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In vitro antiprogestational/antiglucocorticoid activity and progestin and glucocorticoid receptor binding of the putative metabolites and synthetic derivatives of CDB-2914, CDB-4124, and mifepristone☆

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Abstract

In determining the biological profiles of various antiprogestins, it is important to assess the hormonal and antihormonal activity, selectivity, and potency of their proximal metabolites. The early metabolism of mifepristone is characterized by rapid demethylation and hydroxylation. Similar initial metabolic pathways have been proposed for CDB-2914 (CDB: Contraceptive Development Branch of NICHD) and CDB-4124, and their putative metabolites have been synthesized. We have examined the functional activities and potencies, in various cell-based assays, and relative binding affinities (RBAs) for progesterone receptors (PR) and glucocorticoid receptors (GR) of the putative mono- and didemethylated metabolites of CDB-2914, CDB-4124, and mifepristone and of the 17α-hydroxy and aromatic A-ring derivatives of CDB-2914 and CDB-4124. The binding affinities of the monodemethylated metabolites for rabbit uterine PR and human PR-A and PR-B were similar to those of the parent compounds. Monodemethylated mifepristone bound to rabbit thymic GR with higher affinity than monodemethylated CDB-2914 or CDB-4124. T47D-CO cells were used to assess inhibition of R5020-stimulated endogenous alkaline phosphatase activity and transactivation of the PRE_2-thymidine kinase (tk)-luciferase (LUC) reporter plasmid in transient transfections. The antiprogestational potency was as follows: mifepristone/CDB-2914/CDB-4124/monodemethylated metabolites (IC_{50}'s$\sim 10^{-9}$ M) > aromatic A-ring derivatives (IC_{50}'s$\sim 10^{-8}$ M) > didemethylated/17α-hydroxy derivatives (IC_{50}'s$\sim 10^{-7}$ M). Antiglucocorticoid activity was determined by inhibition of dexamethasone-stimulated transcriptional activity in HepG2 cells. The mono- and didemethylated metabolites of CDB-2914 and CDB-4124 had less antiglucocorticoid activity (IC_{50}'s$\sim 10^{-6}$ M) than monodemethylated mifepristone ($IC_{50}\sim 10^{-8}$ M) or the other test compounds. At 10^{-6} M in transcription assays, none of these compounds showed progestin agonist activity, whereas mifepristone and its monodemethylated metabolite manifested slight glucocorticoid agonist activity. The reduced antiglucocorticoid activity of monodemethylated CDB-2914 and CDB-4124 was confirmed in vivo by the thymus involution assay in adrenalectomized male rats. The aromatic A-ring derivatives-stimulated transcription of an estrogen-responsive reporter plasmid in MCF-7 and T47D-CO human breast cancer cells but were much less potent than estradiol. Taken together, these data suggest that the proximal metabolites of mifepristone, CDB-2914, and CDB-4124 contribute significantly to the antiprogestational activity of the parent compounds in vivo. Furthermore, the reduced antiglucocorticoid activity of CDB-2914 and CDB-4124 compared to mifepristone in vivo may be due in part to decreased activity of their putative proximal metabolites.

Keywords: Antiprogestin; Antiglucocorticoid; Alkaline phosphatase; Transactivation; Progestin receptor; Glucocorticoid receptor; T47D-CO cells; HepG2 cells

1. Introduction

Antiprogestins, of which the prototype is mifepristone, have potential uses for both regular and emergency contraception and for treatment of hormone-related pathological conditions such as breast cancer, endometriosis, and leiomyomata. For these purposes, several progesterone antagonists with 11β-aryl substitutions have been synthesized and characterized in recent years. Mifepristone (RU486) was the first steroid of this class to show high affinity binding to the progestin receptor (PR) [1–5]. However, mifepristone, as well as other 11β-aryl substituted antiprogestins, also binds with high affinity to glucocorticoid receptors (GR), resulting in antiglucocorticoid activity which could produce undesirable side effects in vivo for therapies requiring chronic administration [1,6,7]. Other antiprogestins

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have been synthesized and tested in an attempt to identify relatively pure progesterone antagonists with little or no antiglucocorticoid activity. Two such potential drugs, CDB-2914 (CDB: Contraceptive Development Branch of NICHD) and CDB-4124, also with 11β-aryl substitutions, are derivatives of 19-norprogesterone, unlike mifepristone which is derived from 19-nortestosterone. CDB-2914 and CDB-4124 have been shown to be potent antiprogestins in a variety of in vivo and in vitro assays, but to have less antiglucocorticoid activity than mifepristone [8–10]. As proximal metabolites may contribute significantly to the pharmacological activity profile of antiprogestins, it is important to assess the activity, selectivity, and potency of these metabolites. In vivo mifepristone undergoes rapid demethylation at the 11β-4-dimethylaminophenyl group to mono- and didemethylated mifepristone and hydroxylation at the 17α-propynyl group [11]. Similar pathways of metabolism have been proposed for CDB-2914 and CDB-4124, and reference preparations of these putative metabolites have been synthesized. In the present study, we compared activities and potencies of mifepristone, CDB-2914, CDB-4124, and their putative metabolites and synthetic derivatives in a series of in vitro assays. The following metabolites and derivatives were tested: monodemethylated mifepristone (CDB-4628); monodemethylated CDB-2914 (CDB-3877); monodemethylated CDB-4124 (CDB-4453); didemethylated CDB-2914 (CDB-3963); 17α-[3′-hydroxy-propynyl]-mifepristone (CDB-4163);17α-hydroxy CDB-2914 (CDB-3236); 17α-hydroxy CDB-4124 (CDB-4644); aromatic A-ring derivative of CDB-2914 (CDB-4183); and aromatic A-ring derivative of CDB-4124 (CDB-4641). We were unable to obtain didemethylated mifepristone, and didemethylated CDB-4124 has been difficult to synthesize. We investigated PR and GR binding affinities, inhibition of PR-mediated transactivation or alkaline phosphatase activity in T47D-CO human breast cancer cells, and inhibition of GR-mediated transactivation in HepG2 human hepatoblastoma cells. A limited number of in vivo studies were also performed for compounds available in sufficient quantity.

2. Materials and methods

2.1. Chemicals

Structures of the three progestin antagonists examined in this study and their putative metabolites and synthetic derivatives are depicted in Fig. 1. The antiprogestins, CDB-2914 (17α-acetoxy-11β-[4-*N*,*N*-dimethylaminophenyl]-19-norpregna-4,9-diene-3,20-dione), CDB-4124 (17α-acetoxy–21-methoxy-11β-[4-*N*,*N*-dimethylaminophenyl]-19-norpregna-4,9-diene-3,20-dione: 21-methoxy CDB-2914), and mifepristone all contain the 11β-[4-*N*,*N*-dimethylaminophenyl] moiety. CDB-2914 and CDB-4124 and their derivatives and putative metabolites were synthesized in the laboratory of Dr. P. N. Rao (Southwest Foundation for Biomedical Research, San Antonio, TX) under contract NO1-HD-6-3255. The purity of these compounds was assessed by high performance liquid chromatography (HPLC) and ranged from 93 to 99.5%. Mifepristone was obtained from Sigma (St. Louis, MO) and was 99% pure based on HPLC analysis. Monodemethylated mifepristone and 17α-[3′-hydroxy-propynyl]-mifepristone were provided by Dr. Rao and Dr. Martine Gaillard (Aventis, France), respectively. Methylprednisolone (MP) and 17β-estradiol (E_2) were purchased from Steraloids (Newport, RI). Stock solutions (10^{-2} M) were prepared in absolute ethanol, except for mono- and didemethylated CDB-2914 which were dissolved in DMSO, and diluted in the same solvent. CDB-2914 is also known variously as RTI 3021-012, RU 44675, and HRP 2000. The majority of other chemicals were purchased from Sigma.

2.2. Animals

New Zealand White (NZW) rabbits (ILAR Strain Designation Hra:(NZW)SPF) were purchased from Covance Research Products (Denver, PA) and housed in stainless steel cages. Rabbits were fed Purina (St. Louis, MO) laboratory rabbit diet (#5321) and fresh kale daily as a dietary fiber supplement. Immature female rabbits were primed with E_2 (5 μg per rabbit per day, injected sc for 6 days) for determining antiprogestational activity (see Section 2.7). Sprague–Dawley CD rats (Crl:CD(SD)IGS BR Stock) were purchased from Charles River Laboratories (Kingston, NY), housed in polycarbonate solid floor cages with Bed-o-Cob bedding (Andersons Industrial Products Group, Maumee, Ohio), and fed Purina laboratory rodent diet (#5001) ad libitum. All rats were group-housed. Animals received tap water ad libitum, except for adrenalectomized male rats which received 0.9% saline ad libitum. The photoperiod for the rabbit rooms was 12 h light/12 h dark and for the rat rooms was 14 h light/10 h dark. The environmental conditions of the animal rooms were maintained as recommended in the *Guide for the Care and Use of Laboratory Animals* to the maximum extent possible (National Research Council, 1996). All study protocols were approved by BIOQUAL's IACUC.

2.3. Steroid hormone receptor assays

Competitive binding assays for steroid hormone receptors were performed using cytosolic preparations from tissues or cells. Cytosols containing PR or GR were prepared from uterus or thymus, respectively, of E_2-primed immature rabbits. Recombinant human PR-A or PR-B (rhPR-A, rhPR-B) were assayed in cytosolic extracts from Sf9 insect cells infected with recombinant baculovirus expressing either hPR-A or hPR-B (provided by Dr. Dean Edwards, University of Colorado Health Science Center, Denver, CO; [12]). For binding to rabbit uterine PR, cytosol was prepared in TEGMD buffer (10 mM Tris, pH 7.2, 1.5 mM EDTA,

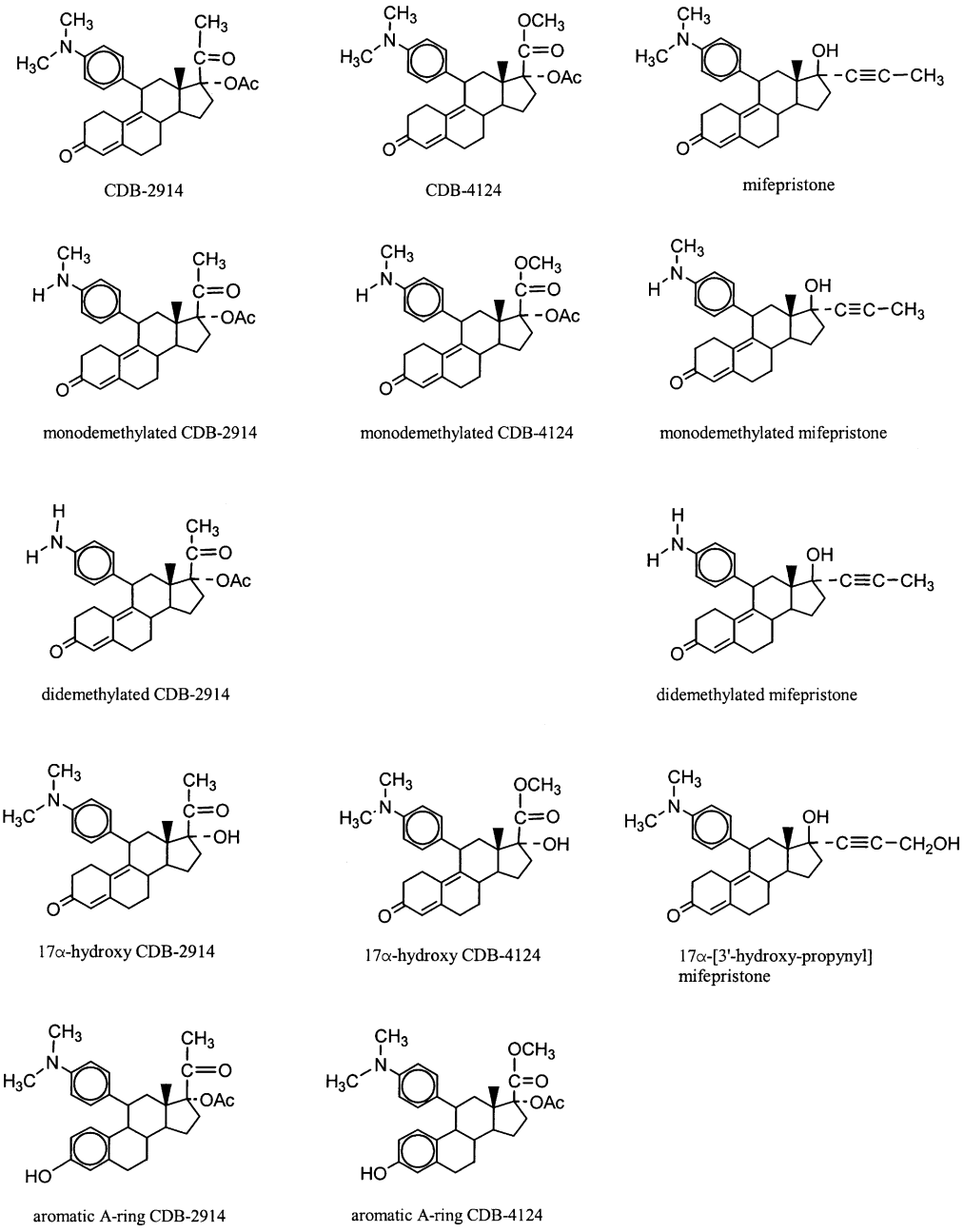

Fig. 1. Structures of the antiprogestins, mifepristone, CDB-2914, CDB-4124, and their putative metabolites and synthetic derivatives. All were included in this study except didemethylated mifepristone (CDB-4162), which was no longer available.

0.2 mM sodium molybdate, 10% glycerol, 1 mM DTT) and incubated with 6 nM 1,2-[^{3}H] progesterone (Perkin-Elmer Life Sciences Inc., Boston, MA; 52 Ci/mmol); competitors were added at concentrations from 2 to 100 nM. For binding to rhPR-A or rhPR-B, cytosol from Sf9 cells (prepared in TEGMD buffer containing the following protease inhibitors: bacitracin at 100 μg/ml, aprotinin at 2 μg/ml, leupeptin at 94 μg/ml, pepstatin A at 200 μg/ml) was incubated with 6.8 nM 1,2,6,7,16,17-[^{3}H] progesterone (Perkin-Elmer; 143 Ci/mmol); competitors were added at concentrations from 1 to 100 nM. For binding to rabbit thymic GR, cytosol was prepared in TEGMD buffer and incubated with 6 nM 6,7-[^{3}H] dexamethasone (Perkin-Elmer; 35 or 40 Ci/mmol); competitors were added at concentrations from 2 to 100 nM. After overnight incubation at 4 °C, bound and unbound [^{3}H] steroids were separated by addition of dextran-coated charcoal and centrifugation at 2100 × g for 15 min at 4 °C. Supernatants from GR assays were decanted and counted in a Beckman LS-1800 liquid scintillation counter. Supernatants containing PR were pipetted into 24-well microplates and

counted in a Packard TopCount liquid scintillation counter. In all steroid receptor competitive binding assays, cpm were entered into Packard's RIASmart™ for calculation of EC_{50}'s using a 4-parameter logistic curve fit. Relative binding affinities (RBA, %) for each compound were calculated as follows: EC_{50} of standard/EC_{50} of competitor × 100. The standards for the PR and GR assays were progesterone and dexamethasone, respectively.

The assay for human estrogen receptors (hER) α and β1-long form employed purified recombinant hER purchased from PanVera (Madison, WI). ER (0.45 pmol per well) was incubated with 10 nM 6,7 or 2,4,6,7 –[^{3}H] E_2 (Perkin-Elmer; 40–99 Ci/mmole) in 10 mM Tris, pH 7.5, 10% glycerol, 1 mg/ml ovalbumin, and 2 mM DTT in the absence or presence of 2 μM E_2 to measure total or non-specific binding, respectively. The final ethanol concentration was 5%. Competitors were added at concentrations from 0.5 to 10,000 nM. Incubations were carried out in Millipore Multiscreen plates (Millipore, Burlington, MA) at 4 °C overnight. [^{3}H] E_2–ER complexes were separated from unbound radioligand by addition of 25 or 50 μl 50% hydroxylapatite to each well, incubation for 15 min at 4 °C on a plate shaker, filtration on a vacuum manifold, and washing three times with cold wash buffer (ERα: 40 mM Tris, pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM EGTA; ERβ-1 long: 40 mM Tris, pH 7.5). Plates were dried, and the wells were punched out and transferred to scintillation vials. Receptor-bound cpm were determined in the Beckman LS-1800 or Wallac 1209 Rackbeta scintillation counter and entered into Packard's RIASmart™ for calculation of EC_{50}'s as described above.

2.4. Cell culture and transfection of plasmid DNAs

Cell culture reagents were obtained from Invitrogen (Carlsbad, CA), unless otherwise specified. T47D-CO human breast cancer cells were a gift of Dr. Kathryn Horwitz (University of Colorado Health Science Center, Denver, CO). They were grown routinely in monolayer culture in phenol red-free DMEM supplemented with 10% fetal bovine serum (FBS), 10 U/ml penicillin G, and 10 μg/ml streptomycin sulfate (pen/strep). HepG2 human hepatoblastoma cells purchased from ATCC (Manassas, VA) and MCF-7 cells (from Dr. Sanford Simon at the Rockefeller University, New York, NY) were grown in monolayer culture in phenol red-free MEMα supplemented with 10% FBS and pen/strep. For transient transfection assays, cells were plated in 6-well dishes at ∼ 0.5–1 × 10^6 cells per well in the corresponding medium containing 10% dextran-coated charcoal (DCC)-stripped FBS (Hyclone Laboratories Inc., Logan, UT) and pen/strep and used 24–48 h later at 60–80% confluency. Approximately, 1–2 h prior to transfection, the medium was withdrawn, and fresh medium was added. T47D-CO cells, which express approximately equimolar concentrations of constitutively produced hPR-A and hPR-B (K. Horwitz, personal communication) were used to assess progestational agonist or antagonist activities in an estrogen-free environment [13]. These cells were transfected with the PRE_2-thymidine kinase (tk)-luciferase (LUC) reporter plasmid, containing two copies of the progestin/glucocorticoid/androgen response element upstream of the thymidine kinase promoter and the firefly luciferase reporter gene (from Dr. Dean Edwards), using FuGENE 6 transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's instructions (ratio of FuGENE 6 to DNA: 6 to 1). HepG2 cells were used to assess glucocorticoid agonist or antagonist activity. These cells were cotransfected with PRE_2-tk-LUC and a rat GR expression plasmid (6RGR from Drs. Roger Miesfeld and Keith Yamamoto, UCSF, San Francisco, CA) at a ratio of 20:1 using FuGENE 6 transfection reagent as described previously [10]. Both T47D-CO and MCF-7 cells were used for assessing estrogenic activity of the aromatic A-ring derivatives of CDB-2914 and CDB-4124 using a 3XERE-LUC reporter plasmid (from Dr. Donald McDonnell, Duke University, Durham, NC). Briefly, for each transfection experiment, a sufficient quantity of FuGENE 6–DNA complexes was prepared to dispense to all control and hormone-treated wells, in duplicate or triplicate. After 6 h, cells were washed with DPBS and incubated in fresh medium containing test compounds or vehicle for 20 h. In every experiment, separate wells were transfected with a control vector, pGL3-LUC (Promega Corp., Madison, WI), to monitor transfection efficiency and interassay variability. Cell lysates were prepared in Passive Lysis Buffer (Promega) and analyzed for protein content [14]. LUC activity was determined in 20 μl aliquots of the lysates using Promega's Luciferase Assay System. Light emission was measured in a microplate luminometer (Fluoroskan Ascent FL, Labsystems, Franklin, MA) and expressed as relative light units (RLU). Data were normalized for differences in protein content per well.

2.5. Alkaline phosphatase assay

Inhibition of alkaline phosphatase activity in T47D-CO cells was assessed as described previously [10]. Briefly, cells were transferred to DMEM containing 10% DCC-stripped FBS and pen/strep. After 24 h, they were seeded in 96-well plates at 5 × 10^4 cells per well and incubated at 37 °C. The following day, the medium was aspirated, and fresh medium (150 μl) containing test compounds was added. Antagonist activity was determined by incubating cells with 10^{-8} M R5020 in the presence of antiprogestins, metabolites, or derivatives (10^{-10} to 10^{-6} M) for an additional 72 h. Cells were washed with PBS and lysed by freeze-thawing. Plates were placed on ice, and 50 μl alkaline phosphatase assay mix (5 mM *p*-nitrophenyl phosphate, pNPP: Pierce, Rockford, IL; 0.24 mM $MgCl_2$, 1 M diethanolamine, pH 9.8) was added to each well according to the procedure of Markiewicz and Gurpide [15]. Plates were incubated at room temperature for 2 or 4 h to allow color development, and the OD at 405 nm

was determined in a Molecular Devices microplate reader (Sunnyvale, CA).

2.6. Aromatization reaction

Incubation of test compounds (testosterone, CDB-2914, CDB-4124) with human CYP19 + P450 reductase SUPERSOMES (BD Biosciences, Woburn, MA) was carried out according to the manufacturer's recommendations. A 250 μl reaction mixture contained 25 pmol human CYP19 + P450, 50 μM substrate in ethanol, 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, and 3.3 mM $MgCl_2$ in 100 mM KPO_4, pH 7.4. The mixture was incubated at 37 °C for 40 min during which time production of E_2 from testosterone was linear. After incubation, the reaction was stopped by addition of 125 μl acetonitrile (ACN) and centrifuged at 10,000 × *g* for 3 min. Fifty microliters of the supernatant were injected into a 4.6 mm × 250 mm Phenomenex Luna 5 μ C18 HPLC column (Phenomenex, Torrance, CA) and eluted isocratically with mobile phases empirically determined to separate the substrate from the aromatic A-ring product: testosterone/E_2: 60% H_2O, 30% ACN, 10% methanol; CDB-2914/aromatic A-ring CDB-2914: 50% H_2O, 40% ACN, 10% methanol; CDB-4124/aromatic A-ring CDB-4124: 35% H_2O, 50% ACN, 15% methanol at a flow rate of 1 ml/min. Decrease of substrate and formation of product were monitored by absorbance at 216 nm (testosterone/E_2), 254 nm (CDB-2914/aromatic A-ring derivative), or 248 nm (CDB-4124/aromatic A-ring derivative).

2.7. In vivo antiprogestational activity

Inhibition of progesterone-stimulated endometrial glandular arborization was assessed according to the McPhail scale in E_2-primed immature female rabbits (five per group) treated with the test compound either by the oral or intrauterine route [8,16,17]. Intrauterine administration (0.25–1.0 μg) was employed to examine direct antiprogestational effects of mono- and didemethylated CDB-2914 and monodemethylated CDB-4124 on the uterine endometrium [17]. Oral administration of the putative metabolites (0.4–1.6 mg per rabbit per day for 5 days) to E_2-primed rabbits was used to evaluate the contribution of further metabolism. For oral dosing, rabbits were restrained, and 0.5 ml of vehicle or dosing solution was slowly delivered to the back of the throat using a 16 gauge ball-tipped 4.5 in. curved stainless steel gavage needle attached to a 1 ml airtight syringe. To insure accuracy, the gavage needle was preloaded with the proper dosing solution.

2.8. In vivo antiglucocorticoid activity

To assess antiglucocorticoid activity, young male rats (100–120 g) were adrenalectomized using aseptic surgical technique and treated orally with monodemethylated CDB-2914, monodemethylated CDB-4124, or mifepristone (1.5, 3, or 6 mg per rat per day for 3 days; 10 rats per group), or vehicle, in an attempt to block sc administered methylprednisolone-induced thymus involution (1 mg MP per rat per day for 3 days). For oral dosing, rats were restrained, and a 16 gauge ball-tipped 3 in. curved stainless steel gavage needle was used to deliver the dosing solution directly to the stomach. Twenty-four hours after the final dose, rats were euthanized, the final body weight recorded, and the thymus gland excised, trimmed, blotted, and weighed [8,18].

2.9. Analysis of data

GraphPad PRISM, versions 2.0 or 3.0 (GraphPad Software, San Diego, CA), was used for graphics and determination of IC_{50}'s and EC_{50}'s for inhibition or stimulation, respectively, of transactivation or alkaline phosphatase activity by antagonists or agonists of PR or GR. Multiple determinations of the IC_{50}'s for different test compounds were compared statistically by one-way analysis of variance (ANOVA) followed by Bonferroni's *t*-test for all pairwise data using SigmaStat, version 2.03 (SPSS Inc., Chicago, IL). Thymus weights in the antiglucocorticoid bioassay were compared by one-way ANOVA followed by the Student–Newman–Keuls multiple range test. Data that did not pass normality were $\log_{10}$ transformed prior to analysis. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. PR and GR binding characteristics

Fig. 1 illustrates the chemical structures of mifepristone, CDB-2914, CDB-4124, and all putative metabolites and synthetic derivatives evaluated in the present study. Table 1 summarizes the EC_{50}'s and relative binding affinities of these compounds for rabbit uterine PR (PR-B; [19]), rhPR-A or rhPR-B, and rabbit thymic GR. Mifepristone, the CDB antiprogestins, and their monodemethylated metabolites bound with high affinity to rabbit uterine PR. In contrast, affinities of mifepristone and CDB-2914 for rhPR-A and rhPR-B were two to three-fold greater than those of CDB-4124 and its putative monodemethylated metabolite. The EC_{50}'s and RBAs for binding to rhPR-A were very similar to those for binding to rhPR-B for all the compounds tested. Some compounds showed higher affinity for rabbit PR than rhPR-A or B, especially monodemethylated mifepristone, CDB-4124, and didemethylated CDB-2914. The 17α-hydroxy derivatives had the lowest affinities for PR. Mifepristone and its monodemethylated metabolite showed the highest affinity for rabbit thymic GR, whereas didemethylated CDB-2914 and the aromatic A-ring compounds showed the lowest (Table 1). Affinities of the other test compounds for GR were similar (RBAs = 40–50%).

Table 1
Binding of antiprogestins, putative metabolites and derivatives to progestin and glucocorticoid receptors

CDB compound	rhPR-B		rhPR-A		Rabbit uterine PR		Rabbit thymic GR	
	EC_{50} (nM)	RBA[a] (%)	EC_{50} (nM)	RBA (%)	EC_{50} (nM)	RBA (%)	EC_{50} (nM)	RBA[b] (%)
Progesterone	8.0 ± 0.3	100[c]	7.7 ± 0.8	100[c]	11.6 ± 0.4	100[c]		
Dexamethasone							8.2 ± 0.4	100[c]
Mifepristone	9.5 ± 0.9	82	10.6 ± 1.3	84	11.5 ± 0.9	99	9.1 ± 0.8	88
Monodemethylated mifepristone	9.7 ± 1.3	76	11.9 ± 1.3	70	7.7 ± 0.5	132	6.7 ± 0.7	105
17α-[3′-Hydroxy-propynyl]-mifepristone	44.9 ± 16.4 (2)	15	25.7 ± 0.8 (2)	31	40.1 ± 18.3 (2)	26	18.8 ± 1.1 (2)	43
CDB-4124	17.5 ± 3.5	42	18.3 ± 3.0	45	13.2 ± 1.0	88	16.6 ± 2.9	41
Monodemethylated CDB-4124	27.0 ± 3.0	27	25.8 ± 4.3	33	10.0 ± 1.4	109	19.2 ± 5.3	38
17α-Hydroxy CDB-4124	204.7 ± 27.9	4	345.7 ± 59.8	2	133.1 ± 22.0	10	17.3 ± 1.4	43
Aromatic A-ring CDB-4124	29.6 ± 4.9	25	35.9 ± 5.2	21	34.8 ± 4.2	39	34.5 ± 2.7	22
CDB-2914	7.7 ± 0.5	99	8.5 ± 0.6	101	13.6 ± 0.6	85	15.4 ± 1.3	53
Monodemethylated CDB-2914	8.8 ± 0.2	78	11.6 ± 1.0	74	11.8 ± 0.9	101	14.7 ± 0.8	55
Didemethylated CDB-2914	83.2 ± 11.9	9	108.5 ± 8.1	8	17.5 ± 2.5	60	73.9 ± 10.3	11
17α-Hydroxy CDB-2914	91.9 ± 14.2	8	149.8 ± 16.8	6	77.5 ± 12.1	15	21.9 ± 0.7	37
Aromatic A-ring CDB-2914	19.5 ± 4.1	36	32.4 ± 1.8	26	72.7 ± 16.1	16	78.2 ± 13.0	10

Values represent the mean ± S.E., $n = 3$–56, or the mean ± S.D., $n = 2$, where indicated.
[a] RBA: relative binding affinity = EC_{50} progesterone/EC_{50} test compound × 100.
[b] RBA: relative binding affinity = EC_{50} dexamethasone/EC_{50} test compound × 100.
[c] Defined.

3.2. Competitive inhibition of transcriptional activity and alkaline phosphatase induction mediated by PR

Antiprogestational activity was assessed in T47D-CO cells by inhibition of R5020-stimulated induction of endogenous alkaline phosphatase activity or of transcription of the PRE_2-tk-LUC reporter plasmid. Representative one-site inhibitor curves for monodemethylated mifepristone and CDB-2914 in the transient transfections are illustrated in Fig. 2A. IC_{50}'s for these and all other compounds tested were calculated and potencies expressed relative to mifepristone (=1) (Table 2). The monodemethylated metabolites of mifepristone, CDB-2914, and CDB-4124 manifested considerable potency as antiprogestins in both assays. The relative potency of the various test compounds was similar in the alkaline phosphatase and transcription assays: monodemethylated > aromatic A-ring derivatives > didemethylated/17α-hydroxy derivatives. Steroidal PR ligands have been reported to demonstrate agonist, antagonist, or mixed agonist activities. Fig. 3A shows that in this cell system, mifepristone, CDB-2914, CDB-4124, and their metabolites/derivatives, even at 10^{-6} M, did not exhibit any agonist activity (≤transcriptional activity of the vehicle control).

3.3. Competitive inhibition of transcriptional activity mediated by GR

In the HepG2 cell system cotransfected with PRE_2-tk-LUC and the 6RGR expression plasmid, glucocorticoid agonists were effective inducers of transcription, whereas androgens,

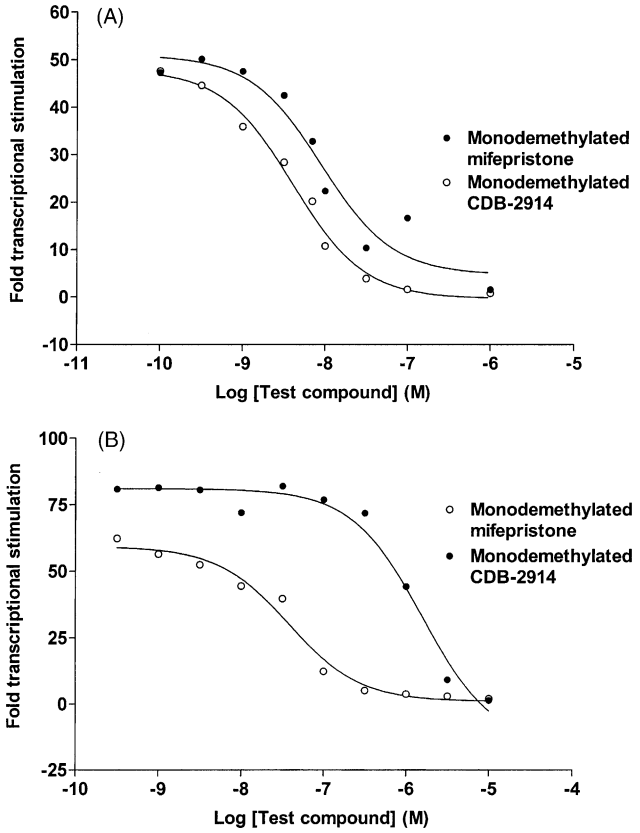

Fig. 2. Representative curves for inhibition of transcriptional activity by monodemethylated mifepristone or monodemethylated CDB-2914. (A) T47D-CO cells treated with 10^{-8} M R5020 (IC_{50}'s: 9.0 and 4.1×10^{-9} M, respectively); (B) HepG2 cells treated with 10^{-8} M dexamethasone (IC_{50}'s: 3.8×10^{-8} and 1.6×10^{-6} M, respectively).

Table 2
Inhibition of R5020 (10^{-8} M)-stimulated transcription or alkaline phosphatase activity in T47D-CO cells or inhibition of dexamethasone (10^{-8} M)-stimulated transcription in HepG2 Cells by antiprogestins, putative metabolites, and derivatives

CDB Compound	IC_{50}[a] for inhibition of R5020-stimulated transcription (M)	Antiprogestational potency compared to mifepristone[b]	IC_{50}[a] for inhibition of R5020-stimulated alkaline phosphatase activity (M)	Antiprogestational potency compared to mifepristone[b]	IC_{50}[a] for inhibition of dexamethasone-stimulated transcription (M)	Antigluco-corticoid potency compared to mifepristone[b]
Mifepristone	$1.3 \pm 0.2 \times 10^{-9}$ c	1	$7.0 \pm 1.3 \times 10^{-9}$ cd	1	$5.9 \pm 1.5 \times 10^{-9}$ c	1
Monodemethylated mifepristone	$7.6 \pm 1.9 \times 10^{-9}$ d	0.17	$3.3 \pm 1.3 \times 10^{-8}$ cef	0.21	$4.5 \pm 0.6 \times 10^{-8}$ d	0.13
17α-[3'-Hydroxy-propynyl] mifepristone	$1.3 \pm 0.2 \times 10^{-7}$ ef	0.01	$7.5 \pm 0.3 \times 10^{-7}$ g	0.009	$5.1 \pm 0.7 \times 10^{-7}$ fh	0.012
CDB-4124	$6.7 \pm 1.7 \times 10^{-9}$ d	0.19	$1.0 \pm 0.3 \times 10^{-8}$ cd	0.68	$1.7 \pm 0.5 \times 10^{-7}$ def	0.035
Monodemethylated CDB-4124	$4.2 \pm 0.6 \times 10^{-9}$ cd	0.31	$2.3 \pm 0.6 \times 10^{-8}$ df	0.31	$1.0 \pm 0.3 \times 10^{-6}$ ghi	0.0059
17α-Hydroxy CDB-4124	$4.5 \pm 1.4 \times 10^{-7}$ f	0.0029	$7.4 \pm 0.8 \times 10^{-7}$ g	0.0095	$2.3 \pm 0.5 \times 10^{-7}$ efg	0.026
Aromatic A-ring CDB-4124	$5.8 \pm 1.1 \times 10^{-8}$ e	0.022	$5.2 \pm 0.4 \times 10^{-8}$ efh	0.13	$2.7 \pm 0.6 \times 10^{-7}$ efg	0.022
CDB-2914	$2.0 \pm 0.4 \times 10^{-9}$ cd	0.65	$8.2 \pm 2.2 \times 10^{-9}$ cd	0.85	$7.3 \pm 1.8 \times 10^{-8}$ de	0.081
Monodemethylated CDB-2914	$3.2 \pm 1.1 \times 10^{-9}$ cd	0.41	$4.5 \pm 1.8 \times 10^{-9}$ d	1.55	$1.3 \pm 0.1 \times 10^{-6}$ hi	0.0045
Didemethylated CDB-2914	$2.0 \pm 0.6 \times 10^{-7}$ ef	0.0064	$1.3 \pm 0.2 \times 10^{-7}$ egh	0.052	$2.5 \pm 0.3 \times 10^{-6}$ i	0.0024
17α-Hydroxy CDB-2914	$3.6 \pm 0.4 \times 10^{-7}$ f	0.0036	$2.7 \pm 1.1 \times 10^{-7}$ gh	0.026	$3.8 \pm 1.3 \times 10^{-7}$ fgh	0.016
Aromatic A-ring CDB-2914	$5.9 \pm 1.4 \times 10^{-8}$ e	0.022	$5.4 \pm 1.7 \times 10^{-8}$ de	0.13	$3.2 \pm 0.7 \times 10^{-7}$ efgh	0.018

[a] Values represent the mean ± S.E., $n = 3$–9.
[b] IC_{50} mifepristone/IC_{50} test compound.
cdefghi Means with different letters are significantly different ($P < 0.05$).

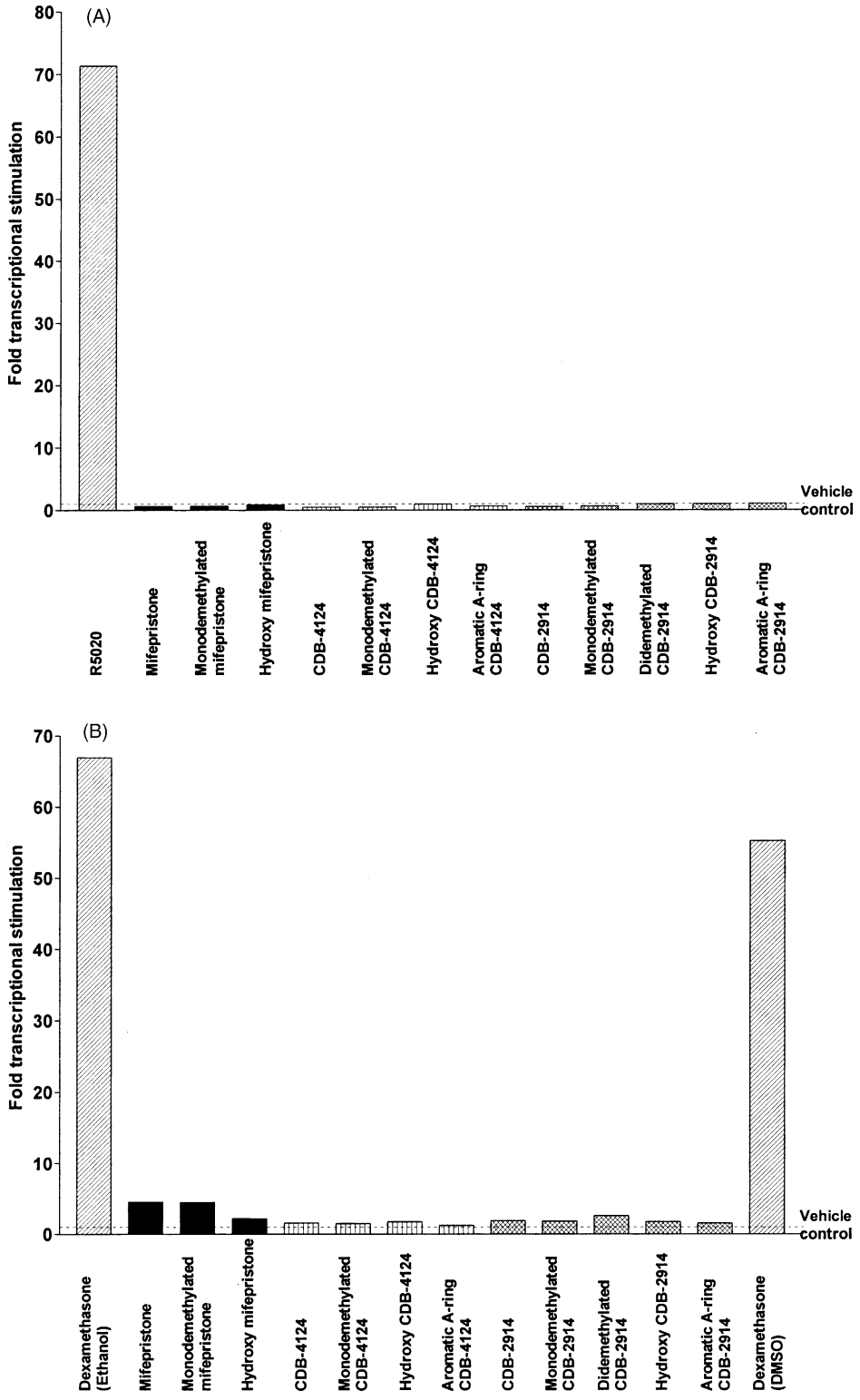

Fig. 3. Progestin (A) or glucocorticoid (B) agonist activity of mifepristone, CDB-2914, CDB-4124 and their putative metabolites and synthetic derivatives. T47D-CO (A) or HepG2 (B) cells were transfected in duplicate or triplicate with the PRE_2-tk-LUC reporter plasmid as described in Section 2.4 and treated with the indicated compounds at 10^{-6} M for 20 h. Luciferase activity was normalized for protein content and expressed as fold stimulation.

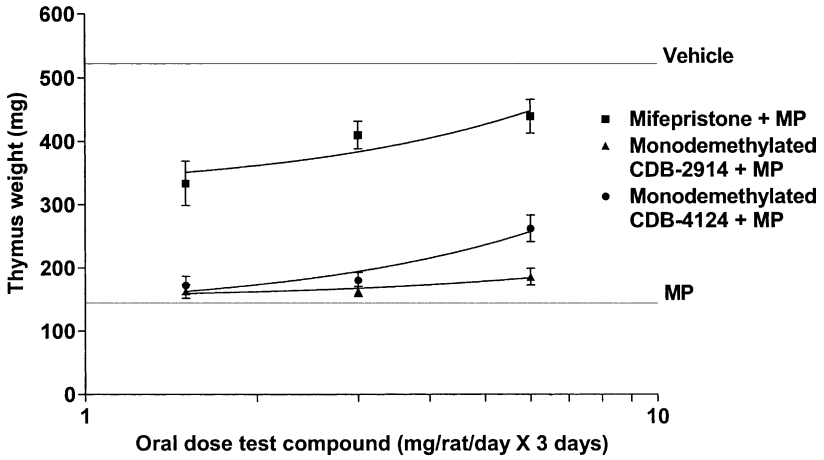

Fig. 4. Reversal of MP-induced thymus involution in the adrenalectomized male rat by mifepristone, monodemethylated CDB-2914, and monodemethylated CDB-4124.

E_2, and progestins showed little or no induction of LUC activity [10]. The antiglucocorticoid potency of the various antiprogestins, metabolites, and derivatives was assessed in HepG2 cells by dose-dependent inhibition of dexamethasone (10^{-8} M)-stimulated transcription of PRE_2-tk-LUC. Representative one-site inhibitor curves for monodemethylated mifepristone and CDB-2914 are illustrated in Fig. 2B. IC_{50}'s were calculated, and potencies expressed relative to mifepristone (=1) (Table 2). Monodemethylated mifepristone, like mifepristone itself, was considerably more potent as a glucocorticoid antagonist than mono- or didemethylated CDB-2914 or monodemethylated CDB-4124. The antiglucocorticoid activity of the aromatic A-ring and 17α-hydroxy derivatives of CDB-2914 or CDB-4124 was equivalent, but low. Possible glucocorticoid agonist activity was investigated by treating transfected HepG2 cells with test compounds at 10^{-6} M, dexamethasone at 10^{-8} M, or vehicle (ethanol or DMSO) (Fig. 3B). Mifepristone and monodemethylated mifepristone at 10^{-6} M stimulated luciferase activity four to five-fold over basal; however, the effect was much less than that of dexamethasone at 10^{-8} M. All other compounds had negligible glucocorticoid agonist activity.

3.4. Estrogenic activity of the aromatic A-ring derivatives of CDB-2914 and CDB-4124

Aromatic A-ring derivatives of CDB-2914 and CDB-4124 were prepared for this study by organic chemists to determine whether these synthetic derivatives possess estrogenic activity. Both compounds stimulated transcription of 3XERE-LUC, but with low potency (not shown). The EC_{50}'s for transactivation in T47D-CO cells were $2–3 \times 10^{-7}$ M, about four orders of magnitude higher than that for E_2 ($EC_{50} = 2.5 \pm 0.8 \times 10^{-11}$ M, mean ± S.D., $n = 2$). In MCF-7 cells, the response to aromatic A-ring derivatives was biphasic: transcription of 3XERE-luciferase was stimulated at concentrations $\leq 10^{-7}$ M and inhibited at higher concentrations (not shown). RBAs for both hERα and hERβ-1 of the aromatic A-ring derivatives of CDB-2914 and CDB-4124 were <1 and <0.1%, respectively (compared to E_2 as reference standard at 100%). Mifepristone, CDB-2914, and CDB-4124 did not bind to hER (EC_{50}'s > 10,000 nM; RBAs < 0.1%). Using human CYP19 (aromatase) + P450 reductase SUPERSOMES, we attempted to detect A-ring aromatization of CDB-2914 and CDB-4124 using conditions established for demonstrating aromatization of testosterone to E_2. There was no evidence for formation of the aromatic A-ring derivative of either CDB-2914 or CDB-4124 in this system during a 40 min incubation (data not shown).

3.5. In vivo studies with metabolites of CDB-2914 and CDB-4124

In most cases, the quantities of putative metabolites/synthetic derivatives available were too limited to perform in vivo assays. However, when possible, compounds were tested for antiprogestational activity in rabbits, following intrauterine or oral administration, and for oral antiglucocorticoid activity in the adrenalectomized male rat. Neither monodemethylated CDB-2914 nor monodemethylated CDB-4124 demonstrated significant antiprogestational activity after oral dosing. However, inhibition (10–60%) of endometrial gland arborization based on the McPhail index was observed for mono- and didemethylated CDB-2914 and monodemethylated CDB-4124 following intrauterine administration (data not shown). These results support the notion that the metabolites possess some antiprogestational activity in vivo but are rapidly metabolized further following systemic administration. In the antiglucocorticoid assay (Fig. 4), mifepristone, at all doses tested (4.5, 9, or 18 mg total dose), significantly ($P < 0.05$) reversed MP-induced thymus involution. In contrast, thymus weights from rats

treated with monodemethylated CDB-2914, at all dose levels, were not different ($P > 0.05$) from those of MP-treated rats. Monodemethylated CDB-4124 significantly ($P < 0.05$) reversed the MP-induced thymus weight decrease only at the highest dose, but thymus weight was significantly lower than that from vehicle-treated rats ($P < 0.05$).

4. Discussion

The metabolism of mifepristone has been studied extensively although there has been little prior examination of the functional activity and potency of the metabolites in cell systems. In humans, rats, and monkeys, mifepristone undergoes rapid demethylation at the 11β-4-dimethylaminophenyl group to mono- and didemethylated mifepristone and hydroxylation at the 17α-propynyl group ([11]; reviewed in [20]). In humans there are seven "possible" metabolites, but the three mentioned above have been identified. The *N*-monodemethylated product is the primary metabolite, and after oral administration of 100–800 mg mifepristone, its concentration exceeds that of the parent compound [21,22]. At higher dose levels of mifepristone (>400 mg), the other metabolites also accumulate at high concentrations [21]. Both the demethylated and hydroxylated metabolites are eventually further degraded and excreted into bile [11]. From metabolic studies performed on rat liver or hepatoma cells, Chasserot–Golaz et al. [23,24] concluded that demethylation of mifepristone is mediated by cytochromes P450 2B and 2C, whereas CYP450 3A4 is responsible for the hydroxylation steps. CYP 3A4 has also been shown to be the primary CYP enzyme involved in oxidative metabolism of mifepristone in human liver microsomes [25].

The half-life ($t_{1/2}$) for elimination of mifepristone has been reported to vary between 24 and 48 h when measured by HPLC and between 54 and 90 h when measured by radioimmunoassay (RIA) or radioreceptor assay (RRA) (see [20]). The longer apparent $t_{1/2}$ obtained by RIA or RRA is likely due to cross-reactivity with various metabolites. As described previously [26], monoclonal antibodies to mifepristone provided by Dr. Fortune Kohen (Weitzman Institute of Science, Rehovot, Israel) exhibited 86, 57, and <1% cross-reactivity with monodemethylated, didemethylated, and hydroxylated mifepristone, respectively. By analogy to the authenticated pathway for metabolism of mifepristone, putative metabolites have been proposed for CDB-2914 and CDB-4124 involving monodemethylation, didemethylation, and hydroxylation at the 17α position. We have developed an RIA for CDB-2914 [26] which employs rabbit polyclonal antibodies. This antiserum cross reacts extensively with several of the putative metabolites/synthetic derivatives of CDB-2914 (monodemethylated, 90%; didemethylated, 62%; aromatic A-ring, 25%; 17α-hydroxy, <1%). Currently, an assay involving HPLC separation of CDB-2914 and metabolites is under development by HRA Pharma (Paris, France) which is expected to provide more extensive information about the nature and time-course of appearance of various metabolites [27]. Preliminary HPLC and LC/MS results have confirmed that the mono- and dimethylated metabolites of CDB-2914 are present in rat plasma and bile after intravenous or oral administration of CDB-2914 (5 mg/kg) to female rats. In addition to the parent compound, three other metabolites were found in plasma and a total of six, in bile [Gainer, HRA Pharma, personal communication].

It is clear from the foregoing that the biological activity and potency of these early major metabolites are important considerations. In the present study, we investigated the binding to PR and GR and the functional activities in cultured cells of the currently available metabolites/derivatives of mifepristone, CDB-2914, and CDB-4124. The mono- and didemethylated and the hydroxylated metabolites of mifepristone were shown previously to bind to human uterine PR and placental GR [28]. Mifepristone, CDB-2914, and their monodemethylated metabolites bound to rhPR-A and rhPR-B and rabbit PR with the highest affinity. The affinities of the other compounds for PR were ranked as follows: CDB-4124/mondemethylated CDB-4124 > aromatic A-ring derivatives > didemethylated/17α-hydroxy derivatives. The monodemethylated metabolites of mifepristone, CDB-2914, and CDB-4124 manifested considerable potency as antiprogestins in the alkaline phosphatase and transcription assays. The relative potency of the various test compounds was similar in both assays: mifepristone/CDB-2914/CDB-4124/monodemethylated metabolites (IC_{50}'s$\sim 10^{-9}$ M) > aromatic A-ring derivatives (IC_{50}'s$\sim 10^{-8}$ M) > didemethylated/17α-hydroxy derivatives (IC_{50}'s$\sim 10^{-7}$ M). These results indicate that the relative inhibitory potency of these metabolites/derivatives depends on their relative binding affinities for PR.

Mifepristone and its mondemethylated metabolite showed the highest affinity for rabbit thymic GR, whereas didemethylated CDB-2914 and the aromatic A-ring compounds showed the lowest. Affinities of the other test compounds for GR were similar. Monodemethylated mifepristone, like mifepristone itself, was considerably more potent as a glucocorticoid antagonist than monodemethylated CDB-2914 or CDB-4124 or didemethylated CDB-2914. The antiglucocorticoid activity of the aromatic A-ring and 17α-hydroxy derivatives of CDB-2914 or CDB-4124 was equivalent, but low. RBAs for GR were not predictive of functional activity confirming that other factors, such as interactions with coregulators, are probably of importance in modulating transactivation/transrepression [29]. Relative to mifepristone (=1), the ratio of antiprogestational to antiglucocorticoid activity was highest for the putative monodemethylated metabolites of CDB-2914 and CDB-4124 (=50–100), followed by CDB-2914, CDB-4124, and didemethylated CDB-2914. This ratio approximated 1 for monodemethylated mifepristone and the aromatic A-ring derivatives, whereas it was 0.1–0.2 for the 17α-hydroxy derivatives.

In most cases, it was not possible to confirm activity of the metabolites in vivo as the amounts of compounds available were too limited, and further metabolism would likely occur. The results of the oral versus intrauterine antiprogestational activities of the putative monodemethylated metabolites of CDB-2914 and CDB-4124 support the notion that these metabolites possess some antiprogestational activity in vivo, but are rapidly metabolized further to less potent derivatives following systemic administration. However, the monodemethylated metabolites of CDB-2914 and CDB-4124 both showed very reduced antiglucocorticoid activity in vivo, as well as in vitro, compared to mifepristone and its monodemethylated metabolite. In addition, the low level of glucocorticoid agonist activity observed here in transcription assays in HepG2 cells is in agreement with our in vivo results. Mifepristone, CDB-2914, and CDB-4124 had negligible glucocorticoid agonist activity in the adrenalectomized male rat thymolytic assay at doses up to 3.33–4 mg per day (10–12 mg total dose).

The biological activity of the antiprogestin metabolites, especially the proximal ones, is of great importance due to the rapid metabolism of the parent compounds in vivo. The data presented here strongly suggest that certain metabolites of the antiprogestins, particularly the monomethylated derivatives, may contribute quite significantly to the in vivo antiprogestational/ antiglucocorticoid activity profiles of the parent compounds. Currently, CDB-2914 and CDB-4124 are being tested, both in animal models and clinical trials, for emergency and long-term, low dose contraceptive use and for treatment of hormone dependent diseases such as breast cancer, endometriosis, and uterine fibroids. In particular, the reduced antiglucocorticoid activity of CDB-2914 and CDB-4124 (and their monodemethylated metabolites) may provide considerable advantage over mifepristone for gynecological and contraceptive uses that require chronic administration.

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