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The selective progesterone receptor modulator, telapristone acetate, is a mixed antagonist/agonist in the human and mouse endometrium and inhibits pregnancy in mice

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Running Title: CDB-4124 inhibits decidualisation and implantation

Key words SPRM, Progesterone, Estradiol, Implantation, decidualisation, DNA synthesis

ABSTRACT

Objective

To investigate the actions of selective progesterone receptor modulator, telapristone acetate (CDB-4124) on endometrial biology and reproductive outcomes. Ovariectomized and hormone treated CD1 female mice, CD1 female mice with xenotransplants of reconstructed human endometrial tissue, mated wild type female mice, and cultured human endometrial stromal cells (hESC) were treated with CDB-4124 followed by the assessment of endometrial cell DNA proliferation, stromal decidual response and embryo implantation.

Design

Experimental study

Setting

Academic research laboratory

Patients

Healthy volunteer women were recruited for endometrial biopsies from the community .

Intervention

Treatment of mice and hESC with CDB-4124.

Main outcome measure

The effect of CDB-4124 on endometrial cell morphology and DNA synthesis, decidual response and mouse embryo implantation.

Results

CDB-4124 inhibits E2-induced epithelial DNA synthesis in the mouse uterus and the xenotransplanted human endometrium. This anti-proliferative effect was less than that of P4 and was observed when CDB-4124 was administered alone or concomitantly with P4. In the uterine epithelium CDB-4124 is a P4 agonist and partial antagonist. In contrast, CDB-4124 acts as a complete P4 antagonist in the uterine stroma where it blocked P4's action to induce decidual response in the mouse pseudopregnant uterus and the wildtype mouse uterus after copulation. In the mated female mice CDB-4124 impaired embryo implantation. Similarly, CDB-4124 inhibited morphological and biochemical transformation of hESC to decidual cells *in vitro* .

Conclusion

CDB-4124 exerts mixed P4 antagonistic/agonistic effects in the human and mouse endometrium that result in failed embryo implantation due to absence of stromal decidualization.

Capsule: The effects of CDB-4124 on progesterone action are both antagonistic and agonistic in the endometrial epithelium but only antagonistic in the stroma that together results in the inhibition of implantation.

INTRODUCTION

Progesterone receptor modulators (PRMs) were developed to modulate the actions of progesterone receptor (PR) in progesterone (P4) responsive tissues where they exert mixed agonist/antagonist responses (1). The first member of this class was mifepristone (2) that predominantly has P4 antagonistic activity. Second-generation PRMs were developed in an attempt to identify relatively pure P4 antagonists with minimal or no anti-glucocorticoid activity and these include synthetic ligands such as asoprisnil, ulipristal acetate (UPA, CDB-2914) and telapristone acetate (CDB-4124). CDB-4124, a 21-subsitituted-19-norprogestin, has high PR specificity and marginal anti-glucocorticoid, estrogenic and androgenic properties (3). By antagonizing P4 effects, PRMs have considerable therapeutic promise for hormone-dependent gynecological disorders, such as leiomyomata, endometriosis and menorrhagia (4). Phase 2 clinical trials of telapristone acetate for the treatment of uterine fibroids in were halted due to liver toxicity in 2006; however, the trials were restarted using lower oral dose and vaginal administration (1). In addition to treatment of benign reproductive conditions, PRMs can be

82 used to prevent undesired pregnancy. Ulipristal acetate (Ella^{TM}, Watson Pharmaceuticals), a derivative
83 compound of CDB-4124, is in clinical use as effective post-coital contraceptive with efficacy lasting up
84 to 5 days after unprotected intercourse or contraceptive failure (5, 6). Besides delaying ovulation, when
85 taken prior to ovulation, administration of UPA may impair endometrial maturation, which can
86 contribute to its longer contraceptive benefit ((7) (8). Ulipristal acetate, a derivative compound of
87 CDB-4124, is in clinical use as effective post-coital contraceptive (5, 6).
88
89 Clinical studies have reported that daily administration of PRMs induces anovulation and most women
90 become amenorrhoeic (9, 10). Lack of ovulation is the main reason for amenorrhea, but endometrial
91 changes may play a role. Observations point to untypical endometrial changes after PRM exposure and
92 endometrial biopsies sampled from premenopausal women after treatment with mifepristone, UPA and
93 CDB-4124 demonstrated altered architectural features of the endometrium that did not represent
94 premalignant or malignant changes (9-12). Subsequently, these changes that include extensive cystic
95 dilatation of the glands with the glandular epithelium showing low levels of mitotic activity, and
96 elevated incidence of apoptosis were designated as progesterone receptor modulator-associated
97 endometrial changes (PAEC) and encountered in ~70% of women after short-term exposure (3 months)
98 (9, 13-15). While the simultaneous presence of mitotic activity and apoptotic bodies in the glandular
99 epithelium indicates activation of apoptotic cell death to counteract cell proliferation (9), the net effect
100 of epithelial cell proliferation is unknown. Consequently, the endometrial safety of the long-term PRM
101 therapy remains a concern.
102
103 The human and mouse endometrium undergoes extensive cyclic changes in the preparation for embryo
104 implantation in response to changes in circulating estradiol (E2) and P4 that are essential for
105 implantation. E2 elicits epithelial cell proliferation, whereas after copulation in mice and after
106 ovulation in women P4 antagonizes this E2 induced epithelial proliferation. In the mouse E2 activates
107 two pathways that act in parallel to induce DNA synthesis and cell division, and both pathways are
108 blocked by P4 (16, 17). In the first pathway, the paracrine signaling of stromal insulin growth factor
109 (IGF) acting through the receptor, IGFR1 in the uterine epithelium activates a P13-kinase dependent
110 cascade that leads to cyclin D/cyclin-dependent kinase 4 (CDK4) complexes being retained in the
111 nucleus followed by phosphorylation of retinoblastoma (RB) family of proteins by cyclin E and then A
112 in combination with CDK2 and subsequent orderly progression of the canonical cell cycle pathway
113 (18-20). The activation of the second pathway leads to DNA replication licensing through control of
114 the activity of the hexameric minichromosome maintenance (MCM) protein pre-initiation complex (17).
115 Similar mechanisms appear to operate in humans although the details remain less clear (21).
116
117 In mice and humans, another prerequisite for successful implantation is differentiation of endometrial
118 stromal cells into secretory tissue, known as the decidua, which is essential to support the growth of the
119 embryo. This transformation process, called decidualization is driven by E2 and P4 in both species, but
120 also some critical differences exist. In the mouse uterus, stromal differentiation starts with cell
121 proliferation followed by final decidualization in the presence of embryonic signal. In contrast, human
122 stromal decidualization occurs during the secretory phase of each menstrual cycle. Decidualization is
123 guided by the activation of several P4 regulated genes that in part activate paracrine signaling between
124 epithelial and stromal compartments (22). In cultured hESC UPA inhibits P4 induced *in vitro* stromal
125 decidualization (23) however, similar studies have not been performed using CDB-4124. Moreover,
126 whether PRMs prevent embryo implantation remains unknown.
127
128 The unique morphological changes of the endometrium by PRMs led us to systematically investigate
129 the effects of CDB-4124 on DNA synthesis and cell morphology in different cell compartments of the
130 mouse and human endometrium. We also sought to investigate the impact of CDB-4124 on

131 reproductive outcomes in the mouse uterus. Our studies suggest that CDB-4124 acts as a partial P4
132 agonist in the mouse and human endometrial epithelium and as a complete P4 antagonist in the mouse
133 and human stroma.
134
135
136 **MATERIALS AND METHODS**
137
138 **Animals and treatment**
139 Female CD-1 mice (Charles River, Wilmington, MA) were maintained in accordance with the NIH
140 Guidelines and the Animal Institute Committee of the Albert Einstein College of Medicine approved all
141 procedures and protocols. 8 weeks old mice were ovariectomized, rested for two weeks. These mice
142 were subjected to a variety of hormonal regimens optimized to reproduce natural estrus cycles, pre-
143 implantation development and decidualization in mice (16). First all mice were primed with a
144 subcutaneous (SC) injection of E2 as described (16). Two days later they were randomly assignment to
145 one of the following treatments: (i) peanut oil on day 4, (ii) 50 ng E2 alone on day 4 (iii) 1 mg P4 for
146 four days and 50 ng E2 on the last day, (iv) a range of concentrations (0.25-4.0 mg/mouse) as specified
147 of CDB-4124 (provided as a gift from Repros Therapeutics, Inc., The Woodlands, TX) and 50 ng E2,
148 (v) CDB-4124 2.0 mg/mouse alone for four days or (vi) 1 mg P4 for four days plus CDB-4124 2.0 mg
149 with E2 50ng on the fourth day (Figure 1). E2 was dissolved in ethanol and P4 in peanut oil. In all
150 cases except when noted mice were euthanized 15 h after the last E2 injection. In some cases, to assess
151 the percentage of cells in S-phase, bromodeoxyuridine (BrdU) was given intra-peritoneally 2h before
152 sacrifice. Uteri were removed and processed for immunohistochemistry (IHC).

153 The artificial induction of decidualization has been previously described (24). Briefly, 8-week old CD-
154 1 female mice were ovariectomized, rested for 2 weeks and primed with 100 ng of E2 for 2 days. After
155 2 days of rest, animals received daily injections of (i) 10 ng E2 and 1 mg P4 (control group) or (ii) 10
156 ng E2, 1 mg P4 plus 2 mg CDB-4124 (experimental group) for 3 days. Six hours after the last injection,
157 mice were anesthetized and peanut oil (10 μl) was infused intra-luminally in one uterine horn; the
158 contralateral horn served as a control with PBS injection (10 μl). Mice were euthanized 48 hours later,
159 and weights of the uterine horns were recorded to assess the extent of decidualization. After fixation in
160 10% v/v buffered formalin, transverse sections (5 μm) through the mid-point of the uterus were
161 prepared for Hematoxylin and Eosin (H&E) staining and the presence of a decidual response was
162 assessed.

163 To assess implantation, 10-week CD1 male and female mice with proven fertility were caged and
164 female animals were assessed daily for vaginal plugs. The plugged females (plug Day 1) were
165 separated and injected subcutaneously with 2mg CDB-4124 from Day 4-6 to specifically target the
166 time period after ovulation while the control animals received peanut oil vehicle injections. Animals
167 were randomly ascribed to groups. All female mice were euthanized on Day 7 of pregnancy.
168 Reproductive outcomes were assessed by recording uterine weights and visually counting numbers of
169 implantation sites (embryo chambers). Uteri were fixed for histology and H&E staining.

170 **Human Endometrial Biopsies and Treatment**

171 Healthy volunteer women, ages 18-45, with regular menstrual cycles were recruited from the
172 community. The study was approved by Albert Einstein College of Medicine, IRB #2007-537 and
173 conducted in accord with the Declaration of Helsinki for medical research involving human subjects at
174 the General Clinical Research Center. Written informed consent was obtained from each study
175 participant. Participants were selected with no history of infertility or gynecological condition, had not

176 used hormonal contraception within 3 months prior to study inclusion and were at least 90% normal
177 weight for height with exclusions as described. The average age of the participants was 32 years.
178 Screening was done solely for study purposes included history, physical exam, negative urine
179 pregnancy test and normal saline hysterosonography to rule out any intrauterine pathology (25, 26).
180 Endometrial samples were collected during proliferative phase of the menstrual cycle using a Pipelle
181 catheter and placed in Hank's balanced salt solution (Corning, Manassas, VA) for transport and
182 processing as below.

183 Human endometrial tissues was xenotransplanted into the kidney capsule of Nude immune-
184 incompetent mice as described before (25) with minor modifications (27). Briefly, the freshly isolated
185 stromal and epithelial cells were recombined in rat-tail collagen gel and surgically placed under the
186 kidney capsule, the animals were ovariectomized and E2 pellets were placed subcutaneously. After 6
187 weeks of tissue outgrowth, the E2 pellets were removed and mice were randomly assigned to groups of
188 3 and subjected to the following treatments: (i) 5 days of daily SC injection of E2 125 ng, (ii) 5 days of
189 daily SC injection of E2 125 ng and P4 1mg, or (iii) 5 days daily SC injection of E2 125 ng and P4
190 1mg plus CDB-4124 2mg. Mice were euthanized 15 hours after the last injection, xenografts were
191 removed and fixed for IHC.

192 For human decidualization studies, stromal cells were isolated using established protocols with slight
193 modifications (28). Minced endometrial tissue was digested in McCoy 5A media (Gibco/Thermo
194 Fisher Scientific, Waltham MA) supplemented with 2%v/v chicken serum, type I collagenase
195 (2mg/mL, Worthington, Lakewood, NJ) and deoxyribonuclease I (200 IU/mL, Sigma-Aldrich, St. Lois,
196 MO) while rotating at 37°C for 1 hour. After centrifugation the cells were resuspended to 5 mL of
197 Hank's balanced salt solution followed by filtration through a 70 µm nylon mesh for cell separation.
198 The flow through containing stromal and red blood cells (RBC) was resuspended in 1X RBC lysis
199 buffer (BioLegend, San Diego, CA) to lyse RBCs. Human endometrial stromal cell (hESC) were
200 cultured in F12-DMEM supplemented with 10%v/v fetal bovine serum (FBS) and 50 µg/ml
201 penicillin/streptomycin in a 5% v/v CO_2 incubator at 37 ˚C. For in vitro decidualization, hESC were
202 seeded in 6-well plates (Costar/Corning) at 4 x 10^5 per well in DMEM/F12 without phenol red
203 supplemented with 10% v/v heat-inactivated charcoal/dextran-treated FBS (GE Healthcare Life
204 Sciences, Hyclone Laboratories, Logan, UT) and 50 µg/ml penicillin/streptomycin. When hESC
205 reached 80% confluency, the media was changed to the differentiation media of F12/DMEM
206 supplemented with 2% w/v heat-inactivated charcoal/dextran-treated FBS, antibiotics, 30 nM E2
207 (Sigma-Aldrich), 1µM P4 (Sigma-Aldrich) and one of the following treatments: i) 0.1µM CDB-4124,
208 ii) 1µM CDB-4124, iii) 3µM CDB-4124, or iv) 9µM CDB-4124. Control cells received 0.1%v/v
209 ethanol vehicle control. Cells were incubated for 9 days with treatment media changes every 48 hours
210 until harvest. The morphology of decidualization was monitored by live cells microscopy.

211

212 **Immunohistochemistry**

213

214 Uteri or endometrial biopsies were fixed overnight in 10% v/v buffered formalin phosphate and
215 processed for paraffin embedding. Transverse sections (5 µm) through the mid-point of the uterus were
216 prepared and immunostained for BrdU incorporation, or for cyclin A or MCM-2. Briefly, 5 µm paraffin
217 sections were de-paraffinized and subjected to antigen retrieval by boiling the samples in 0.01 M
218 sodium citrate buffer. Primary antibody was applied overnight at 4° C after which sections were
219 washed in phosphate-buffered saline (PBS) and then incubated with biotin-conjugated secondary
220 antibodies (Vector Laboratories). Positive signals were visualized as brown precipitates utilizing 3, 3′-

221 diaminobenzidine tetrahydrochloride (Sigma). Sections were counterstained with hematoxylin.
222 Negative controls included omission of the primary antibody. Polyclonal antibodies to BrDU (Roche),
223 cyclin A (1:100, sc-596) and MCM-2 (1:300, sc-983) were obtained from (Santa Cruz Biotechnology,
224 Santa Cruz, CA, USA). 5μm sections of the xenografted endometrial tissue were stained as above with
225 anti-MKI67 antibody to assess DNA synthesis. The nuclear labeling or proliferative index was
226 represented as a percentage of nuclear positive luminal and glandular epithelial cells for each antibody
227 stain. At least 100 total cells were counted on slides that contained tissue sections that were at least 20
228 μm apart so that the same area was not counted twice.
229
230

231 **RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)**

232 Mouse uteri were homogenized in 1 ml TRIzol (Ambion, Austin, TX, USA). In human studies, the
233 harvested hESC were stored in TRIzol (Life Technologies, Grand Island, NY) at -80°C. In each case,
234 total RNA was isolated and purified using miRVana miRNA Isolation kit (Life Technologies) and total
235 RNA extracted as described (29). The RNA yield and quality were assessed using the Agilent
236 Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA) and spectophotometric analysis. For
237 cDNA synthesis, one milligram of RNA was reverse transcribed using Supercript III first Strand
238 Synthesis System (Life Technologies). Quantitative real-time PCR was run in triplicate using Power
239 SYRB Green master mix (Life Technologies) according to the manufacturer's instructions and using
240 ABI Prism 7900HT (Amersham-Pharmacia, Piscataway, NJ). The oligonucleotide sequences for
241 primers were *Ihh* forward 'tatggactgctggcgcgctt' and reverse "gcggccgaatgctcagacttga"; *Hand2*
242 forward 'taccagctacatcgcctacct" and reverse 'tcactgcttgagctccaggg", *PRL* forward
243 'catggaaagggtccctcctg' and reverse 'gcggtcaaacaggtctcgaa'; *IGFBP1* forward 'tcacaggagacagtgtgag'
244 and reverse 'ccattccaagggtagacgca'. The qrtPCR data was normalized to expression levels of the mouse
245 and human housekeeping gene *gapdh* and *GAPDH*, respectively.

246

247 **Statistical analysis**

248 Statistical analysis was performed using ANOVA for normally distributed data or Kruskal-Wallis for
249 non-parametric data followed by Tukey's post-hoc analysis to determine the significance of the effect
250 of CDB-4124 on the proliferative index at various doses versus the control groups. For single time
251 point comparisons between controls and CDB-4124 treatments for the oil induced decidualization and
252 embryo implantation, Student's *t*-test was used and data presented as mean ± S.D. Relative gene
253 expression was calculated using $2^{-\Delta\Delta CT}$ method (30) and data are presented as mean ± SEM. *P*-value
254 <0.05 was considered significant. GraphPad Prism 5 (La Jolla, CA) was used for statistical analysis.
255
256 **RESULTS**
257
258 **CDB-4124 is a mixed P4 agonist/antagonist in the endometrium**
259
260 *Mouse uterine DNA synthesis*
261 The uterine responses in ovariectomized mice provide a controllable *in vivo* model to assess the
262 action of female steroid hormones and their derivatives. In this model E2 stimulates DNA synthesis
263 in the luminal and glandular epithelium with a peak 15 hours post-administration while P4
264 completely inhibits this response. In contrast, P4 permits E2 induction of DNA synthesis in the

265 uterine stroma with a similar time course to that observed in the epithelium (18). Thus, we used this
266 model to assess the action of CDB-4124 in the uterus (Figure 1A). As previously reported E2
267 stimulates ~80% of the epithelial cells to enter DNA synthesis as measured by BrDU incorporation
268 at 15 hours post-treatment (Figure 1B,C). This induction of DNA synthesis is completely inhibited
269 by P4 to below the level found in hormone untreated ovariectomized mice (Fig 1C). To test the
270 progestogen action of CDB-4124 we treated primed mice with different doses in the same regimen
271 as P4 (Fig1). There was a progressive dose-dependent inhibition of E2 induced DNA synthesis with
272 the maximum inhibition at 4.0 mg ($P>0.001$). This finding indicated that CDB-4124 is a P4 agonist
273 in the mouse epithelium. 4.0 mg per 100 μL injection was at the limit of solubility for this drug in
274 arachis oil, and therefore we chose 2.0mg/100μL subcutaneous injection in subsequent studies to
275 obtain complete solubility and also as the anti-proliferative effect of the 2 mg CDB-4124 was
276 similar in effect to the 4.0 mg dose. When given alone, CDB-4124 at the 2mg dose had no effect on
277 DNA synthesis being equivalent to the control untreated regimen (Figure 1B,E, H).
278
279 In the uterine epithelial compartment, we compared the effect on DNA synthesis of CDB-4124 to that
280 of P4 alone or in combination. As previously reported there is a low level of DNA synthesis in the
281 control hormone naïve mice (18) and that this DNA synthetic response is dramatically enhanced by E2
282 (Fig 1B, C, E, F). P4 pretreatment suppressed this DNA synthesis to below control levels (Fig 1 C, G).
283 CDB-4124 given at 2mg per dose to the E2 treated group also suppressed this response (Fig 1B,C, I)
284 but not with equivalent potency as P4. Furthermore CDB-4124 given together with P4 resulted in less
285 suppression of E2-induced DNA synthesis than when P4 is given alone (Fig 1C,J). Thus, in the uterine
286 epithelium CDB-4124 is a P4 agonist and acts as a partial antagonist to P4.
287
288 In the stroma, E2 alone does not induce DNA synthesis (Fig 1D, F) but pre-treatment with P4 induced
289 ~50% of the cells to go through a single synchronized wave of DNA synthesis in response to E2 as
290 shown in Figure 1D, G (18). P4 alone induces a small increase in DNA synthesis with ~10% of the
291 cells engaged in this process (Figure 1D). CDB-4124, however, does not act as a P4 agonist in this
292 tissue as it could not support E2 to induce stromal cell DNA synthesis nor did it induce DNA synthesis
293 on its own (Figure 1D,I). To test whether CDB-4124 would act as a PR antagonist or agonist in the
294 stroma it was given in combination with P4 and E2 in different regimens. CDB-4124 when given with
295 P4 resulted in a complete abolition of the P4 response in the stroma (Figure 1D, J). CDB-4124 was also
296 unable to induce any stromal cell DNA synthesis on its own with levels equivalent to control oil treated
297 mice. Furthermore, it displayed no synergistic action with E2 (Fig 1D). In the stroma therefore CDB-
298 4124 is a complete antagonist.
299
300
301 Two genes essential for DNA synthesis that are regulated by the two parallel pathways controlling this
302 process in the E2 stimulated luminal epithelium are Cyclin A and MCM2. Thus, we used IHC to assess
303 the nuclear localization of each of these proteins as described before (17, 18). E2 induces cyclin A and
304 MCM2 in most epithelial cells as previously reported (Fig2A, C, E, H) whilst E2P4 treatment blocks
305 this nuclear accumulation (Fig 2A, C, F,I) consistent with its effect on DNA synthesis. CDB-4124
306 when given with E2 had a partial but significant inhibitory effect on both cyclin A and MCM 2 nuclear
307 accumulation indicating that it is an impaired P4 agonist in the uterus (Fig 2A, C G, H). In contrast in
308 the stroma E2 has little effect on nuclear accumulation of Cyclin A or MCM2 while P4 pre-treatment
309 resulted in ~60% of cells being positive consistent with the DNA synthetic response (Fig 2B,D, F, I).
310 CDB-4124 however exhibited no activity in the stroma according to these two markers (Fig 2B, D, G,
311 J) that is also consistent with its lack of effect on DNA synthesis. Thus, in the epithelium CDP-4214
312 acts as an impeded progestin inhibiting both the canonical and DNA replication licensing pathways. In
313 contrast in the stroma CDB-4124 has no progestin activity.

314
315 *Human Endometrial Epithelial DNA synthesis.*
316
317 Xenotransplants of endometrial tissue into the kidney capsule of immunocompromized mice allows
318 assessment of hormone action in the human epithelium (25, 27). In this model E2 induced a DNA
319 synthesis response as assessed by HKI67 staining in the epithelial cells that peaks on day 5 of treatment
320 (Figure 3) (25) but not an effect on stromal proliferative responses. Hence in these experiments, only
321 epithelial tissue was analyzed (22). This E2-induced response is completely suppressed by P4 to a
322 level equivalent to the hormone naïve tissue (Figure 3). CDB-4124 acts as a partial P4 agonist
323 reducing the E2 induced response but not to the extent of P4, while when give in combination with P4
324 it acts a partial antagonist of the P4 inhibition of E2-induced DNA synthesis (Figure 3). Thus, in human
325 endometrial epithelia CDB-4124 acts in a similar manner to that observed in the mouse.
326
327 *CDB-4124 inhibits embryo implantation and decidualization*
328 The antagonist effect of CDB-4124 to P4 in the stroma was intriguing because in the mouse uterus
329 stromal proliferation is required for stromal decidualization and endometrial preparation for
330 implantation (16). Thus, we investigated the impact of CDB-4124 on embryo implantation in CD1
331 mice at day 7 of pregnancy, a time when well-developed decidual chambers are evident in untreated
332 naturally mated mice. When CDB-4124 was administered after ovulation the CD1 mice failed to
333 conceive (Figure 4 A and B). The uterine weight of these animals did not increase as would have been
334 expected for a uterus with normal stromal decidual response to P4 as was seen in the control uteri (Fig
335 4 A). Furthermore, in these natural mating's, there were no implantation sites in the CDB-4124 treated
336 mice compared to approximately 13 in the controls (Figure 4B). In addition, there was no sign of
337 decidualization in transverse sections of the uteri of CDB-4124 treated mice compared to the controls
338 that had well defined decidual chambers with embryos inside (Fig 4C, D). These results show that the
339 CDB-4124 administration after ovulation completely inhibited embryo implantation and
340 decidualization.
341
342 To further address the mechanism by which CDB-4124 impairs embryo implantation, we used a
343 pseudo-pregnant mouse model in which artificial decidualization was induced in appropriately
344 hormone treated ovariectomized mice by intrauterine oil infusion in one uterine horn and the
345 contralateral PBS infused horn served as a control. The uterine horns in animals treated with CDB-
346 4124 failed to decidualize in response to oil induction and demonstrated no change in morphology nor
347 increase in uterine weight as compared to the control uterine horn (Figure 5A and 5B). As stromal cell
348 decidualization is guided in part by the activation of several P4 regulated genes that mediate paracrine
349 signaling between the epithelial and stromal cell compartments, we investigated the expression of two
350 such genes, heart and neural crest derivatives-expressed transcript 2 (*Hand-2*) and Indian Hedgehog
351 (*Ihh*), in the mouse E2 plus P4 plus CDB-4124 treated uterus compared to E2 plus P4 treated uterus.
352 *Hand-2* is a P4 regulated gene exclusively localized to the stromal cells and has an important role in
353 decidualization (31). In addition, mouse uteri lacking *Hand-2*, maintain epithelial proliferation and
354 stimulate E2-induced pathways, resulting in impaired implantation (31, 32). *Ihh* has been shown to be
355 rapidly stimulated by P4 in the mouse uterus and expression of *Ihh* is critical in mediating the
356 communication between the uterine epithelium and stroma required for embryo implantation (32).
357 Furthermore, a conditional ablation of *Ihh* in the murine uterus results in mice that are sterile because
358 of defects in embryo implantation (33). The mRNA expression of both *Hand-2* and *Ihh* was
359 significantly reduced in CDB-4124 treated pseudopregnant mouse uterus given intraluminal oil
360 injection compared to the control uteri (Figure 5C). Collectively, these results indicate that CDB-4124
361 impairs embryo implantation by antagonizing P4 induced stromal decidual response.
362

363 **CDB-4124 inhibits hESC decidualization in vitro**
364
365 In vitro decidualization of isolated hESC in culture was monitored by measuring the mRNA expression
366 of two decidualization markers, prolactin and insulin-like growth factor binding protein 1 (IGFBP1),
367 and by monitoring cell morphology by microscopy. After 9 days of culture, hESC treated with
368 differentiation media changed from fibroblast-shaped cells to rounded decidualized cells and expressed
369 high levels of mRNA for prolactin and IGFBP1 (Figure 6A). In contrast, the expression of both
370 decidualization markers remained at the level of control cells in the cells treated with differentiation
371 media supplemented with CDB-4124 at 1μM or greater concentration (Figure 6A). In addition, the
372 morphological progression of these cells was halted and they sustained fibroblast-like phenotype of the
373 undifferentiated control cells (Figure 6B). A portion of the cells cultured in differentiation media
374 supplemented with 0.1 μM CDB-4124 changed to round decidualized cells, but not to the extent than
375 the fully decidualized hESC. In these cells the expression of prolactin and IGFBP1 was decreased by 2-
376 fold ($p= 0.15$) and 3-fold ($p=0.07$), respectively, compared to the expression levels of the fully
377 decidualized cells. However, prolactin and IGFBP1 expression levels were significantly lower in cells
378 treated with greater than 0.1 μM CDP-4124 than in the control cells. Thus, there is a dose dependent
379 inhibition of human stromal cell decidualization *in vitro* by CDB-4124.
380
381
382 **DISCUSSION**
383
384 Selective progesterone receptor modulators (PRMs) are synthetic compounds with mixed P4
385 agonist/antagonist properties in sex steroid responsive tissues, including the endometrium.
386 Understanding the precise PRM effects on endometrial functions will assist in evaluation of their safety.
387 Here, we investigated the action of telapristone acetate, CBD-4124, a derivate of ulipristal acetate (3)
388 on endometrial biology in mice and xenotransplanted human tissue. Like other PRMs, CDB-4124
389 induced PAEC changes in the mouse uterus but only in approximately 5% of mice and this lack of a
390 complete response precluded a full-scale analysis of this morphological change. However, consistently
391 in the mouse uterus, CDB-4124 suppressed E2 induced epithelial DNA synthesis less than P4, whether
392 CDB-4124 was administered alone or together with P4. Similarly, the treatment of host mice with E2
393 and CDB-4124 decreased nuclear MKI67 staining to half of the epithelial cells of the xenotransplanted
394 human endometrium compared to the treatment with E2 and P4. In a previous study, intrauterine
395 administration of UPA caused significant but not complete suppression of E2 induced epithelial DNA
396 synthesis in non-human primates (34). In both cases CDB-4124 also antagonized P4's effect on
397 epithelial DNA synthesis when administered together with P4 suggesting that it is of lower potency
398 than P4 and competed with P4 for PR binding. Whitaker et al. showed that 9-12 week treatment of
399 UPA in women reduced both glandular and stromal cell proliferation to the level observed in secretory
400 endometrium (35). This is more profound inhibition of epithelial proliferation than described in other
401 studies and the reason for it remains unclear but maybe the duration of treatment. However, taken
402 together CDB-4124 and other PRMs display P4 agonist effect in the epithelial endometrium and are
403 also antagonistic in the presence of P4 suggesting they are impeded progestins. The anti-proliferative
404 effect of PRMs on the epithelium may at least partially explain the absence of increased rates of
405 endometrial neoplasia after 3-4 months of treatment with PRM.
406
407 Endometrial decidualization of stromal cells, a P4 driven process is a necessary step for
408 endometrial receptivity and establishment of pregnancy. Our results both in the mouse uterus and hESC
409 cultures support the P4 antagonist effect of PRMs on endometrial differentiation. First, in
410 ovariectomized and hormone treated mouse CBD-4124 completely inhibited PE induced stromal

proliferation, an event required for decidual response in mouse uterus, and it also reduced expression of P4 dependent genes, *Hand-2 and Ihh,* essential for decidualization and embryo implantation, respectively (32). Further, CDB-4124 inhibited transformation of stroma to decidua after artificial stimulation in pseudopregnant mice or in the presence of embryos in the wild type mice. As previously demonstrated, the abundance of stromal tissue of the human reconstructed endometrium is limited. It also appears to be hormonally unresponsive thus preventing the evaluation of sex steroid and CDB-4124 effects on the stroma (27). Therefore, to translate our findings in mouse to human we investigated *in vitro* decidual transformation of hESC. In concordance with a previous study using another PRM, UPA (33), in the current study CDB-4124 in dose dependent fashion halted the morphological and biochemical transformation of cultured hESC to decidual cells. Furthermore, in wild type mice CDB-4124 treatment after copulation and following follicular rupture prevented embryo implantation by blocking P4 induced stromal decidual response. Using artificially induced hormonal decidualization regimens in mice CDB-4124 also blocked the stromal decidual response. Collectively, our results demonstrate CDB-4124 acts as P4 antagonist in the stroma where it blocks decidualization in humans and mice resulting in impaired embryo implantation in mice. We propose that CDB-4124, like mifepristone, elicits its P4 antagonist stromal actions through competitive binding to PR in the endometrium.

The mixed agonist/antagonist effects CDB-4124 in different endometrial cell types suggest that the engagement with the PR and its co-factors differs between these cells type. This differential binding can provide insight into the mechanism of action of P4 in controlling proliferation. While this is beyond the scope of the current study, proteomic analysis of receptor chromatin immuonoprecipitation (ChIP) of the complexes in P4 and P4/CDB-4124 groups would be of great value. Similarly, Chip sequencing (seq) of the different complexes would reveal differentially regulated target genes that may be responsible for the different proliferative status in the tissue.

Beside proven efficacy as an emergency contraceptive, PRMs also have potential application as long-term contraceptives. To this end, intrauterine delivery of UPA in rhesus monkeys that were treated with continuous E2 and cyclic P4 implants resulted in amenorrhea, thin endometrium, absence of decidual changes and partially reduced Ki67 staining of the glandular epithelium (34). After the removal of P4 implants the monkeys did not menstruate suggesting UPA inhibited P4 dependent stromal decidual response. Also, UPA alone did not induce stromal decidual response in the monkeys (34). These findings in non-human primates are almost identical to what we describe here in the mouse: PRM exhibits partial P4 agonist effect on epithelial proliferation and P4 antagonist effect on P4 induced stromal decidualization. Hence, the profound anti-decidualization effect makes PRMs promising compounds for contraceptive use while potentially providing additional medical benefits, such as reduced menstrual bleeding. However, clinical studies with longer duration of PRM administration are required to ensure safety.

Acknowledgements

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463 Center. All the animals were maintained in the animal maintenance facilities and all animal studies
464 were performed under the guidelines and protocols of the Institutional Animal Care and Use
465 Committee of the Albert Einstein College of Medicine. We thank all the women who participated in the
466 studies.
467

Figure Legends

Fig 1 CDB-4124 is a mixed Progesterone agonist/antagonist for DNA Synthesis

A. Experimental procedures described in the materials and methods for treatment of ovariectomized mice to study the effects of CDB-4124 (CDB) on P4 and E2 regulation of uterine DNA synthesis. In all cases BrDU was administered to measure cells in DNA synthesis two hours before mice were killed 15hrs after the last treatment.
B. Effects of CDB-4124 (CDB) on E2 induced uterine epithelial DNA synthesis. The proliferation index is the percentage of cells in DNA synthesis as determined by incorporated BrDU assessed by immunohistochemistry in this and panels C and D. CDB alone does not affect DNA synthesis in hormone naïve mice but acts as an antagonist to E2 in a dose dependent fashion as shown. Error bars are SEM, N= 5-15.
C. Effect of CDB on P4 antagonism to E2 for uterine DNA synthesis. CDB acts as an antagonist to E2 induced DNA synthesis but also acts as a partial antagonist to P4 ($P<0.0001$) in combined treatments. Error bars are SEM, N= 5-15.
D. CDB acts as an antagonist to P4E2 induced stromal cell DNA synthesis and in turn, did not support E2 induced DNA synthesis. Error bars are SEM, N= 5-15.
E-H: Representative transverse section of uterine epithelia stained with anti-Brdu antibody (brown stain) and counterstained with haematoxylin showing the epithelium and stroma treated as indicated on panels in referenced to the treatment regimens shown in A. Bar = 20μm

Fig 2. Effects of CDB-4124 on Cyclin A and MCM2 in the mouse uterus under different hormonal treatments.

Ovariectomised mice were treated according to the regimens shown in Figure 1A and described in the "Materials and Methods". 5 μM transverse sections of uteri derived from the experimental cohorts in Fig 1 were immunostained stained (brown) with either anti-cyclin A (A,B,E,F,G) or anti-MCM2 antibody (C,D,H,I,J).
A-D the % positive cells with nuclear stain were determined in the Luminal (LE) and glandular (GE) epithelium (A,C) or in the stroma (B,D). Error bars are SEM n = 5 for all groups. CDB-4124 antagonized E2 in the LE/GE for cyclin A and MCM2 nuclear localization in a similar fashion to P4 but to a lesser extent. In contrast CDB-4124 was not a P4 agonist in the uterine stroma.
E-J Representative transverse sections for each treatment group Bar = 20 μm

Fig 3. CDB-4124 acts as a P4 agonist and antagonist in the human endometrial transplants.

Endometrial xenotransplants were performed as described in the material and methods into nude mice. These mice after ovariectomy and removal of the E2 pellet were treated with the hormonal regimens as described in Fig 1A except that E2 was given for 5 days the point of maximal DNA synthesis and P4 and CDB-4124 were also given for the 5 days. Subsequently, the transplants were harvested and 5uM transverse sections were made and immunostained with anti-HKI67 antibody, counterstained with haematoxylin and the % of positive epithelial cells counted. E2 stimulated DNA synthesis was suppressed by P4. CDB-4124 suppressed E2 induced DNA synthesis ($p= 0.0066$) but also antagonized P4's effect ($p= 0.0147$). Error bars SEM, N=2-5 mice for each group and repeated at least twice.

Fig 4. CDB-4124 fails to support decidualization and pregnancy

516 Proven fertile female mice were mated and the day of the copulation plug noted. From 4 to 6 days post
517 plugging mice were treated daily with either a SC injection of oil alone or with 2.0 mg CDB-4124 in oil.
518 Mice were killed at day 7 and the uterine weights measured (A) and the number of implantation sites
519 counted (decidual swellings) N= 5 Error bars SEM (B).
520 C and D: Subsequently the uteri were fixed, processed and transverse sections were prepared and
521 stained with H&E. Controls showed well-formed implantations sites with embryos in the middle (C)
522 while no signs of decidualization could be found in the CDB-4124 treated mice (D). Bar = 200 uM
523
524
525 *Fig 5. CDB-4124 suppresses decidualization*
526
527 Ovariectomized mice were treated with hormonal regimens that induce pseudo-pregnancy. One group
528 was given P4E2 while the other received P4E2 and 2.0mg CDB-4124. After the final nidatory E2
529 injection, one uterine horn of each mouse was injected intra-luminally with oil to induce
530 decidualization while the other horn was injected with PBS as described in the "Materials and
531 Methods". 48hrs later uteri were retrieved, weighed, fixed and sectioned at 5μM followed by H&E
532 staining.
533 A, Representative transverse sections from the treatment groups shown were stained with H&E. Bar =
534 200um
535 B, Uterine weights for the treatment groups, N=5-15 Error bars SEM
536 C, mRNA was isolated from total uteri and prepared for QRTPCR for the following genes *Hand-2* and
537 *Ihh* and the relative mRNA expression shown normalized by *Gapdh* expression. N=5 Error bars SEM
538
539 *Figure 6. CDB-4124 blocks in vitro decidualization of human endometrial stromal cells*
540
541 Human endometrial stromal cells (hESC) were cultured as described to 80% confluency followed by
542 culture in differentiation media to induce decidualization. Cultures were maintained with P4 and E2
543 and doses of CDB-4124 as described in the "Materials and Methods" and indicated in the figure. Cell
544 morphology was monitored over 9 days and at this time point RNA isolated from the cells for
545 QRTPCR analysis.
546
547 A, Effects of different doses of CDB-4124 on P4-induced mRNA expression of the decidualization
548 markers, prolactin (*PRL*) and insulin like growth factor 1 (*IGFB1*) normalized with *GAPDH* and
549 expressed relative to the maximum expression in the fully decidualized hESC. Stromal cell isolates
550 cultured from 5 different women. N=5 Error bars show the SEM.
551
552 B, Morphology of hESC cells by phase contrast microscopy given the different hormonal regimens as
553 shown. Isolated hESC cultured in differentiation media transformed to decidualized cells with typical
554 morphology of round decidualized cells. Supplementation of the media with CDB-4124 at 1 μM or
555 higher concentration completely halted this transformation and cells remained morphologically
556 fibroblast-type cells. Bar = 80 μm.
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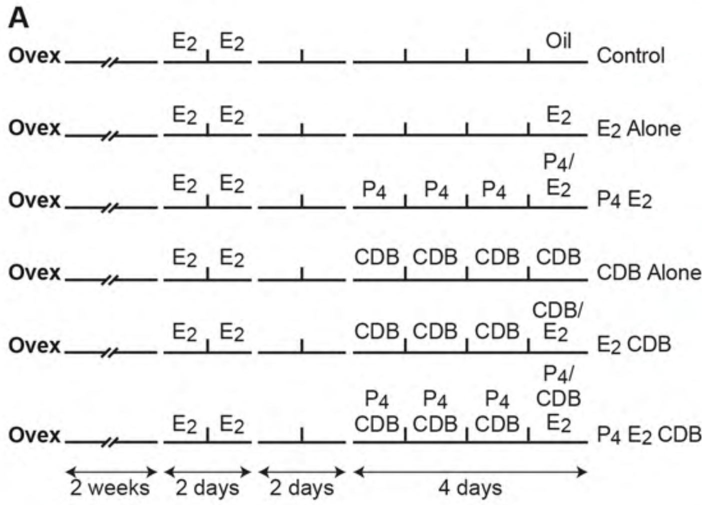
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Ovex
E2 E2
Oil
Control
E2 Alone
P4 P4 P4
P4/ E2
P4 E2
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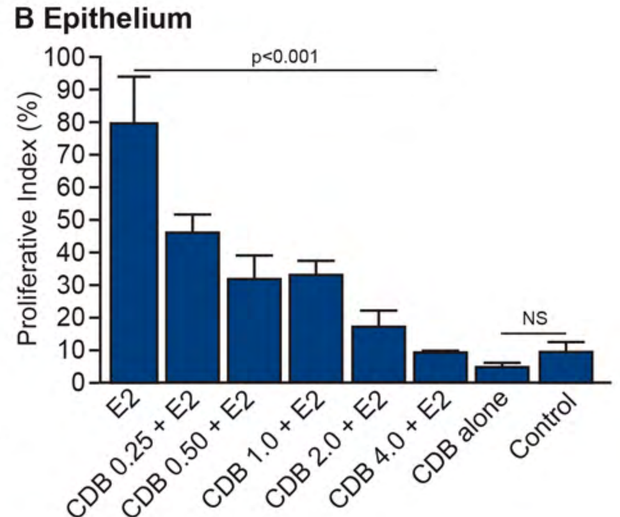
2 days
2 days
4 days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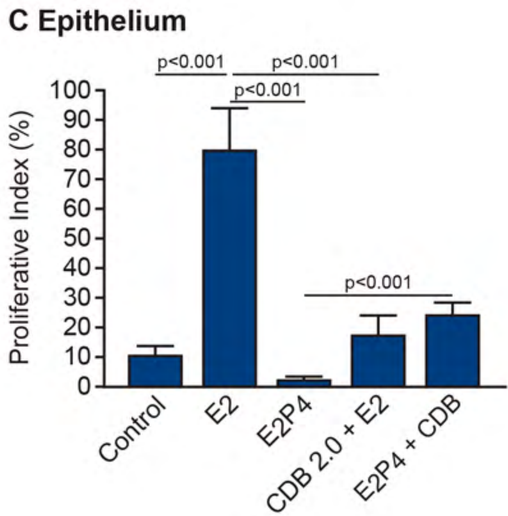
CDB 2.0 + E2
CDB 4.0 + E2
CDB alone
Control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Proliferative Index (%)
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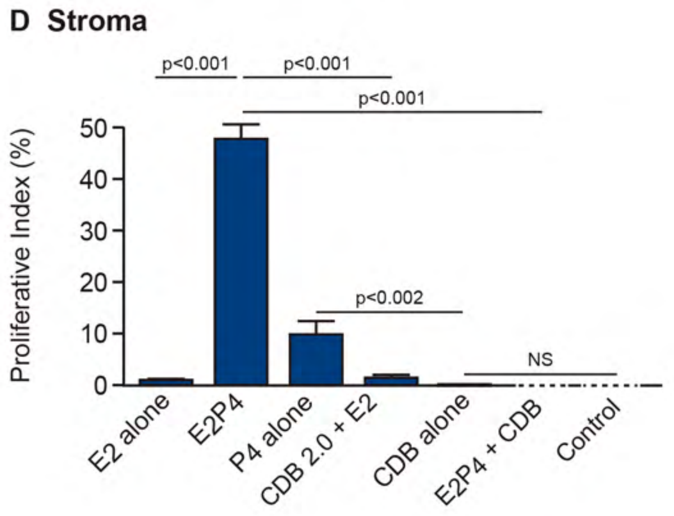
CDB 2.0 + E2
E2P4 + CDB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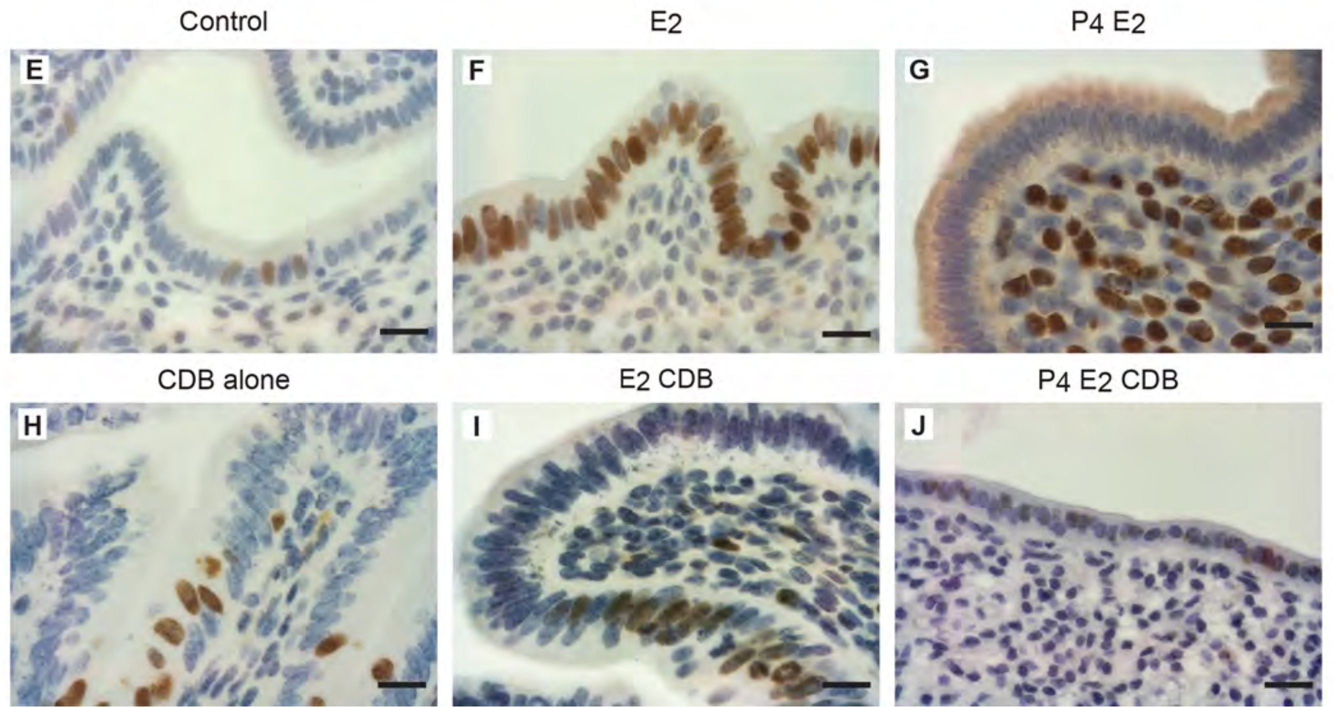
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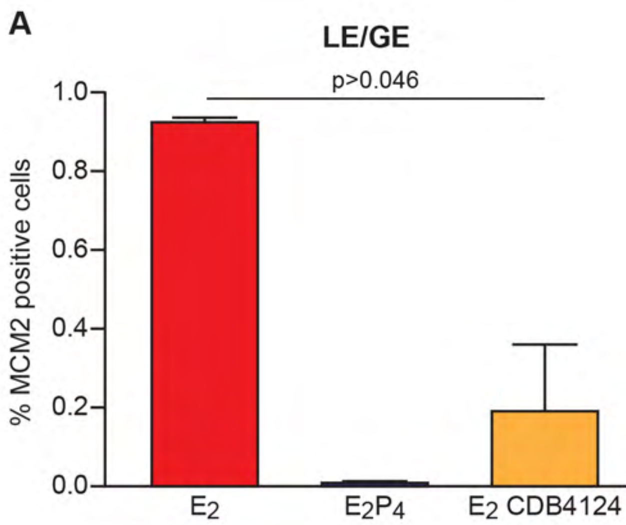

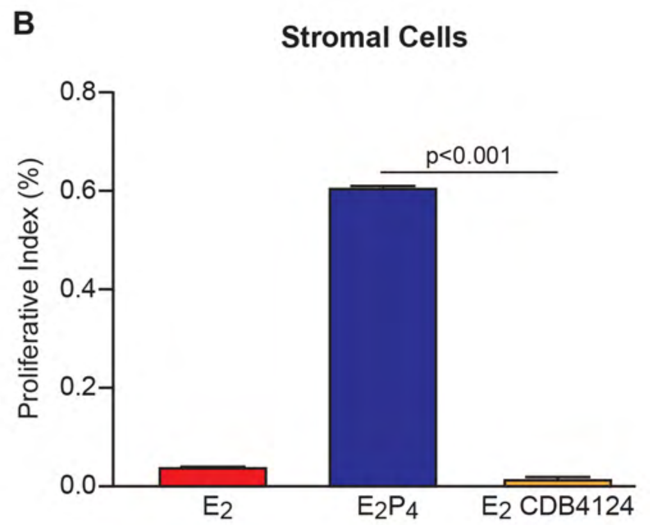

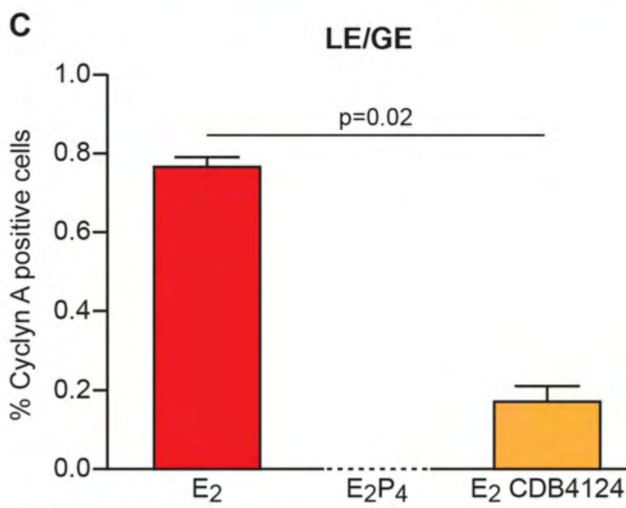

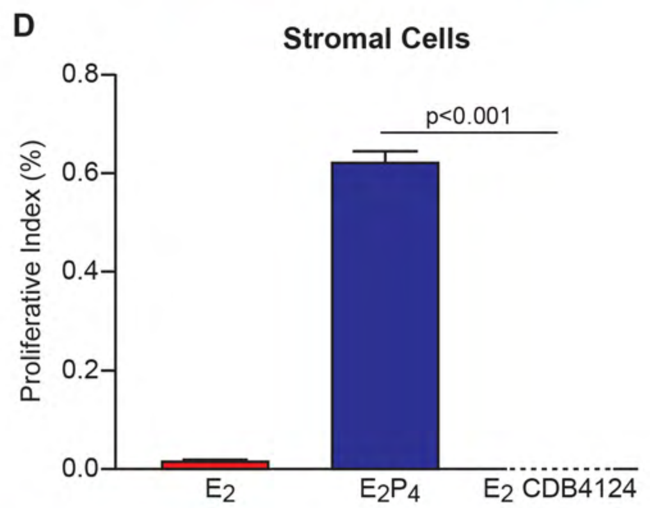

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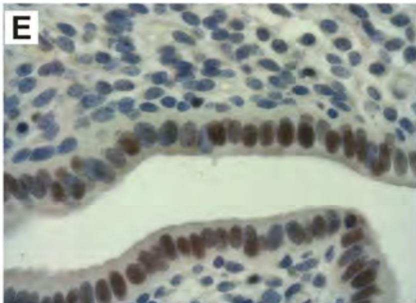
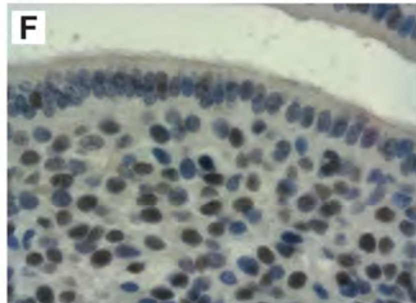
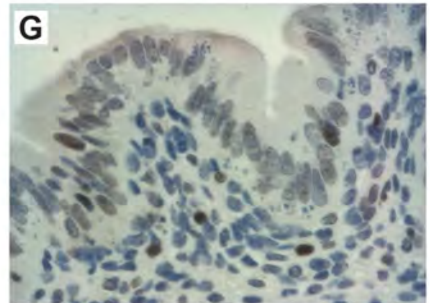
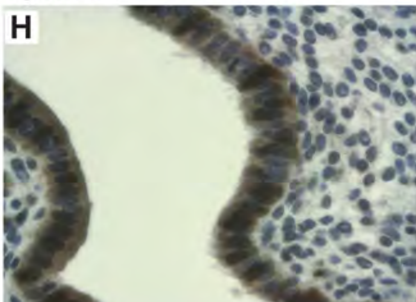
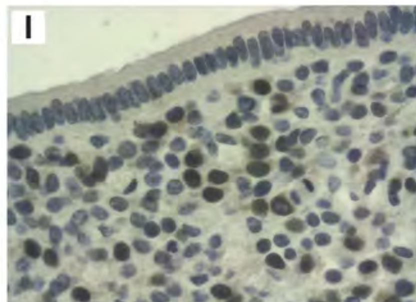


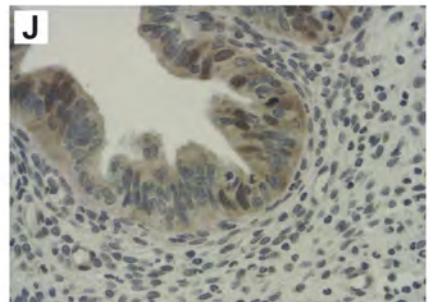


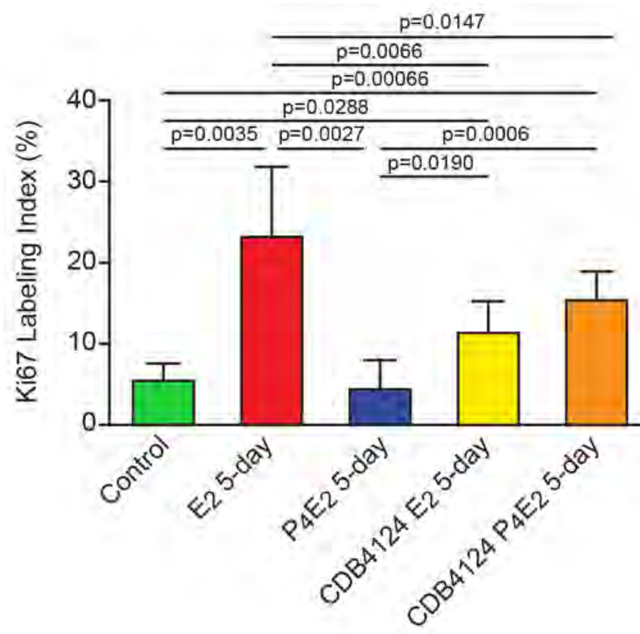


Cyclin A





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20
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Ki67 Labeling Index (%)
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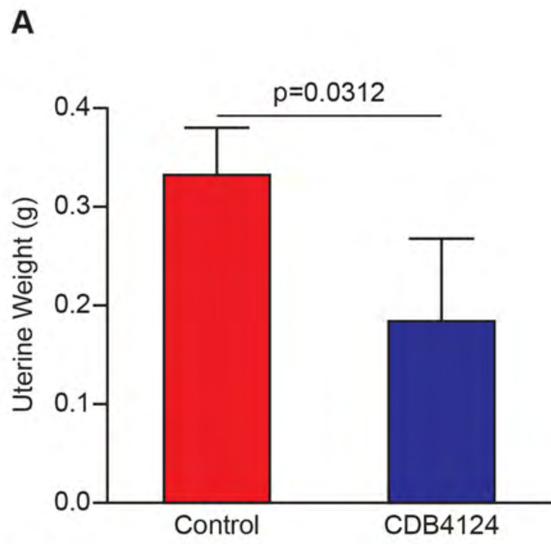
CDB4124 P4E2 5-day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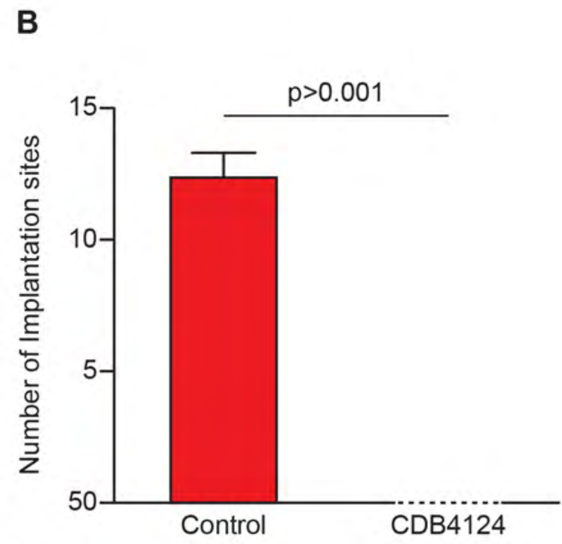
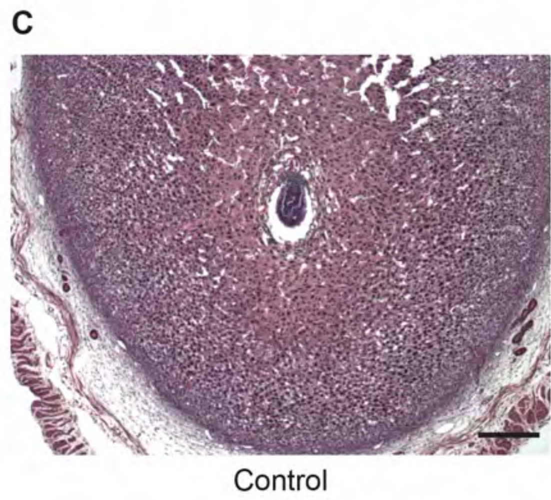
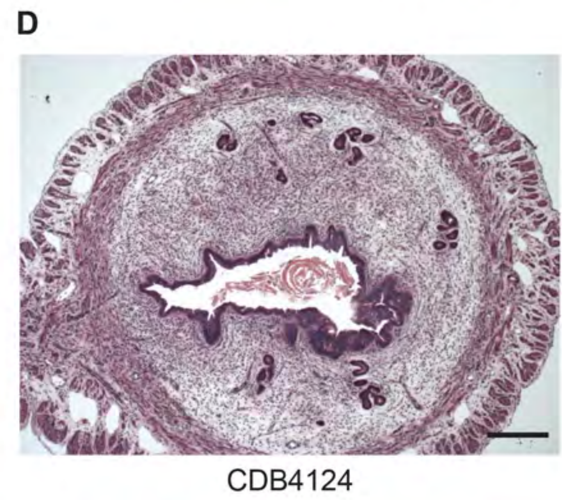
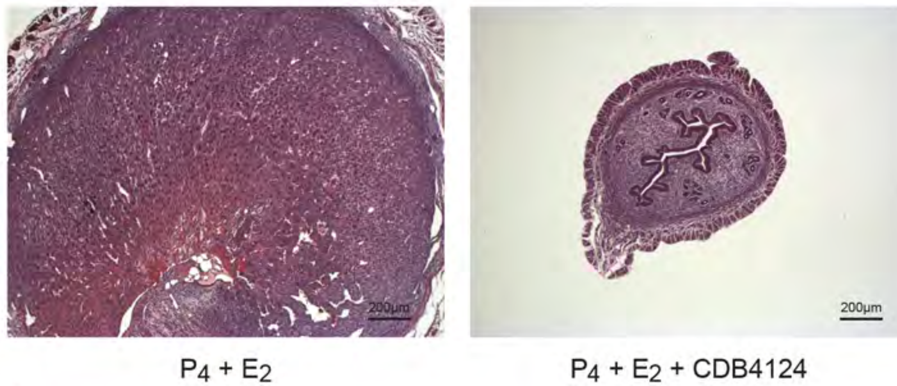
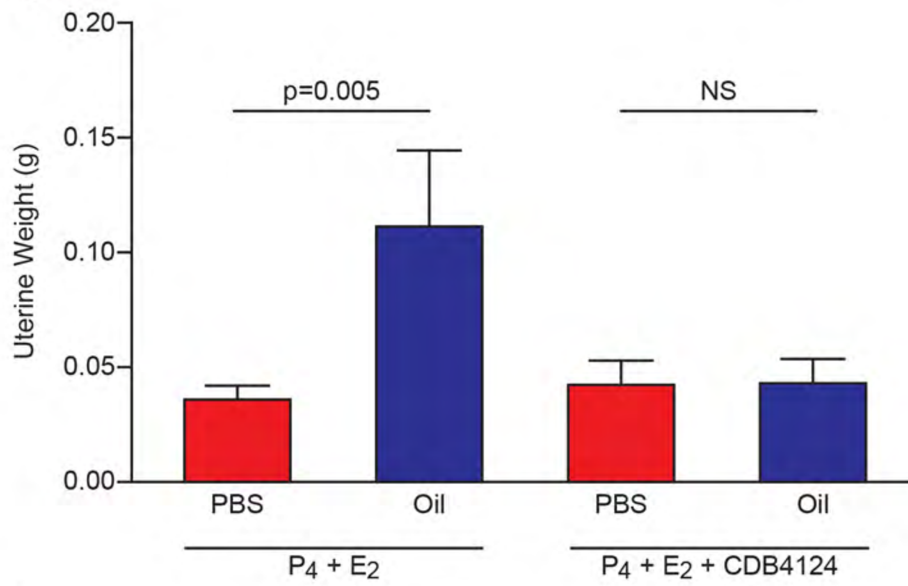
0.1
0.0
Control
CDB4124

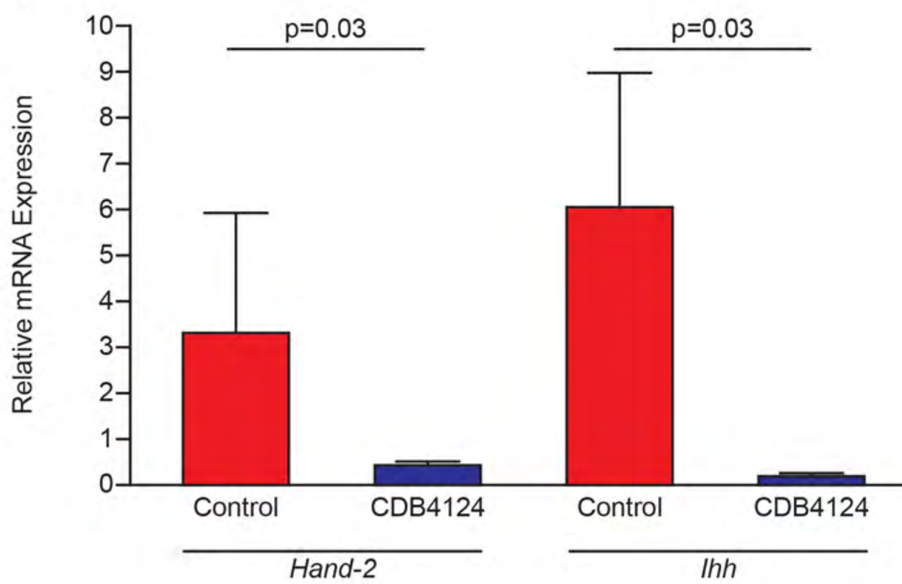

B
p>0.001
Number of Implantation sites
15
10
5
50
Control
CDB4124


C
Control

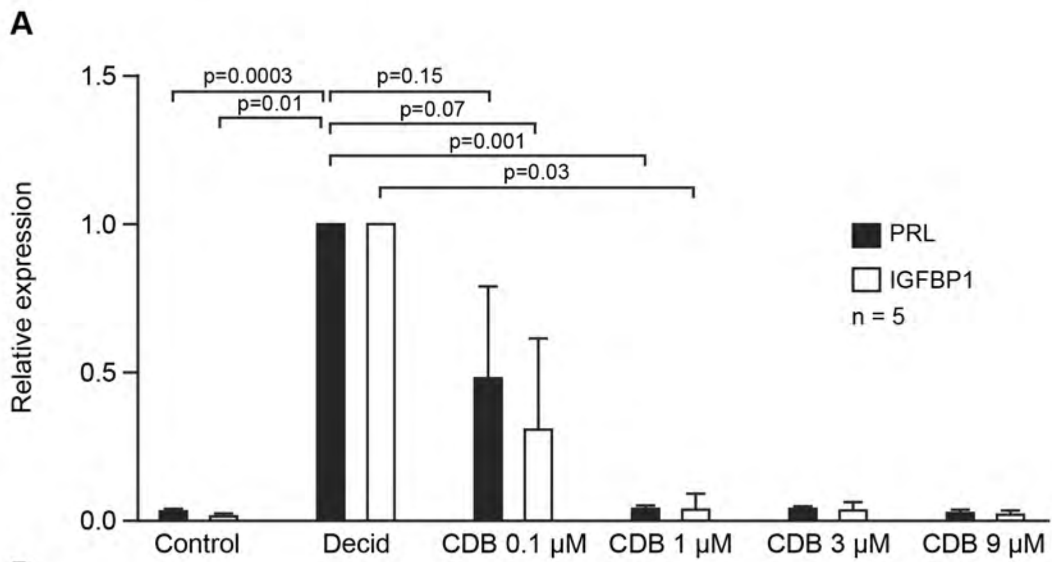

D
CDB4124


A
200µm
200µm
P4 + E2
P4 + E2 + CDB4124


B
Uterine Weight (g)
0.20
0.15
0.10
0.05
0.00
p=0.005
NS
PBS
Oil
PBS
Oil
P4 + E2
P4 + E2 + CDB4124


C
Relative mRNA Expression
10
9
8
7
6
5
4
3
2
1
0
p=0.03
p=0.03
Control
CDB4124
Control
CDB4124
Hand-2
Ihh

A



B

i

ii

Control cells

Decidualized cells

iii

iv

CDB 0.1 μM

CDB 1 μM

v

vi

CDB 3 μM

CDB 9 μM