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Novel orally active selective progesterone receptor modulator CP8947 inhibits leiomyoma cell proliferation without adversely affecting endometrium or myometrium

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Abstract

Context—Uterine leiomyomas are highly prevalent and often symptomatic. Current medical therapies are limited. A novel, potent, selective, orally active therapy is needed.

Objective and Methods—To determine the progesterone receptor (PR) specificity and activation, endometrial response, and impact on proliferation and extracellular matrix (ECM) production of the novel non-steroidal selective progesterone receptor modulators (SPRMs) CP8863 and CP8947 in human immortalized leiomyoma and patient-matched myometrial cells. Receptor binding in vitro was assessed using LNCaP, Ishikawa, T-47D, and HeLa cell extracts for AR, ER- α , PR, and GR, respectively. Progestational activity assessed by alkaline phosphatase assay in T47D cells and ER- α expression in human leiomyoma and myometrial cells. In vivo progestational activity assayed by the McPhail assay. Proliferation and gene expression studies (q RT-PCR and western blot) were performed in immortalized leiomyoma and myometrial cells.

Results—Both CP8863 and CP8947 is highly selective for PR but not for ER- α , AR, and GR. Both induced alkaline phosphatase comparably to progesterone, while CP8947 induced ER- α in leiomyoma cells but not myometrial cells. CP8947 was progestational in rabbit endometrium. Nanomolar CP8947 treatment inhibited human leiomyoma but not myometrial cell proliferation. The decreased proliferation correlated with increased TRAIL and caspase -7, suggesting induction of apoptosis in leiomyoma cells. ECM components were decreased in leiomyoma cells, including COL1A1 and COL7A1 at nanomolar concentrations.

Conclusions—CP8947 was a potent novel non-steroidal SPRM that was selective for PR, showed progestational activity in endometrium, inhibited leiomyoma cell proliferation (potentially via induction of apoptosis), and decreased ECM component production, without disrupting myometrial cell proliferation.

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WHC, MM, PD, JS, and JD have nothing to declare. SC is employed by Tokai Pharmaceuticals, Inc. and supplied CP8863 and CP8947 to WHC.

Key Terms

Leiomyoma; selective progesterone receptor modulator; endometrium; apoptosis; myometrium; extracellular matrix

INTRODUCTION

Uterine leiomyomas, or fibroids, are common uterine tumors found in over 70% of women by the age of 50 (1). While a proportion of women with leiomyomas may remain asymptomatic, many women suffer from menorrhagia, dysmenorrhea, dyspareunia, infertility, miscarriage, placental abruption, malpresentation, and preterm labor (2–4). Despite the high prevalence and symptomatic nature of these tumors, the etiology of leiomyomas is incompletely understood. Consequently, therapeutic medical options remain limited.

Currently, the definitive therapy for leiomyomas is hysterectomy (5). However, for women who desire pregnancy or who wish to avoid surgery, hysterectomy is not a viable therapeutic option. Newer therapies, including uterine artery ablation and MRI-guided high frequency ultrasound, are contraindicated for women who wish to preserve fertility (6,7). Myomectomy, or the surgical removal of the leiomyomas while preserving the uterus, is the standard surgical procedure if future pregnancy is desired. However, myomectomy is associated with significant morbidity including hemorrhage, adhesion formation, leiomyoma recurrence, blood transfusion, bowel injury, and rarely hysterectomy (8). Effective medical therapies are needed.

Leiomyoma growth is regulated in part by estrogen and progesterone. Growth with sex steroids is illustrated by the increase in leiomyoma size beginning with menarche and their regression after menopause (9). Based upon this insight, various hormonal therapies have been developed for leiomyoma treatment.

Selective Progesterone Receptor Modulators (SPRMs) have shown promise for the treatment of leiomyomas. Examples of such SPRMs include mifepristone, and the mifepristone congeners CDB-2914, CDB-4124, and asoprisnil. Mifepristone itself is efficacious in reducing leiomyoma size (10), but due to its anti-progestational effect, this agent has been associated with endometrial hyperplasia (11). Furthermore, mifepristone is limited by a lack of progesterone receptor specificity, in that it can also interact with the glucocorticoid receptor (12).

CDB 2914 and CDB 4124 are metabolites of mifepristone that exhibit less glucocorticoid activity and reduce leiomyoma size, but the anti-progestational effect in the endometrium results in endometrial hyperplasia (12–14). Asoprisnil also possesses limited anti-glucocorticoid activity, and causes a reduction in leiomyoma size (15). However, investigation of asoprisnil was halted due to endometrial changes including abnormal vascular growth (16). The greatest challenge in identifying an effective SPRM for the treatment of leiomyomas is to identify a compound with exquisite progesterone receptor selectivity that can selectively act as a progestin in the endometrium, while acting as an anti-progestin within the leiomyoma.

Tabata and colleagues (17) have developed several progesterone receptor inhibitors from the microorganism *Penicillium oblatum* that are based upon an eremophilane-type sesquiterpene carbon skeleton. These compounds are unique because they are not derived from steroidal derivatives (18). CP8863 is a semi-synthetic orally active derivative that demonstrates progestational activity similar to natural progesterone in the endometrium (19). This

compound also inhibits estradiol-mediated epithelial cell proliferation but does not effect stromal cell proliferation (20). The major metabolite of CP8863 is CP8947. The effects and role of CP8947 in leiomyoma treatment have not previously been reported.

We hypothesized that two novel nonsteroidal PR ligands, CP8863 and CP8947, would have PR-specific selectivity and will inhibit leiomyoma cell proliferation. Furthermore, since symptoms caused by leiomyomas are due to increasing bulk which is directly related to the excessive and disorganized extra cellular matrix production (21), further hypothesize that these compounds regulate critical extracellular matrix components which define the leiomyoma phenotype (22). We found that both compounds bound with high affinity to the progesterone receptor, but did not bind with high affinity to the estrogen receptor, glucocorticoid receptor, or androgen receptor. The compounds demonstrated partial agonist activity in PR-regulated gene induction and in rabbit endometrium decidualization. Finally, CP8947 inhibited leiomyoma cell proliferation but did not impact myometrial cell proliferation, and inhibited extra cellular matrix production in leiomyoma cells. Taken together, CP8947 is a novel SPRM which has direct and specific inhibitory effects on leiomyoma cells while providing progestational stimulation to the endometrium and no glucocorticoid receptor activation at therapeutic concentrations.

MATERIALS AND METHODS

All studies were approved by the Institutional Review Board of the Uniformed Services University of the Health Sciences.

Receptor Binding Assays

Cultured cells were washed once with pre-warmed (37°C) 1X PBS and then detached from substrate with pre-warmed (37°C) cell dissociation buffer (3 mM EDTA in 1X PBS without Ca^{2+} and Mg^{2+}). The cell pellet was recovered by centrifugation at 500 rpm in an Eppendorf 5804 R centrifuge for 10 min at 4°C. The cells were resuspended in 5 packed volumes of 1X lysis buffer (20 mM TrisHCl (pH 7.4), 1.0 mM EDTA, 10 mM sodium molybdate, 10% glycerol, 1.0 mM DTT, and complete protease inhibitors (Roche, Branchburg, NJ)). Cells were disrupted by brief sonication and centrifuged at 100,000 g for 60 min at 4°C. Supernatants containing steroid receptors were aliquoted and stored at -80°C. Cell lines used as sources of steroid receptors were LNCaP prostate carcinoma cells (androgen receptor; American Type Culture Collection, Manassas, VA), Ishikawa endometrial adenocarcinoma cells (estrogen receptor; generous gift of Dr. Erlio Gurpide), T-47D mammary ductal carcinoma cells (progesterone receptor; ATCC), and HeLa cervical adenocarcinoma cells (glucocorticoid receptor; ATCC). Ishikawa cells were cultured in DMEM/F12 containing 10% charcoal-stripped fetal bovine serum. HeLa cells were cultured in DMEM containing 10% charcoal-stripped fetal bovine serum and T-47D cells were cultured in RPMI1640 containing 10% charcoal-stripped fetal bovine serum.

Aliquots of the respective lysates were incubated with [6, 7- ^3H (N)]-Dexamethasone (Amersham, Quebec, Canada) for 24 h to detect binding of glucocorticoid receptors, [17 α -Methyl- ^3H]-Mibolerone (Amersham) for 24 h to detect specific binding of androgen receptor, [1,2,6,7- ^3H (N)]-Progesterone (Amersham) for 1 h to detect binding of progesterone receptor, or [2,4,6,7- ^3H (N)]-Estradiol (Amersham) for 20 h to detect binding of estrogen receptor. Progesterone, estradiol, hydrocortisone, and dexamethasone were obtained from Sigma-Aldrich (St. Louis, MO). Mibolerone was from Perkin Elmer (Waltham, MA). CP8863 and CP8946 were generous gifts from Tokai Pharmaceuticals, Inc. (Cambridge, MA; Figure 1). Binding reactions were then treated with dextran-coated charcoal to remove unbound steroids, centrifuged, and binding of radiolabeled steroid was

determined by scintillation counting. Nonspecific binding was that observed in the presence of a molar excess of unlabeled steroid.

Cell proliferation studies—Immortalized myometrial and leiomyoma cells (22) maintained in DMEM-F12 supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂ were sub-cultured in DMEM-F12 phenol-free media containing 10% charcoal-stripped FBS to 50% confluence. Cells were trypsinized, plated in 96-well plates, and monolayer cultures of 50% confluence were treated with graded concentrations of CP8863 and CP8947 in serum-free, phenol red-free DMEM-F12 for times indicated. The plates were collected each day for up to five days. The final concentration of ethanol vehicle in culture medium or control cultures was < 0.01%. Cell proliferation was measured using sulforhodamine-B method (Sigma-Aldrich) according to manufacturer's protocol and repeated in triplicate.

RNA and Protein Protocol—Immortalized myometrial and leiomyoma cells maintained in DMEM-F12 supplemented with 10% FBS were plated in 6-well plates and allowed to reach 20% confluence before media was replaced with DMEM-F12 phenol-free media containing 10% charcoal-treated FBS. Cells were allowed to reach 60% confluence before treatment with CP8863 and CP8947 at 10⁻⁹M to 10⁻⁵M. Experiments were repeated in triplicate. After 24hr treatment with the agents, cells were collected for RNA and protein analysis.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis—

Real time reverse transcriptase-polymerase chain reaction (qRT-PCR) was used to quantify expression of extra cellular matrix (ECM) genes, COL1A1, COL7A1, versican, connective tissue growth factor, TGF-β3, and fibronectin, as described previously (23–25). The 18S ribosomal RNA gene was used as an internal control and each sample was analyzed in triplicate. Bio-Rad iCycler software version 3.1 was used for data analysis.

Western Blot—Protein was isolated using a RIPA lysis and extraction buffer (Pierce Biotech., Rockford, IL) containing 1X Halt protease inhibitor (Pierce Biotech.) as described previously (25,26). Briefly, aliquots of the proteins extracted from treated cultured cells were electrophoresed on a SDS-PAGE under reducing conditions, blotted onto nitrocellulose, and apoptosis related proteins were detected overnight with mouse monoclonal antibody against Caspase 3 (sc-7272; dilution 1:200) at 4°C, rabbit polyclonal antibody against Caspase 7 (sc-33773, dilution 1:200), or TRAIL (sc-7877, dilution 1:200) as indicated. All antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Horseradish peroxidase (HRP)-conjugated secondary antibody (ImmunoPure, Pierce Biotech) in combination with the SuperSignal West Pico (Pierce Biotech) was used for detection of the proteins. Beta-actin (sc-1616; Santa Cruz Biotechnology) was an internal control for protein loading.

Alkaline Phosphatase Assay

Methods for determining progesterone-dependent induction of alkaline phosphatase activity were similar to those described by Madaus and colleagues (27). Briefly, 8 × 10⁴ T47D cells were plated per well in 48-well plates in DMEM/F12 medium containing 2% charcoal-stripped FBS and incubated at 37°C overnight. The next day, progesterone, increasing concentrations of test compound alone, or 10nM progesterone plus increasing concentrations of test compound were added to cells and incubation at 37°C was continued overnight. After 20 h incubation, cells were washed with 1X TBS, lysed by addition of 100μL 0.1 M Tris-HCl, pH 9.8 containing 0.2% Triton X-100 with shaking for 15 minutes at room temp. 300μL of 0.1 M Tris-HCl, pH 9.8 containing 4 mM p-nitrophenyl phosphate was then added

to each well. Vmax measurements were recorded at 405 nM during 2 min intervals over a 30 min period using a Bio-Rad Benchmark Plus microplate spectrophotometer.

McPhail Study: Experimental Design—Endometrial decidualization and agonist and antagonist activity of the two antiprogestins, CP8863 and CP8947 were determined in estrogen-primed immature rabbits. The protocol was approved by the USUHS Animal Use Committee. Immature female rabbits weighing 1.2–1.8 kg were purchased from Covance Research Products Inc. (Denver, PA). A stock solution of each drug, CP8863 and CP8947, and estradiol (Sigma-Aldrich, St. Louis, MO) was made in absolute alcohol. Medroxyprogesterone 17-acetate (MPA, Sigma) was dissolved in chloroform. Further dilutions for subcutaneous injections (Experiment 1) or oral delivery (Experiment 2) to the rabbits was performed using sesame oil (Sigma).

Experiment 1—Rabbits were primed with estrogen (5 µg/kg/day) for 6 days, and then treated with subcutaneous administration of either CP8863 or CP8947 (0, 0.1, 1.0 or 10 mg/kg/day) or MPA (0.15 mg/kg/day) for 5 consecutive days. Each group consisted of five rabbits.

Experiment 2—Five rabbits per group were primed with estrogen (5 µg/kg/day) for 6 days, and then orally administered the test compound (0, 0.04, 0.2, 1.0 or 3.0 mg/kg/day) or the test compound in combination with MPA (0.15 mg/kg/day) for 5 consecutive days.

All rabbits were sacrificed on the day following the final administration of test medication or progesterone. The uteri were excised and fixed in 10% buffered formalin. Six transverse sections, three each from proximal (closest to the uterus), medial and distal parts of the right and left uterine horns were stained with haematoxylin and eosin, and examined histologically. The grading of endometrial transformation was based on the previously described McPhail method (28).

Statistical analysis—For q RT-PCR data, results are reported as mean $\pm$ SEM. For each result the average expression of three replicates was calculated and normalized against 18S RNA. Relative expression was calculated based on Pfaffl method (29). Wilcoxon-signed rank test was used for nonparametric statistical evaluation. For proliferation data, statistical significance was calculated by ANOVA or student's t-test. For western blot analysis, calculations were done using QualityOne software from Bio-Rad. Data are presented as fold difference between relative density units of treated and untreated samples, normalized for loading. Receptor binding and alkaline phosphatase assays were analyzed by student's t-test. McPhail assays were analyzed by Mann-Whitney-U test. P values < 0.05 were considered significant.

RESULTS

Binding affinities for CP8863 and CP8947 for AR, ER- α , PR, and GR in LNCaP, Ishikawa, T-47D, and HeLa lysates are shown in Table 1. The IC₅₀ value for CP8863 was 57 nM and for CP8947 was 8 nM compared to the medroxyprogesterone acetate (MPA) value of 2 nM. The IC₅₀ values for the two compounds were greater than 1000 nM for all other receptors. Both CP8863 and CP8947 showed high specificity for progesterone receptor binding.

Progestational activity was assessed by induction of ER- α mRNA transcripts in human leiomyoma and myometrial cells. CP8863 increased steady-state levels of ER- α transcript compared to the untreated cells in both leiomyoma and myometrial cell lines at 100 nM (Fig. 2a,b). There was a 5.2-fold increase in expression of ER- α in leiomyoma cells

treated with CP8863 at 10^{-6} M. In contrast, CP8947 increased ER- α transcripts in leiomyoma cells in a dose dependent manner but did not affect myometrial cell steady-state levels of ER- α mRNA (Fig 2c,d). A 6.9-fold increase in mRNA expression was noted at a concentration of 1nM, which was significantly greater than the expression seen in CP8863 treated cells. At potentially cytotoxic concentrations (10 μ M), ER- α transcripts were decreased.

To confirm progesterone receptor specific activation, we performed alkaline phosphatase assays in T47D human breast cancer cells. Progesterone concentration at 10nM significantly increased alkaline phosphatase activity (Fig 3A,B). Both CP8863 and CP8947 increased alkaline phosphatase production in a dose dependent fashion; both CP8863 and CP8947 demonstrated significant progestational activity at 10–50nM treatment concentrations (Fig. 3A,B). When these cells were treated with both CP8863 and progesterone, alkaline phosphatase production was increased to levels greater than with progesterone alone with CP8863 concentrations of 100nM or greater (Fig 3C). At high concentration (500nM), CP8947 inhibited progesterone-mediated alkaline phosphatase induction (Fig 3D), but maintained induction at rates greater than untreated leiomyoma cells. These results suggest that CP8947 acts as a less potent progestin relative to progesterone, obtaining 70% efficacy at 50x concentration relative to progesterone.

One limitation of SPRM therapy is the antiprogestational activity on the endometrium. We therefore assessed the in vivo endometrial progestational activity of CP8863 and CP8947 in estrogen-primed rabbits. Both CP8863 and CP8947 showed a dose-dependent increase in the McPhail index, demonstrating a progestational effect on the uterine lining with both subcutaneous and oral dosing (Fig. 4). A greater progestational effect was observed with oral CP8947 compared to subcutaneous CP8947 and oral CP8863 (Fig. 4A and 4B). Concomitant treatment with progesterone demonstrated a McPhail score equivalent to progesterone alone, suggesting that neither CP8863 nor CP8947 exhibited antiprogestational effects in rabbit endometrium (Fig. 4C).

Next we evaluated the influence of CP8863 and CP8947 treatment on immortalized human leiomyoma and patient-matched myometrial cells at doses ranging from 1nM to 10 μ M (Fig. 5). Neither compound significantly altered myometrial cell proliferation (Fig 5A). Leiomyoma growth was also not influenced by CP8863 in a dose-dependent matter (Fig. 5B). However, leiomyoma cell growth was inhibited with CP8947 at a concentration of 10^{-9} M and further inhibited at greater concentrations (Fig. 5D). Treatment for 48 hours with CP8947 demonstrated efficacy at 0.1nM concentrations in leiomyoma cells, while not significantly impacting myometrial cell proliferation at 1,000-fold greater concentrations (Figure 6). These results suggest that CP8947 selectively inhibited leiomyoma cell proliferation at 0.1–1nM concentration while having negligible effects on myometrial cell growth at these concentrations.

To determine the mechanism of anti-proliferative effect of CP8947, we evaluated markers of apoptotic activity. Western blots demonstrated an increase in caspase 7, and TRAIL expression in cells treated with CP8947, without an increase in caspase 3 (Fig. 7). Caspase 7 expression was increased by treatment with CP8947 at concentrations as low as 10nM (Fig. 7B). TRAIL expression was increased significantly with treatment concentrations of 100nM CP8947 (Fig. 7C). Protein concentration of caspase 3, caspase 7, and TRAIL increased with CP8863 treatment at 0.1–1 μ M concentrations.

Ultimately, symptoms from leiomyomas result from increasing bulk due to overproduction of a disorganized extracellular matrix (21,30). We therefore evaluated the influence of CP8947 on extracellular matrix genes known to be altered in human leiomyomas (31).

CP8947 at 1nM or greater concentration had a no effect on myometrial expression of COL 1A1 (Fig. 8A). In leiomyoma cells, COL 1A1 initially increased by up to 12-fold at 24 hours with 1nM CP8947 treatment, but returned to expression levels identified in untreated myometrial cells at 48 hours after treatment (Fig. 8B). COL 7A1 expression was induced in myometrial cells by CP8947 concentrations of 1nM, but inhibited at 1 μ M or greater concentrations (Fig. 8B). In leiomyoma cells, COL 7A1 expression was reduced with CP8947 at 1nM. These findings demonstrate that CP8947 decreased transcripts encoding ECM components in leiomyoma cells at nanomolar concentrations. Neither CP8863 nor CP8947 altered expression of fibronectin, connective tissue growth factor, nor TGF β 3 (data not shown).

Discussion

We found that the novel compounds CP8863 and CP8947 are highly selective and potent selective progesterone receptor modulators, with minimal activity on estrogen, glucocorticoid, or androgen receptors. These compounds can bind to and activate the progesterone receptor as demonstrated by the alkaline phosphatase assay at nanomolar concentrations as demonstrated by the alkaline phosphatase assay. Furthermore, both compounds are orally active, and induced progestational changes in the rabbit endometrium. They were unable to block progesterone-mediated endometrial changes. In human leiomyoma cells, CP8947 specifically inhibited proliferation while not influencing myometrial cell proliferation at nanomolar to micromolar concentrations. Inhibited proliferation may involve apoptosis, as demonstrated by alterations in TRAIL and caspase 7, but not in caspase 3. Finally, CP8947 altered expression of critical extracellular matrix genes involved in the fibrotic phenotype of leiomyomas, inhibiting both COL1A1 and COL7A1 expression. Collectively, these results show that CP8947 is a highly potent, highly selective, orally active selective progesterone receptor modulator that selectively inhibited both leiomyoma cell proliferation and extracellular matrix gene expression without disrupting myometrial cell growth or acting as an antiprogestin in the endometrium. As such, CP8947 represents a compound that could have therapeutic efficacy in human leiomyomas.

Current medical therapy for uterine leiomyomas is based upon the finding that human leiomyomas are hormonally-dependent tumors and involves the use of gonadotropin releasing hormone agonists (32) or antagonists (33). While these therapies are effective in reducing leiomyoma size from 30–50% (34), they therapies also induce a hypoestrogenic state, with side effects of hot flashes, vaginal dryness, and bone loss (35). In addition, these therapies cannot be dosed orally. As a result of the significant side-effects, therapy is often limited to 3–6 months, with longer treatment requiring a complex regimen of hormonal add-back medication (36).

As an alternative to GnRH analogue therapy, SPRMs have been developed for leiomyoma therapy: mifepristone (11), and the congeners of mifepristone, asoprisnil (14) and CDB-2914 (16). While each of these compounds showed promise, two innate characteristics of these compounds limited their applicability: interaction with glucocorticoid receptor and endometrial hyperplasia. Progesterone is able to interact with and activate the glucocorticoid receptor (37), due to the similarities in structure between the two hormones. Mifepristone and mifepristone congeners, which have a similar structure, exhibit glucocorticoid binding activity (38). Furthermore, while mifepristone and congeners act as anti-progestins on the leiomyoma, they also act as anti-progestins on the endometrium. As a result, therapy results in endometrial stimulation. After several months, the estrogen-induced endometrial changes may lead to hyperplasia and atypia (39).

The novel compounds CP8863 and CP8947 are semi-synthetic compounds that were isolated from the fermentation broth of *Penicillium oblatum*. Among the prototypical compounds isolated, PF1092A possessed an IC₅₀ of 30nM for the porcine uterine progesterone receptor (17). Further compound development resulted in the development of CP8863, which inhibited estradiol-mediated epithelial cell proliferation (20). The CP8863 IC₅₀ for the progesterone receptor was 40nM, while the IC₅₀ was >9uM for the glucocorticoid receptor, estrogen receptor, and androgen receptor (20). Furthermore, oral dosing of CP8863 exerted a progestational action in the rabbit endometrium (19). Analysis of CP8863 metabolism resulted in the identification of CP8947, which we show here to be a more potent and efficacious selective progestin receptor modulator.

In conclusion, we have characterized a novel selective progesterone receptor modulator, CP8947, which exhibits characteristics favorable for development as a human leiomyoma therapy. In particular, CP8947 was active at nanomolar concentrations, was selective to the progesterone receptor, was orally active, had progestational activity in the endometrium, and inhibited leiomyoma cell proliferation and extracellular matrix gene expression while having no appreciable effect on myometrial cell proliferation. Clinical trials will be necessary to determine the promise of CP8947 as an orally active therapy for uterine leiomyomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Chemical structure of CP8863 (left) and its metabolite CP8947 (right). These novel compounds are not structural derivatives of known steroids, but have a unique structure among the selective progesterone receptor modulators.

Figure 2.

Effects of graded concentrations of CP8863 (A) and CP8947 (B) on estrogen receptor-alpha (ER-α) mRNA levels in immortalized human leiomyoma and patient-matched myometrial cells as assessed by real time RT-PCR. Comparative expression of the two compounds and cell types were assessed at 24 hours. (A) In the absence of treatment, ER-α expression was comparable between myometrial and leiomyoma cells. However, 10nM concentration (10⁻⁸M) of CP8863 induced increased ER-α transcripts in myometrial cells, and at 1nM concentrations in leiomyoma cells. Increasing concentration of CP8863 to 10μM did not result in further ER-α expression in myometrial cells, while there was a greater expression of ER-α in leiomyoma cells only 1μM concentration. At concentrations of 10uM CP8863, ER-α expression dramatically decreased in both cell types. *, P< 0.05 vs. untreated myometrial cells or untreated leiomyoma cells. (B) In the absence of treatment, ER-α expression was comparable between myometrial and leiomyoma cells. In myometrial cells, CP8947 did not induce ER-α expression at concentrations ranging from 1nM to 10μM. However, 1nM concentration (10⁻⁹M) of CP8947 dramatically induced ER-α expression in leiomyoma cells. Increasing concentration of CP8947 resulted in greater expression of ER-α in leiomyoma cells at concentrations up to 1μM. At 10μM CP8947, ER-α expression dramatically decreased. *, P< 0.05 vs. untreated leiomyoma cells.

Figure 3.

Effects of CP8863 and CP8947 on expression of alkaline phosphatase activity in T47D breast cancer cells. Induction of alkaline phosphatase activity was assessed by measurement of the hydrolysis of p-nitrophenyl phosphate (PNPP). Alkaline phosphatase activity was measured following treatment for 20 hours without compound, with 10 nM progesterone alone, or with increasing concentrations of either CP8863 (A) or CP8947 (B), as indicated. Progesterone alone, CP8863, and CP8947 effectively induced alkaline phosphatase activity 10 nM treatment concentrations. Alkaline phosphatase induction by CP8863 approached progesterone induction levels, while CP8947 demonstrated 60% potency relative to 10nM progesterone, even at 1 μ M concentration. In competition assays with progesterone, T47D cells were treated with 10 nM progesterone for 20 hours in the presence of increasing concentrations of either CP8863 (C) or CP8947 (D), as indicated. Concomitant treatment of progesterone and CP8863 demonstrated an additive induction at concentrations of 100nM or greater, while CP8947 partially inhibited progesterone-mediated alkaline phosphatase stimulation at 500nM concentrations. * = $p < 0.01$ relative to untreated cells. ** = $p < 0.01$ relative to progesterone-treated cells.

Figure 4.

Progestational effects of CP8863 and CP8947 on rabbit endometrium assayed by McPhail Index after estradiol priming. In the absence of compound, no progestational influence was noted (X, red line). In the presence of progesterone, maximal progestational histologic changes were noted (●, green line). (A) When either CP8863 (yellow) or CP8947 (blue) were provided subcutaneously, there was a dose-dependent increase in progestational histologic findings that were comparable. (B) With oral dosing, the progestational effect of CP8947 was greater than CP8863 at maximal dose, approaching the progestational effect demonstrated with progesterone. (C) In competition studies using 10nM progesterone and increasing oral concentrations of either CP8863 or CP8947, neither compound demonstrated anti-progestational effect, even at concentrations as high as 3mg/rabbit/day. * = $p < 0.01$ relative to untreated animals. ** = $p < 0.01$ relative to progesterone-treated animals.

Figure 5.

Effects of graded concentrations of CP8863 and CP8947 on the number of viable cultured human myometrial and leiomyoma cells, as assessed by sulforhodamine-B method. At concentrations ranging from 1nM to 10 μ M, CP8863 had no discernable effect on either myometrial (A) or leiomyoma (B) cell viability. CP8947 also had no discernable impact on myometrial cell proliferation (C). However, compared with untreated control leiomyoma cultures, 48-h treatment with 1nM (10^{-9} M) CP8947 significantly decreased the number of viable leiomyoma cells. Results represent the mean + SE of at least three independent experiments performed in triplicate. * = p<0.025 relative to untreated cells).

* = $p < 0.025$ vs. untreated cells

Figure 6.

Effects of graded concentrations of CP8947 on the number of viable cultured human myometrial and leiomyoma cells, as assessed by sulforhodamine-B method after 72 hours treatment. At concentrations ranging from 10pM to 1 μ M, CP8947 inhibited myometrial cell proliferation only at 1 μ M treatment concentrations. However, compared with untreated control leiomyoma cultures, 72-h treatment with 0.1nM (10^{-10} M) CP8947 significantly decreased the number of viable leiomyoma cells. Results represent the mean + SE of at least three independent experiments performed in triplicate. * = $p < 0.025$ relative to untreated cells).

* = $p < 0.01$ vs. untreated cells

Figure 7.

Effects of graded concentrations of CP8863 and CP8947 on caspase-3 expression (A), caspase-7 expression (B), and TRAIL induction in cultured human leiomyoma cells. Compared with untreated control cells, treatment with CP8863 induced caspase-3 activity at 100nM (10^{-7} M) concentration, and stimulated further induction in a dose-dependent manner, while CP8947 had no discernable impact on caspase-3 expression at treatment concentrations as high as 10 μ M (10^{-5} M). Caspase-7 was induced by CP8863 and CP8947 at 1 μ M concentration (B). TRAIL was induced with 100nM CP8947 treatment and 1 μ M CP8863 treatment (C). Results represent the mean + SE of at least three in triplicate. * = $p < 0.01$ relative to untreated controls).

Figure 8.

Effects of graded concentrations of CP8947 on COL1A1 and COL7A1 expression in human leiomyoma and patient-matched myometrial cells, as assessed by real time RT-PCR analysis. COL1A1 expression in human myometrial cells (A) was unaffected by CP8947 treatment to concentrations as high as 10 μ M (10⁻⁵M). Leiomyoma cells treated with CP8947 initially demonstrated a dramatic increase in COL1A1 expression by 24 hours at concentrations as low as 1nM, and a rapid diminution of COL1A1 expression to expression level seen in untreated myometrial cells by 48 h (B). COL7A1 expression in human myometrial cells was increased with 1nM treatment concentrations, but decreased by CP8947 treatment at concentrations of 1 μ M or greater (C), while expression was inhibited at concentrations of 1nM (10⁻⁹M) in human leiomyoma cells. Results represent the mean + SE of at least three in triplicate. * = p<0.01 relative to untreated myometrial cells. ** = p<0.01 relative to untreated leiomyoma cells.

Binding affinities of CP8863 and CP8947 for steroid receptors. The results represent the means derived from three independent experiments.

Table 1

Compounds	IC ₅₀		
	Progesterone Receptor	Estrogen Receptor	Glucocorticoid Receptor
			Androgen Receptor
			nM
CP8863	57	>1000	>1000
CP8947	8	>1000	>1000
MPA	2		