$  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, Fig. 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. Histologic 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 MNU tumors of control and CDB-4124-treated animals. CDB-4124 significantly suppressed Ki-67-positive cells in mammary tumors, from  $30.5 \pm 5.4\%$  in the placebo-treated group to  $25.4 \pm 14.4\%$  in low-dose group, and further to  $10.3 \pm 4.5\%$  ( $P < 0.001$ ) in the high-dose group of treated animals (Table 3). Both doses of CDB-4124 also induced apoptosis in mammary tumors, where  $0.7 \pm 0.4\%$  apoptotic cells were detected in the control group versus  $1.4 \pm 0.8\%$  in low-dose group ( $P < 0.05$ ) and  $1.6 \pm 0.8\%$ , 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 whether 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 ( $3 \mu\text{mol/L}$ ) treated animals, and high-dose ( $30 \mu\text{mol/L}$ ) treated animals are summarized in Table 5. The data show that CDB-4124 progressively decreased the serum progesterone level from  $14.6 \pm 9.5$  ng/mL in the placebo-treated group to  $12.5 \pm 7.9$  ng/mL in the low-dose group, and further to  $7.9 \pm 4.4$  ng/mL ( $P < 0.05$ ) in high-dose group. CDB-4124 had little effect on serum estradiol at both dose levels.



**Figure 1.** Morphological changes in mammary gland of rats treated for 2 years with CDB-4124. A: Development of lobular hyperplasia in control animals with single cystic formations; B: In CDB-4124 treated animals, cystic formations predominated in mammary gland; C and D: In some control animals lobular hyperplasia and especially in D, formation of adenomas was found. Note, the high functional activity of lobular cells with milk-like product secreted in lobular lumen; E and F: Continuous treatment with CDB-4124 reduced the ductal lateral branching (arrow) with prevalence of small lobules. Hematoxylin-Eosin staining x 400.

**CDB-4124 suppressed PR expression in mammary tumors.** Because antiprogestins have been shown to modulate PR signaling, we assumed that this might affect both ER and PR expressions in mammary tumors. To test this hypothesis, we evaluated both receptors by IHC in parallel sections of tumor tissue. As shown in Table 5, CDB-4124 decreased the proportion of PR<sup>+</sup> cells from  $48 \pm 11\%$  in placebo-treated animals to  $32 \pm 12\%$  in CDB-4124 (30 mg/kg) treated animals ( $P < 0.01$ ). Although trending lower, CDB-4124 did not significantly affect the proportion of ER<sup>+</sup> 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  $\mu\text{mol/L}$  CDB-4124 for 3 days or 6 days, and cell number in triplicate was determined by cell counter (Fig. 3). CDB-4124 at 0.1  $\mu\text{mol/L}$  did not affect cell growth after either 3 days or 6 days of treatment, whereas doses of 1.0  $\mu\text{mol/L}$  and 10.0  $\mu\text{mol/L}$  suppressed cell growth in a dose-dependent manner. After 3 days of treatment with CDB-4124, the cell number decreased from  $138.4 \pm 4.2 \times 10^3$  cells/mL in the placebo-treated culture to  $112.5 \pm 10.5 \times 10^3$  cells/mL at 1.0  $\mu\text{mol/L}$  CDB-4124 and to  $68.2 \pm 6.7 \times 10^3$  cells/mL at 10.0  $\mu\text{mol/L}$  CDB-4124. After 6 days of treatment, the difference in cell number further decreased from,  $560 \pm 32.6 \times 10^3$  cells/mL in placebo-treated culture

**Table 2.** Development of fibroadenomas, lobular hyperplasia, atypical lobular hyperplasia and cystic formations in mammary gland of animals followed for 23.5 months

Lesions (number)	CDB-4124 Dose in mg/kg body weight			
	0	20	70	200
N at study end	16	16	22	22
Fibroadenomas	11 <sup>a</sup>	0 <sup>a</sup>	3 <sup>a</sup>	2 <sup>a</sup>
Lobular hyperplasia	40 <sup>a</sup>	21 <sup>a</sup>	25 <sup>a</sup>	22 <sup>a</sup>
Atypical lobular hyperplasia	11 <sup>a</sup>	3 <sup>a</sup>	1 <sup>a</sup>	2 <sup>a</sup>
Cystic structures	3 <sup>a</sup>	n/a	n/a	10 <sup>a</sup>

NOTE: These lesions were identified by pathologist on formalin fixed, paraffin-embedded tissue sections stained by H&E.

<sup>a</sup>Significant difference in the values between CDB-4124-treated and placebo-treated animals ( $P < 0.05$ ).

Abbreviation: n/a, not available.

to  $464 \pm 24.3 \times 10^3$  cells/mL in 1.0  $\mu\text{mol/L}$  CDB-4124 and further to  $48.6 \pm 6.5 \times 10^3$  cells/mL in cells treated with 10.0  $\mu\text{mol/L}$  CDB-4124.

**CDB-4124 inhibits G<sub>1</sub>/S cell cycle progression.** We also examined whether CDB-4124 differentially affected various cell cycle phases. T47D cells were treated for 3 or 6 days with 1.0  $\mu\text{mol/L}$  CDB-4124, a dose that suppressed cell growth, as shown in the text. As shown in Table 6, CDB-4124 decreased the percentage of cells in S phase from  $12.2 \pm 1.8\%$  in 3-day placebo-treated cells to  $8.5 \pm 1.6\%$  in CDB-4124-treated cells, and from  $14.1 \pm 1.7\%$  in 6-day placebo-treated cells to  $9.3 \pm 2.3\%$  in CDB-4124-treated cells ( $P < 0.01$ ). In the 6-day CDB-4124-treated group, a significant increase in G<sub>1</sub>/G<sub>0</sub> cells was also observed ( $P < 0.05$ ). These data suggest that CDB-4124 may inhibit the transition of cells from G<sub>1</sub> to S phase of the cell cycle.

**CDB-4124 decreased ER $\alpha$ , but had no effect on PRA and PRB expressions.** To support the data showing the effect of CDB-4124 on ER and PR expressions in mammary tumors, T47D cells were treated for 3 or 6 days with 1.0  $\mu\text{mol/L}$  CDB-4124 and PRA, PRB, and ER $\alpha$  expressions was deter-

mined by Western blot (Fig. 4A). Both PRA and PRB were differentially expressed at both time points, but CDB-4124 at 1.0  $\mu\text{mol/L}$  failed to affect the expression of either PR. Conversely, CDB-4124 decreased ER $\alpha$  expression in both, 3-day and 6-day treated cells.

**CDB-4124 inhibits cdk2 and cdk4 but not cyclin D1 and CDK6 expressions in T47D cells.** To corroborate the findings from the cell cycle analysis, cells treated for 3 or 6 days with 1.0  $\mu\text{mol/L}$  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 (Fig. 4B). Analysis of the blot implied that CDB-4124 did not affect cyclin D1 or CDK6 expressions, but decreased CDK2 and CDK4 expressions 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, premalignant, and spontaneous tumors when the agent is provided

**Table 3.** Effects of CDB-4124 on cell proliferation and apoptosis

	CDB-4124 (mg/kg)	n	Ki-67 (%)	P	Apoptosis (%)	P
Mammary Lobules						
Placebo	0	16	$16.5 \pm 5.4$		$1.0 \pm 0.7$	
CDB-4124	200	22	$9.1 \pm 3.4$	0.001	$1.2 \pm 0.7$	ns
MNU Tumors						
Placebo	0	19	$30.5 \pm 7.1$		$0.7 \pm 0.4$	
CDB-4124	3	20	$25.4 \pm 14.4$	ns	$1.4 \pm 0.8$	0.05 <sup>a</sup>
CDB-4124	30	20	$10.3 \pm 4.5$	0.001	$1.6 \pm 0.8$	0.01 <sup>a</sup>

NOTE: 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 Materials and Methods). At least 1,000 lobular cells were examined in toxicity study and the values compared with those of placebo-treated animals. In mammary tumors peripheral tumor areas free of necroses were examined for Ki-67 and apoptotic cells.

<sup>a</sup>The differences in the values are significant ( $P < 0.05$ ) as compared with those of the control animals (Student-Fisher *t* test).

Abbreviation: ns, not significant.

**Table 4.** Tumor latency, incidence, multiplicity, and burden in CDB-4124–treated mammary tumors

Dose (mg/pellet)	<i>n</i>	First tumor (days)	Latency (days)	Incidence (%)	Multiplicity	Burden (g)
0	20	49	66.4 ± 23.5 <sup>a</sup>	85 <sup>b</sup>	3.0 <sup>b</sup>	2.16 ± 4.40 <sup>a</sup>
3	20	55	73.5 ± 21.1	60	2.2	0.62 ± 1.87 <sup>a</sup>
30	20	59	87.1 ± 19.5 <sup>a</sup>	35 <sup>b</sup>	1.1 <sup>b</sup>	0.26 ± 0.33 <sup>a</sup>

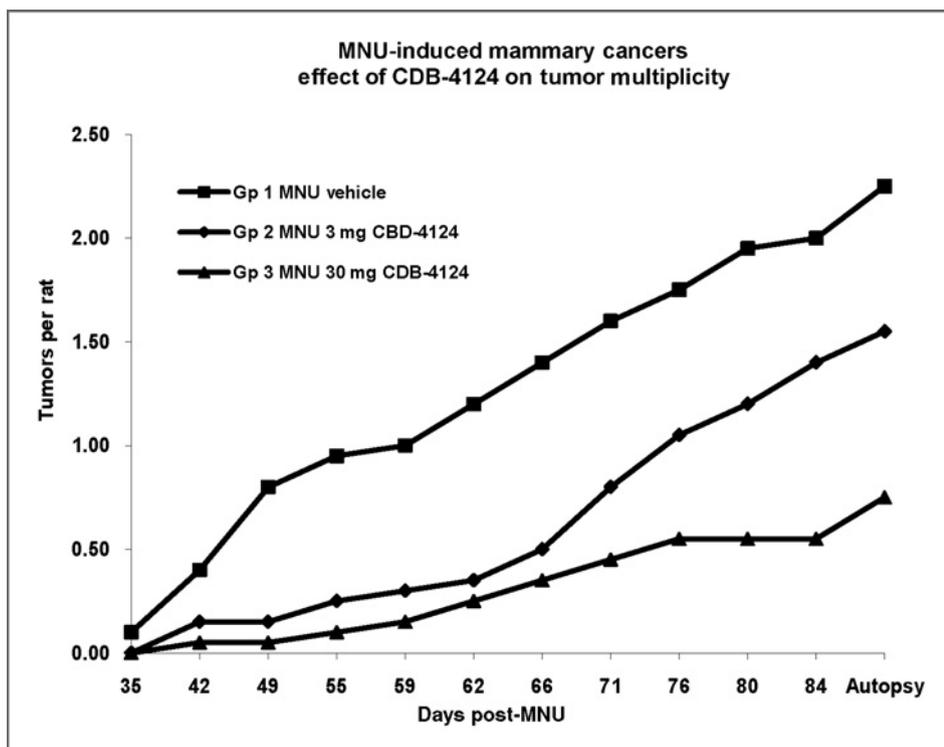
<sup>a</sup>Significant ( $P < 0.05$ , Student–Fisher *t* test).

<sup>b</sup>Significant ( $P < 0.001$ ,  $\chi^2$  test).

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 antiprogesterins, CDB-4124 suppressed cell growth and this correlated with inhibition of CDK2 and CDK4 expressions and consequently with G<sub>1</sub>/G<sub>0</sub>–S cell cycle block. These alterations in cell cycle progression appear to be associated with a downregulation of ER $\alpha$  expression, and a decrease in the population of PR<sup>+</sup> cells in MNU-induced tumors.

The proliferation and apoptotic markers were investigated in the MNU-induced tumors that remained after the treatment period. We did not sacrifice animals at more than

one time period. These investigated tumors 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 later in the text. 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 observed 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



**Figure 2.** Effect of CDB-4124 on tumor multiplicity. The number of tumors per animal was determined at the end of experiment after sacrifice and histological examination of mammary gland lesions. On the abscissa is presented the frequency of mammary tumor as function of time after injection of carcinogen. On the ordinate is given the number of tumors per rat. There is clear dose dependent decrease in tumor number in CDB-4124–treated vs. control animals. Filled boxes represent animal given MNU plus vehicle. Filled diamonds represent animals given MNU plus the lower dose of CDB-4124 in a pellet (3 mg over almost 90 days). Filled triangles represent animals given MNU and the higher dose of CDB-4124 in a pellet (30 mg over almost 90 days).

**Table 5.** Effects of CDB-4124 on serum<sup>a</sup> estradiol, serum progesterone<sup>b</sup>, and hormone receptors in MNU-induced mammary tumors<sup>c</sup>

Dose ( $\mu\text{mol/L}$ )	Progesterone (ng/mL)	Estradiol (pg/mL)	% PR <sup>+</sup>	% ER <sup>+</sup>
0	14.6 $\pm$ 9.5	36.1 $\pm$ 5.1	48 $\pm$ 11 ( <i>n</i> = 13)	58 $\pm$ 14 ( <i>n</i> = 13)
3	12.5 $\pm$ 7.9 <sup>d</sup>	38.3 $\pm$ 6.3 <sup>d</sup>	ND	ND
30	7.9 $\pm$ 4.4 <sup>e</sup>	34.7 $\pm$ 7.6 <sup>d</sup>	33 $\pm$ 12 ( <i>n</i> = 14) <sup>f</sup>	49 $\pm$ 21 ( <i>n</i> = 14) <sup>d</sup>

<sup>a</sup>Blood was collected at the time of animals' sacrifice and the serum was isolated.

<sup>b</sup>As determined by radioimmunoassay

<sup>c</sup>At least  $1 \times 10^3$  cells from the periphery of the tumor were examined.

<sup>d</sup>Not significant.

<sup>e</sup>*P* < 0.05.

<sup>f</sup>*P* < 0.01.

Abbreviation: ND, not determined.

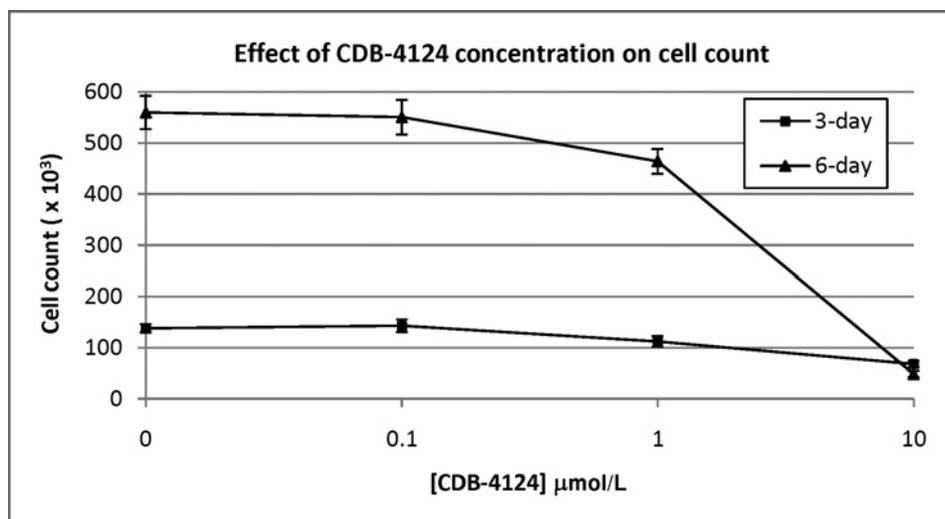
parenchymal organs when examined at the end of experiment. However, histologic examination of mammary gland in CDB-4124-treated rats revealed dose-dependent inhibition of lobular hyperplasia and fibroadenomas that was associated with the development of cystic formations in mammary gland parenchyma. The inhibitory effect of CDB-4124 on mammary carcinogenesis appears to be a consequence of decreased ductal-lateral branching. This hypothesis is supported by recent data on *BRCA1/p53*-transgenic mice indicating that RU-486, another antiprogesterin, suppressed mammary carcinogenesis by inhibiting ductal-lateral branching and lobular differentiation of MECs (29). These data also suggest that patients with mutations in *BRCA1* may benefit from antiprogesterin 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

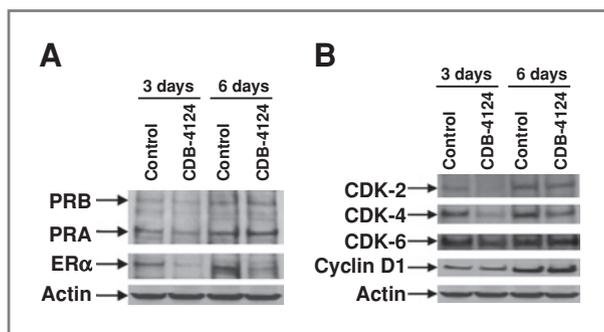
result of CDB-4124 treatment. This has also been observed in the endometrium of women treated for uterine fibroids and endometriosis with the same agent (30).

The inhibitory effect of CDB-4124 on cell proliferation we see in both, long- and short-term (3 month) treatments correlates well with recent data from a clinical trial with mifepristone (RU-486). Women with leiomyoma, treated with 50 mg RU-486 every second day 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 versus placebo-treated patients, suggesting that antiprogesterin treatment can prevent the development and progression of ER<sup>+</sup> and PR<sup>+</sup> breast cancer by inhibiting MEC 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

**Figure 3.** Effect of CDB-4124 on T47D growth. T47D cells were grown for 24 hrs in 6-well plates starting with 104 cells. In each well were put 3 ml of cell suspension. Cells were treated for 3 or 6 days with 0, 0.1, 1.0, 10.0  $\mu\text{mol/L}$  CDB-4124. At the end of experiment the total number of cells measured by cytometer. A dose dependent decrease in cell number was observed at both time points. CDB-4124 at both 1.0 and 10.0  $\mu\text{M}$  significantly decreased (*p* < 0.05) cell number.





**Figure 4.** Effects of CDB-4124 treatment on steroid hormone receptors, CDKs and Cyclins in T47D cells. Cells were plated into 6-well plates at  $10^4$  cells per well and allowed to attach. Cells were then treated for 3 or 6 days with control media (DMSO) or 1.0  $\mu\text{mol/L}$  CDB-4124. Cells were lysed and total protein was isolated. A. Western blot showing effects of CDB-4124 on ER and PR expression. B. Western Blot showing effects of CDB-4124 on CDK2, CDK4, CDK6, and cyclin D1. Actin was used as a loading control.

it has been shown that tamoxifen suppresses the growth of approximately only 50% of ER<sup>+</sup> breast carcinoma (2, 3), antiprogesterins alone or in combination with tamoxifen may improve the clinical outcome (29). Moreover, in the course of breast cancer treatment with tamoxifen, approximately 50% of ER<sup>+</sup> 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 G<sub>1</sub>/G<sub>0</sub> cells, suggesting a G<sub>1</sub>/S cell cycle block. This observation was supported by a reduction in CDK2 and CDK4 expressions as determined by Western blot. CDB-4124 did not appear to affect cyclin D1 and cdk6 expressions, suggesting that antiprogesterins may modulate specific cell cycle targets as part of their antiproliferative action. Because p27<sup>Kip1</sup> and p21<sup>Cip1/Waf1</sup> are inhibitors of CDK2 and CDK4 (19, 20), respectively, their expressions were 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 cdk6 to determine their selective response to antiprogesterins. Because CDB-4124

induced apoptosis in mammary tumors, in addition to inhibiting cell proliferation, we assessed Bcl-2, Bax, caspase 3, and cleaved caspase-3 expressions in control and in cells treated with CDB-4124. However, no significant changes in these biomarkers of apoptosis were found in CDB-4124-treated cells versus placebo-treated cells (data not shown).

We also found that CDB-4124 inhibits ER $\alpha$ , but not PRA and PRB expressions in T47D cells which contradicts the results from MNU-induced mammary tumors where the same antiprogesterin preferentially suppressed PR<sup>+</sup> cells, as determined by IHC. Because ER $\alpha$  has been shown to regulate PR expression (33), we expected that CDB-4124-induced downregulation of ER $\alpha$  in T47D cells would lead to downregulation of PR as well. In fact, in MNU-induced mammary tumors CDB-4124 also appears to decrease ER<sup>+</sup> cells, from  $58 \pm 14\%$  in placebo-treated to  $49 \pm 21\%$  in CDB-4124-treated animals, although the difference between groups was not statistically significant (Table 5). We may speculate that small decreases in ER $\alpha$  expression may trigger more significant decreases in PR expression, as has been previously reported (6, 33). However, various factors may affect ER and PR expressions, such as the dose and duration of CDB-4124 treatment. We may speculate that a dose of 1.0  $\mu\text{mol/L}$  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 anti-progestin 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 postmenopausal women (1–3, 37, 38) and the luteal effects of progesterone in the breast (39) are 2 cases that point to progesterone as a bad player with respect to proliferation. Our own work that reported on the effects of progesterone and antiprogesterins on DMBA-induced tumors (12), in conjunction with that given here,

**Table 6.** Effects of CDB-4124 on cell cycle progression of T47D cells

Phase	3 Days		6 Days	
	Control	CDB-4124	Control	CDB-4124
G <sub>1</sub> /G <sub>0</sub> (%)	80.4 $\pm$ 3.2	85.2 $\pm$ 3.8	75.3 $\pm$ 2.5 <sup>a</sup>	81.9 $\pm$ 3.2 <sup>a</sup>
S (%)	12.2 $\pm$ 1.8 <sup>b</sup>	8.5 $\pm$ 1.7 <sup>b</sup>	14.1 $\pm$ 1.7 <sup>b</sup>	9.3 $\pm$ 2.3 <sup>b</sup>
G <sub>2</sub> M (%)	7.5 $\pm$ 2.1	5.2 $\pm$ 1.2	10.6 $\pm$ 1.4	8.8 $\pm$ 2.0

<sup>a</sup>Significant increase compared with control.

<sup>b</sup>Significant decrease compared with control.

further supports the view that progesterone can stimulate proliferation. In the present case, we have used an anti-progestin in T47D cells at a relatively high culture concentration (1–10  $\mu\text{mol/L}$ ). The observed activity of CDB-4124 could be considered as agonistic in this setting. We have seen earlier in the study by Wiehle and colleagues (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 MNU model used here provides constant but relatively low amounts of the anti-progestin (3 or 30 mg over 90 days) and retains antagonism toward cell growth. Although 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 anti-progestins 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 specific estrogen receptor modulators (SERMs) on the modulation of ER and PR signaling, and/or on potential involvement of coactivators and corepressors. The development of therapy resistance in ER<sup>+</sup> breast carcinomas after treatment with tamoxifen may offer additional possibilities for clinical applications of PR 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.

### Disclosure of Potential Conflicts of Interest

Repros Therapeutics provided CDB-4124 for conducting this study. Dr. R. Wiehle was involved in planning of both studies, in assessment the toxicity and antitumor efficacy of CDB-4124 in MNU carcinogenesis model. Toxicity study was performed at MPI Research Facility, Mattawan, MI, whereas short-term cancer prevention study was performed at the University of Illinois at Chicago Biology Research Laboratory. Daniel Lantvit, BS, was responsible for animal experiments and monitoring tumor development and progression. Dr. Tohru Yamada, PhD., help in western blots and in evaluation the effect of CDB-4124 on ER and PR in T47D cells. Dr. Konstantin Christov, MD., PhD. was responsible for cancer prevention study and biomarkers validation. Both, D. Lantvit and K. Christov were compensated for their work on this project. R. Wiehle is an employee of and a stock holder in Repros Therapeutics.

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# Cancer Prevention Research

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

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