Endocrinological Properties of Two Novel Nonsteroidal Progesterone Receptor Modulators, CP8816 and CP8863

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ABSTRACT

We have isolated PF1092A, B, and C, novel nonsteroidal progestosterone ligands with preferential affinity for the progesterone receptor, from fermentation broth of a fungus [Tabata Y, Miike N, Hatsu M, Kurata Y, Yaguchi T, Someya A, Miyadoh S, Hoshiko S, Tsuruoka T, and Omoto S (1997) J Antibiot 50:304–308; Tabata Y, Hatsu M, Kurata Y, Miyajima K, Tani M, Sasaki T, Kodama Y, Tsuruoka T, and Omoto S (1997) J Antibiot 50:309–313]. The original skeleton of PF1092, tetrahydronaphthofuranone, was modified synthetically to produce a new skeleton, tetrahydrobenzindrone, and in the present study, biological activities of two derivatives, CP8816 [(4aR,5R,6R,7R)-6-(N,N-dimethylaminocarbonyl)oxy-7-methoxy-4a,5,6,7-tetrahydro-1,3,4-furan-5,6,7-tetramethylbenz[f]indol-2(4H)-one] and CP8863 [(4aR,5R,6R,7R)-7-hydroxy-6-(N-methylcarbamoyl)oxy-4a,5,6,7-tetrahydro-1,3,4a,5-tetramethylbenz[f]indol-2(4H)-one], were investigated. Both CP8816 and CP8863 demonstrated selective binding to progesterone receptor and partial agonistic activity in a progesterone-dependent endogenous alkaline phosphatase expression assay. In the Clauberg-McPhail test, progestational activity of CP8816 (0.1 mg/kg s.c. or 10 mg/kg p.o.) was comparable to that of progesterone (0.15 mg/kg s.c.), and oral administration of CP8863 at more than 1.0 mg/kg also exerted similar effects. Anti-estrogenic (antiuterotropic) activity was confirmed on daily oral application of more than 0.1 mg/kg CP8863 for 3 days by inhibition of estrogen-dependent uterine wet weight gain in ovariectomized rats. CP8816 also exerted antiuterotropic activity at doses of 10 mg/kg (s.c.) and 100 mg/kg (p.o.). These results indicate that our nonsteroidal progestosterone ligands have affinity for the progesterone receptor with partial progestational activity in vitro and clear progestational effects in vivo. Thus, these progesterone receptor modulator profiles suggest that CP8863 and CP8816 are good candidate compounds for treatment of hormone-dependent gynecological disorders.

Progestosterone, as well as estrogen, is an essential ovarian steroid hormone playing important roles in female reproductive processes, including the menstrual cycle and maintaining pregnancy (Csapo and Wiest, 1969). Synthetic steroidal progestosterone receptor modulators (PRMs; agonists, antagonists, or mixed agonists and antagonists) have been widely used, alone or in combination with estrogen, in the gynecological field for female contraception, hormone replacement therapy, and new fertility controls, as well as for treatment of hormone-dependent cancer, endometriosis, and uterine adenomyosis (Spitz and Chwalisz, 2000). Crystallographic analyses of the ligand binding domains of the nuclear receptor have allowed structure-based drug design and have generated renewed interest in the development of new PRMs with high selectivity for the progesterone receptor.

A series of novel nonsteroidal progestosterone receptor ligands, PF1092A, B, and C, possessing a tetrahydronaphthofuranone structure, has been discovered in fermentation broth of a rare fungus, Penicillium oblatum. Among these, PF1092A showed the highest binding affinity for progesterone receptor in porcine uteri cytosol preparations, with an IC_{50} of 30 nM (Tabata et al., 2002), and some derivatives demonstrated partial agonistic activities in vitro assays (Tabata et al., 2002). We modified synthetically the original skeleton of a tetrahydronaphthofuranone by a few steps to produce new analog tetrahydrobenzindolone-type compounds with agonistic and antagonistic activity in vivo (Tabata et al., 2002, 2003).

In this report, we describe biological activities of two new nonsteroidal progesterone receptor ligands, the tetrahydrobenzindolone derivatives CP8863 and CP8816 (Fig. 1). Both

ABBRVIATIONS: PRM, progesterone receptor modulator; MPA, medroxyprogesterone acetate; PBS(−), phosphate-buffered saline without calcium and magnesium; PR-A, progesterone receptor-A; PR-B, progesterone receptor-B; CP8863, (4aR,5R,6R,7R)-7-hydroxy-6-(N-methylcarbamoyl)oxy-4a,5,6,7-tetrahydro-1,3,4a,5-tetramethylbenz[f]indol-2(4H)-one; CP8816, (4aR,5R,6R,7R)-6-(N,N-dimethylaminocarbonyl)oxy-7-methoxy-4a,5,6,7-tetrahydro-1,3,4a,5-tetramethylbenz[f]indol-2(4H)-one; CP8868, (4aR,5R,6R,7R)-7-methoxy-6-(N-propylaminocarbonyl)oxy-4a,5,6,7-tetrahydro-1,3,4a,5-tetramethylbenz[f]indol-2(4H)-one.
exhibit preferential affinity for progesterone receptor in comparison to other steroid hormone receptors and display partial agonistic activity in alkaline phosphatase expression assays in vitro and progestational effects in rabbit uterus and antiuterotrophic (antiestrogenic) activity in rats.

**Materials and Methods**

**Chemicals**

CP8816, (4αR,5β,6R,7R)-6-\{(N,N-dimethylaminocarbonyl)oxy-7-methoxy-4,5,6,7-tetrahydro-1,3,4α,5-tetramethylbenz\}[indol-2(4H)-one], and CP8863, (4αR,5β,6R,7R)-7-hydroxy-6-(N-methylcarbamoyl)oxy-4,5,6,7-tetrahydro-1,3,4α,5-tetramethylbenz\}[indol-2(4H)-one], were synthesized from PF1092C in our laboratory. Their structures are shown in Fig. 1. The purity of both compounds was 99% by high-performance liquid chromatographic analysis. Progesterone for in vitro and in vivo tests was obtained from Junsei Chemical (Tokyo, Japan) and Teikoku Hormone (Tokyo, Japan), respectively. Testosterone, 17β-estradiol, and promegestone (R5020), were synthesized from DIPE (Bristol-Myers Squibb, UK). Other chemicals were obtained from Sigma-Aldrich (St. Louis, MO); promegestone (R5020), [1,2,6,7-3H(N)]mibolerone (specific activity 3145 GBq/mmol), and [2,4,6,7-3H(N)]estradiol for 24 h for the estrogen receptor, and [2,4,6,7-3H(N)]estradiol for 20 h for the estrogen receptor. At the end of the incubation period, cold dextran-coated charcoal solution was added, and after incubation at 4°C for 10 min and centrifugation at 5000g, the radioactivity of the supernatant was determined with Aquasol-2 (PerkinElmer Life and Analytical Sciences) using a liquid scintillation counter, LS6500 (Beckman Coulter, Fullerton, CA). Nonspecific binding was defined as that observed when a 10 μM concentration of corresponding nonlabeled steroid was added to the reaction mixture. Experimental conditions for receptor binding assays are summarized in Table 1.

**Progesterone-Dependent Endogenous Alkaline Phosphatase Expression Assay**

Each compound was dissolved in DMSO at a concentration of 10 mM, and then 50% aqueous methanol was added to give a 100 μM concentration. The resultant solutions were then diluted to appropriate concentrations in PBS(−) and added to the assay medium. The final concentration of methanol in the assay medium was less than 0.5%.

Progesterone-dependent modulation of alkaline phosphatase expression was examined using T47D cells as previously described (Tabata et al., 2001). In brief, growth medium was replaced with phenol red-free Dulbecco's modified Eagle's medium containing 5% fetal bovine serum treated with dextran-coated charcoal. After 24 h of cultivation, the cells were plated in 96-well plates at 25,000 cells/well. After 24 h of cultivation, a test compound was added to each well at an appropriate concentration. After 20 h of cultivation, the medium was removed and the cells were washed with 200 μl of PBS(−). The plates were kept at −80°C for 15 min and then thawed at room temperature. This freeze-and-thaw cycle was repeated once again. After 5 min of incubation at room temperature, 50 μl of PBS(−) and 30 μl of CSPD chemiluminescent substrate solution (Great EscAPe SEAP detection kit; BD Biosciences Clontech, Palo Alto, CA) was added to each well with agitation. After 30 min of incubation at room temperature, luminescence was measured with an ARVO luminometer (PerkinElmer Life and Analytical Sciences).

**Progesterone Activity in Estrogen-Primed Immature Rabbits**

The experimental procedure for the rabbit endometrial transformation test complied with the Guidelines for Animal Experimentation approved by the Pharmaceutical Research Center of Meiji Seika Kaisha.

**CP8816.** The aim of this assay was to determine which dose route is superior for agonist activity of this compound, either s.c. or p.o. CP8816 was dissolved in miglyol oil (MIGLYOL 812 Neutral Oil; Mitsubishi Trading, Tokyo, Japan) at 100°C with stirring to obtain a clear solution, and then cooled to room temperature. The final con-
centration was 10 mg/ml. Immature female rabbits, weighing 800 to 1100 g, were used at three animals per group. All rabbits were injected subcutaneously with 17β-estradiol (estrogen; 5 μg/kg/day) dissolved in sesame oil for 6 days and then treated with 0 (vehicle; miglyol oil), 0.1, 1.0, or 10 mg/kg/day (s.c.) or 1.0 or 10 mg/kg/day (p.o.) CP8816 dissolved in miglyol oil, or 0.15 mg/kg/day (s.c.) of progesterone dissolved in sesame oil, for 5 days. Rabbits were sacrificed on the day after the last administration of test compound or progesterone. The uterus were excised, weighed, fixed in buffered formalin, and cut into 2- to 3-mm sections with a razor blade. Six transverse sections, three each (proximal, medial, and distal parts) from the right and left uterine horns, were prepared, stained with hematoxylin and eosin, and examined histologically by microscopic observation. The grade of endometrial transformation as a progestational effect was recorded according to the method described previously (McPhail, 1934).

**CP8863.** We estimated that this compound is more stable in vivo than CP8816. Therefore, determination not only of agonist but also of antagonist activity was performed.

CP8863 was dissolved using the same method as for CP8816. The final concentration was 10 mg/ml. Immature female rabbits, weighing 800 to 1040 g, were used at five animals per group. They were injected subcutaneously with estrogen (5 μg/kg/day) dissolved in sesame oil for 6 days and then treated with 0 (vehicle; miglyol oil), 0.04, 0.2, 1.0, or 5.0 mg/kg/day (p.o.) of CP8863 for 5 days (agonist format). In a separate experiment (antagonist format), estrogen-primed rabbits were administered the test compounds at the same doses as for agonist format in combination with progesterone (0.15 mg/kg/day, dissolved in sesame oil) for 5 days. The uterus were collected on the day after the last administration of test compounds or progesterone and prepared for histological examination as described in the assay of CP8816.

**Antiprostaglandin Effects in Estrogen-Primed Ovariectomized Rats**

The experimental procedure for the rat antiprostaglandin test complied with the Guidelines for Animal Experimentation approved by the Pharmaceutical Research Center of Meiji Seika Kaisha. This assay was used to detect antiprostaglandin (antiestrogenic) activity of CP compounds in estrogen-primed ovariectomized rats.

**CP8816.** For the s.c. and p.o dosing studies, 3- or 4-week-old female Sprague-Dawley rats were ovariectomized bilaterally under pentobarbital sodium anesthesia. One week thereafter, the rats were divided into 11 groups. In the s.c. dosing study, rats in groups 1 to 4 (five rats each) were subcutaneously injected with estrogen (2 μg/kg/day) and simultaneously received daily s.c. administration of 0, 0.1, 1, and 10 mg/kg CP8816 dissolved in miglyol oil, respectively, for 3 days. Rats in group 5 (five rats) were given 10 mg/kg CP8816 without estrogen administration for evaluation of uterotrophic effects. Rats in group 6 (five rats) were given vehicle (miglyol oil) alone. In the p.o. dosing study, animals in groups 7 to 10 (six to eight rats) were orally given 0, 1.0, 10, and 100 mg/kg CP8816 plus estrogen (2 μg/kg/day s.c.) for 3 days. Rats in group 11 were given 10 mg/kg MPA plus estrogen for 3 days as a positive control. We set a 10 times higher concentration was 10 μg/ml. Immature female rabbits, weighing 800 to 1040 g, were used at five animals per group. They were injected subcutaneously with 17β-estradiol (estrogen; 5 μg/kg/day) dissolved in sesame oil for 6 days and then treated with 0 (vehicle; miglyol oil), 0.1, 1.0, or 10 mg/kg/day (s.c.) or 1.0 or 10 mg/kg/day (p.o.) CP8816 dissolved in miglyol oil, or 0.15 mg/kg/day (s.c.) of progesterone dissolved in sesame oil, for 5 days. Rabbits were sacrificed on the day after the last administration of test compound or progesterone. The uterus were excised, weighed, fixed in buffered formalin, and cut into 2- to 3-mm sections with a razor blade. Six transverse sections, three each (proximal, medial, and distal parts) from the right and left uterine horns, were prepared, stained with hematoxylin and eosin, and examined histologically by microscopic observation. The grade of endometrial transformation as a progestational effect was recorded according to the method described previously (McPhail, 1934).

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**CP8863.** Four-week-old female Sprague-Dawley rats were ovariectomized bilaterally under pentobarbital sodium anesthesia. One week thereafter, the rats were divided into 13 groups. Rats in groups 1 to 5 (five rats each) were subcutaneously injected with estrogen (2 μg/kg/day) and simultaneously received daily p.o. administration of 0, 0.01, 0.1, 1.0, and 10 mg/kg CP8863 dissolved in miglyol oil, respectively, for 3 days. Rats in group 6 (six rats) were given 10 mg/kg CP8863 without estrogen administration and used for assessment of uterotrophic effects. Rats in group 7 (six rats) were given vehicle (miglyol oil) alone. Animals in groups 8 to 12 (five rats each) were orally given MPA plus estrogen (s.c.) for an antiuterotrophic study at the same doses as in groups 1 to 5. Group 13 (six rats) rats were used for a uterotrophic study of 10 mg/kg MPA alone. Autopsy was performed the day after the last administration. Uterine horns were excised and weighed.

**Statistical Analysis**

Statistical analysis of the mean values of uterine weights was performed using the unpaired t test. P values less than 0.05 were considered significant.

**Results**

**Binding Affinities for Steroid Receptors.** The results for binding affinities of CP8816 and CP8863 for various steroidal receptors are shown in Table 2. IC50 values of CP8816 and CP8863 for progesterone binding to human progesterone receptor were 25 ± 15 and 40 ± 7 nM, respectively. The IC50 of CP8816 for human progesterone receptor was comparable to values for the other synthetic progesterone receptor agonist, MPA (13 ± 1 nM). Little cross-reactivity of CP8863 or CP8816 was observed in the assays with other steroid receptors, such as rat androgen receptor, human glucocorticoid receptor, and human estrogen receptor.

**Progestrogendependent Alkaline Phosphatase Expression Assay.** The results of the progestrogen-dependent alkaline phosphatase expression assay using the T47D human breast cancer cell line are shown in Fig. 2 and Table 3. The EC50 value with CP8816 (1.6 ± 0.8 nM) was comparable to that for progesterone (1.6 ± 0.8 nM), whereas the value with CP8863 (13 ± 3 nM) was approximately 10 times lower. The relative efficacies of CP8816 and CP8863 were 49 ± 3% and 57 ± 6%, respectively (mean ± S.D.), as percentages of the response induced by progesterone at 10 nM. Therefore, both compounds were found to possess partial agonistic activity.

**Progestational Activity in Estrogen-Primed Immature Rabbits.** The results for progestational action of CP8816 (s.c. or p.o.) are presented in Table 4 and those for the progestational (agonist format) or antiprogestational (antagonist format) action of CP8863 in Table 5.

Dose-related increase in McPhail units (endometrial transformation by progestational activity) was clearly demonstrated in the uterus of estrogen-primed rabbits treated with CP8816. The largest scores were observed at a dose of 1 mg/kg s.c. or more, whereas rather weak progestational activity was noted after oral dosing with CP8816.

Oral administration of CP8863 in the agonist format induced dose-related progestational activity. The McPhail
TABLE 3
Effects of CP8816 and CP8863 on progesterone dependent alkaline phosphatase expression
The results are means ± S.D. Relative efficacy was estimated as 100% at 10 nM progesterone.

<table>
<thead>
<tr>
<th>Test Compounds</th>
<th>EC50 Nm</th>
<th>Relative Efficacy %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP8816</td>
<td>1.6 ± 0.8</td>
<td>49 ± 3</td>
</tr>
<tr>
<td>CP8863</td>
<td>13 ± 3</td>
<td>57 ± 6</td>
</tr>
<tr>
<td>Progesterone</td>
<td>1.6 ± 0.8</td>
<td>100</td>
</tr>
<tr>
<td>Promegestone</td>
<td>0.46 ± 0.22</td>
<td>80 ± 6</td>
</tr>
<tr>
<td>MPA</td>
<td>0.19 ± 0.07</td>
<td>79 ± 6</td>
</tr>
</tbody>
</table>

TABLE 4
Progestational activity of CP8816 in estrogen primed-immature rabbits

<table>
<thead>
<tr>
<th>Test Compounds</th>
<th>Dose (mg/kg)</th>
<th>Number of Rabbits</th>
<th>Route</th>
<th>McPhail Score (0–4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP8816</td>
<td>0</td>
<td>3</td>
<td>s.c.</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>3</td>
<td>s.c.</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>3</td>
<td>s.c.</td>
<td>4.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3</td>
<td>s.c.</td>
<td>4.0 ± 0.0</td>
</tr>
<tr>
<td>CP8816</td>
<td>1.0</td>
<td>3</td>
<td>p.o.</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3</td>
<td>p.o.</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.15</td>
<td>3</td>
<td>s.c.</td>
<td>3.2 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± S.D.

score (3.1 ± 0.1) at 5 mg/kg was comparable to that for progesterone (3.3 ± 0.3, 0.15 mg/kg s.c.). In the antagonist format, antiprogestational effects of CP8863 versus 0.15 mg/kg progesterone were not detected at any of the doses used.

Antiprogestational Effects in Estrogen-Primed Ovariectomized Rats. Antiprogestatic activities of CP8816 are shown in Table 6. Subcutaneous injection of 10 mg/kg CP8816 with estrogen pretreatment significantly decreased uterine wet weight gain when compared with values for estrogen alone (group 1). However, CP8816 orally administered at a 10 times higher dose (100 mg/kg) was needed as compared with s.c administration.

Uterine wet weight gains for each group treated with CP8863 are shown in Table 7. Uterotropic effects were clear in group 1 rats treated with 2 μg/kg (s.c.) estrogen followed by vehicle (estrogen alone). Average uterine wet weight gains of rats that were orally administered doses above 0.1 mg/kg CP8863 plus estrogen were significantly lower than those given estrogen alone. Antiotropic effects of MPA were observed at doses greater than 1 mg/kg. Uterotropic effects were not observed in any of the groups treated with CP8863 alone. On the other hand, oral doses of MPA (1 or 10 mg/kg) inhibited uterine wet weight gain, uterotrophic effects were evident at the dose of 10 mg/kg p.o.
In the present studies, in vitro and in vivo assays revealed that CP8816 and CP8863 exert specific partial agonistic activity for progesterone receptor, with clear progestational effects in the estrogen-primed rabbit uterus. Furthermore, the treatment caused inhibition of uterine weight gain due to estrogen in ovariectomized rats.

In our previous study, CP8861, a tetrahydrobenzindolone-type derivative, and CP8754, a tetrahydroannaphthofuranone-type derivative, exerted antagonist activity and antiprogestational effects without an agonist in the rabbit assay (Tabata et al., 2002). In addition CP8668, a preliminary tetrahydrobenzindolone-type derivative, exerted agonist effects in the rabbit assay (Tabata et al., 2003). Thus, the biological activities of our compounds range from agonist to antagonist with synthetic structural modification, and their lead structures may have further potential for development of new nonsteroidal progesterone modulators. Furthermore, although these compounds, from two core structures, show different alkaline phosphatase expression in vitro, they consistently demonstrate selective binding affinity for progesterone receptors. These endocrine features with a nonsteroidal structure may have great advantages for clinical use without side effects.

Antiuterotrophic activity (i.e., antiestrogenic effects) is a key action for clinical benefit of PRMs for hormone-dependent diseases. Treatments with CP compounds in estrogen-primed ovariectomized rats have demonstrated inhibition of uterine wet weight gain. Progesterone inhibits proliferation in the estrogen-primed endometrium of ovariectomized mice but reciprocally acts as a mitogen in stromal cells of the endometrium (Martin and Finn, 1971; Tachi and Tachi, 1974; Moyer and Felix, 1998). We have already reported that CP8816 exerts antiproliferative effects on both epithelial cells and stromal cells in estrogen-primed mice, while both CP8816 and CP8863 inhibit the development of adenomyosis induced experimentally by pituitary grafting (Mori et al., 2002). Thus, the mode of antiproliferative action of CP8816 on the cell components of the endometrium differs from that of progesterone. In the current experiment, direct antiestrogenic activity of CP8816 and CP8863 was also revealed in the assay of antiuterotrophic effects (Tables 6 and 7), whereas treatment with the derivatives alone did not induce any estrogenic action in the uterus of ovariectomized rats. Furthermore, CP8863 at high doses did not change the estrus cycle in treated rats (unpublished data). We confirmed that CP8816 and CP8863 might inhibit development of adenomyosis through direct antiestrogenic actions and partial agonist activity on the endometrium.

CP8863 showed good progestational activity in in vivo assays, though its in vitro activity was milder than that of CP8816. One of the causes is thought to be the metabolic stability of CP8863. On testing of metabolic stability with female rat and human liver S9 fractions, CP8863 proved more stable than CP8816 (unpublished data).

So far, two progesterone receptor isoforms have been reported, namely, progesterone receptor-A (PR-A) and progesterone receptor-B (PR-B). PR-A is a 94-kDa protein, whereas PR-B is a 114-kDa protein that contains an additional 164 amino acids at its amino terminus (Schrader et al., 1981). These isoforms are due to alternative sites in the same mRNA or transcription from alternative promoters (Kastner et al., 1990). PR-B tends to be a stronger activator of progesterone target genes, whereas PR-A has been shown to act as a dominant repressor of PR-B and other steroid hormone receptors such as those for androgen, glucocorticoid, and estrogen receptors (Vegeto et al., 1993). Progestins and anti-progestins have been shown to inhibit estrogen-stimulated uterine proliferation (Medlock et al., 1994). These antiestrogenic effects could be due to a dominant negative influence through PR-A and/or essential progestational effects through PR-B. In the present study, the discrepancy between partial agonist activities of CP8863 in the alkaline phosphatase expression assay and full agonist potential in the estrogen-primed immature rabbits could not be explained. However, it may depend on variation in progesterone receptor isofrom expression in different species. The relationship between modulation of transcription by progesterone isoforms and specific clinical applications remains to be elucidated. However, the present results show that they are good candidates as nonsteroidal PRMs for treatment of gynecological disorders.

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