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PLASMA CONCENTRATIONS AND RECEPTOR BINDING OF RU 486 AND ITS METABOLITES IN HUMANS

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Summary—Using Chromosorb® chromatography and HPLC, we measured the plasma concentrations of RU 486, and its monodemethylated (RU 42633), didemethylated (RU 42848) and alcoholic non-demethylated (RU 42698) metabolites up to 72 h following oral ingestion of 100 mg of RU 486 by five female volunteers. The peak plasma level of RU 486 (4.5 μmol/l) occurred within 1 h after ingestion of the compound; at this point significant amounts of the metabolites were also present in the plasma. After the initial redistribution within 6 h the plasma concentrations of RU 486 and three of its metabolites measured remained stable for 24 h. Concentrations of the monodemethylated metabolite exceeded those of the parent steroid during the time period measured, whereas the concentrations of the didemethylated and alcoholic metabolites were lower than those of RU 486, but still notable. At 72 h the concentrations of all the four steroids were still in the micromolar range. The relative binding affinities of these metabolites to human endometrial and myometrial progesterone receptors as well as to human placental glucocorticoid receptors were determined *in vitro*. The affinity of RU 486 for the human uterine progesterone receptor ($K_d = 1.3 \times 10^{-9}$ M for RU 486) was higher than that of progesterone but lower than that of ORG-2058, a potent synthetic progestin. The relative binding affinities of the monodemethylated, alcoholic and didemethylated metabolites to the progesterone receptor were 21, 15 and 9%, respectively, compared with the parent compound RU 486; each was lower than that of progesterone (43%). RU 486 had an approx. 4-fold higher relative binding affinity to the glucocorticoid receptor than dexamethasone. Interestingly, the relative binding affinities of the metabolites studied to the human glucocorticoid receptor exceeded those of dexamethasone or cortisol. Compared with the parent compound RU 486, they were 61, 48 and 45% for the monodemethylated, alcoholic and didemethylated metabolites, respectively; each was higher than that of dexamethasone (23%). The affinity of dexamethasone to the human glucocorticoid receptor was 1.6×10^{-9} M. These data indicate that the pool of certain metabolites of RU 486 may contribute to a significant extent to the antiprogestagenic (23–33%) and even greater extent to the antiglucocorticoid (47–61%) effects of RU 486.

INTRODUCTION

RU 486 is a recently described 19-nor-steroid derivative with considerable antiprogestagenic and antiglucocorticoidal properties [1, 2]. When given during the luteal phase of the menstrual cycle, RU 486 is able to induce uterine bleeding [1]. In preliminary clinical studies RU 486 induced abortion in approx. 80% of the subjects when given between weeks 5–8 of pregnancy, at a daily dose of 200 mg for 4 days [3, 4]. Recently, using RU 486, Nieman *et al.* reported successful symptomatic treatment of Cushing's syndrome [5].

of RU 486 is important for antiprogestagenic action [6]. For all mammalian progesterone receptors investigated, RU 486 has a higher affinity than progesterone [4, 7, 8]. The relative binding affinity of RU 486 for the glucocorticoid receptor is either equal to [7] or greater than [4] that of dexamethasone. Synthetic steroids may have biologically active metabolites. Recently, Deraedt *et al.*[9] identified micromolar plasma concentrations of a monodemethylated metabolite after oral ingestion of RU 486. Our earlier studies indicate the presence of additional immunoreactive metabolites [10].

interest. Hence, plasma concentrations of RU 486 and its monodemethylated (RU 42633), didemethylated (RU 42848) and alcoholic nondemethylated (RU 42698) metabolites were measured specifically by high pressure liquid chromatography (HPLC) up to 72 h following oral ingestion of 100 mg of RU 486. Furthermore, their relative binding affinities for human placental glucocorticoid and uterine (myometrial and endometrial) progesterone receptors *in vitro* were compared with those of reference steroids.

EXPERIMENTAL

Chemicals

RU 486 (17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(1-propynyl)-estra-4,9-dien-3-one), the monodemethylated metabolite RU 42633 (17β-hydroxy-11β-(4-monomethylaminophenyl)-17α-(1-propynyl)-estra-4,9-dien-3-one), the didemethylated metabolite RU 42848 (17β-hydroxy-11β-(4-aminophenyl)-17α-(1-propynyl)-estra-4,9-dien-3-one), the alcoholic metabolite RU 42698 (17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(1-propynol)-estra-4,9-dien-3-one) and [6,7-^{3}H]RU 486 (sp. act. 37 Ci/mmol) were kindly donated by the Roussel-Uclaf Research Center, Romainville, France. The molecular structures of the compounds are presented in Fig. 1.

Progesterone (4-pregnene-3,20-dione), dexamethasone (9-fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione) and cortisol (11β,17,21-trihydroxy-4-pregnene-3,20-dione) were purchased from Steraloids Inc., Wilton, NH, U.S.A. ORG-2058 (16α-ethyl-21-hydroxy-19-nor-4-pregnene-3,20-dione) was obtained from Organon Int., Oss, The Netherlands. [6,7-^{3}H] dexamethasone [DXM] (sp. act. 45.8 Ci/mmol) was from New England Nuclear, Boston, MA, U.S.A., and [6,7-^{3}H]ORG-2058 (sp.act. 54 Ci/mmol) from Amersham Int, Ltd, Amersham, U.K.

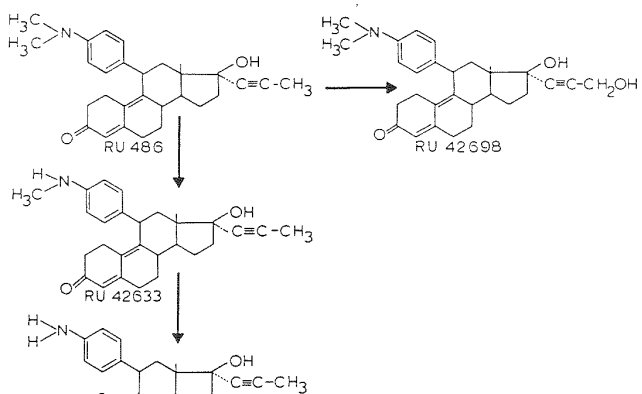
Acetic acid, diethyl ether, ethyl acetate, ethylene glycol, *n*-hexane, gelatin, glycerol, methanol, triethanolamine, titriplex III (EDTA), and HPLC column Hibar LiChrosorb RP-18 (250 × 4 mm int. dia) were purchased from Merck, Darmstadt, West Germany. Tris–HCl, dithiothreitol and Chromosorb® W-NAW 60/80 Mesh were from Sigma, St Louis, MI, U.S.A. Norit A was purchased from Amend, Irvington, NJ, U.S.A., and dextran T70 from Pharmacia, Uppsala, Sweden. Ammonium sulfate was purchased from Schwartz/Mann and scintillation fluid YA-riatuike (70% pseudochumene) was obtained from Yliopiston Apteekki, Helsinki, Finland.

Human samples

Plasma samples were collected from five healthy female volunteers after oral ingestion of 100 mg RU 486 in mid-luteal phase of their cycle. Uteri were obtained from patients undergoing hysterectomy for uterine fibroids. The last menstrual period of the patients had occurred approx. 2 weeks prior to operation. Only non-myomatous uterine tissue was used for the experiments described below. Placentas were obtained from women undergoing elective Caesarean section.

HPLC studies

The Chromosorb® column—HPLC-method described before [10] was modified. Disposable Pasteur pipettes were packed with 3 ml of Chromosorb® W-NAW 60/80 Mesh/20% ethylene glycol. A plasma sample was applied to the column, left for 30 min



and then eluted as follows: (I) 5 ml of ethyl acetate–*n*-hexane 5:95, and (II) 5 ml of ethyl acetate–*n*-hexane 60:40. The eluates were evaporated to dryness, redissolved in the HPLC-eluent used and vortex-mixed. A sample (100 μl) was injected into the HPLC system. The eluent used in HPLC for the assay of RU 486 was methanol–water–triethanolamine, 90:10:0.05, pumped at a rate of 1.5 ml/min; and for the assay of the three metabolites, methanol–water–acetic acid–diethyl ether–triethanolamine, 75:45:30:7.5:0.05, pumped at a rate of 2.2 ml/min.

Preparation of tissue samples

The uterine samples were processed as described by Haukkamaa [11] and placental tissues as described by Kontula *et al.* for adrenal cortical tissue [12]. Cytosol samples were prepared by high-speed centrifugation of tissue homogenates. To remove endogenous steroids from the cytosol samples a Dextran-coated charcoal (DCC) suspension containing 0.5% Norit A, 0.005% Dextran T70 and 0.1% gelatin in 50 mM Tris–HCl-buffer, pH 7.4, was prepared. An aliquot of DCC suspension (the volume corresponding to the cytosolic preparation to be stripped) was centrifuged at 3000 *g* for 10 min. The supernatant was discarded and the cytosol preparation was added to the charcoal pellet. The tubes were vortex-mixed and incubated for 10 min at +4°C. After centrifugation at 3000 *g* for 10 min, the stripped cytosol samples were used for the competitive protein binding assays.

Competitive receptor binding assays

All assays were performed in duplicate or triplicate in disposable glass test tubes and were repeated at least 3 times. For progesterone receptor studies, varying amounts (final concentrations, 10^{-10} to 10^{-5} M) of the steroids investigated (RU 486, RU 42633, RU 42848, RU 42698, ORG-2058 and progesterone) together with 10^{-7} M cortisol (to block binding to corticosteroid-binding globulin and to the glucocorticoid receptor), were pipetted into the tubes and evaporated to dryness. One-hundred microliters of cytosol (diluted to such an extent that approx 50% of the tritiated ligand was bound in the absence of any competitor) and 0.03 μCi of [^{3}H]ORG-2058 (pipetted in 100 μl of 50 mM Tris containing 1% ethanol; final concentration 2.8 nM) were added, the tubes were vortexed-mixed and then incubated overnight at +4°C. After incubation, 200 μl of DCC suspension was added to each tube and the contents vortex-mixed. After 10 min at +4°C, The tubes were centrifuged for 5 min at 3000 *g*. The supernatants

For glucocorticoid receptor studies, similar incubations were carried out, with the following modifications: undiluted placental cytosol was used instead of uterine cytosol; no cortisol was added to the tubes; and [^{3}H]DXM (0.03 μCi/tube; final concentration 3.3 nM) served as tracer instead of [^{3}H]ORG-2058.

Scatchard-plot analysis

To verify the glucocorticoid receptor-nature of the steroid-binding component in placental cytosol, the dissociation constant (K_d) of its interaction with [^{3}H]DXM was measured. Aliquots (0.1 ml) of charcoal-stripped placental cytosol were incubated, in a total volume of 0.2 ml, with varying concentrations (0.3–300 nM) of [^{3}H]DXM dissolved in 50 mM Tris-buffer. The extent of non-specific binding of [^{3}H]DXM was estimated from a parallel set of tubes also containing 10^{-5} M non-radioactive DXM. The tubes were incubated overnight at +4°C. 0.25 ml of DCC was added to separate bound and unbound steroids. Further steps were carried out as described above for the competitive receptor binding assays. The binding data (corrected for non-specific binding) were analyzed according to Scatchard [14].

To measure the K_d of RU 486 for the human uterine progesterone receptor, a partially purified progesterone receptor preparation from human myometrial cytosol was first prepared as described by Kontula *et al.*[15]. Before use, [^{3}H]RU 486 was purified using the Chromosorb® technique [10]. The rest of the analysis was essentially as described above, except that partially purified progesterone receptor preparation and [^{3}H]RU 486 were used instead of placental cytosol and [^{3}H]DXM, respectively, and non-radioactive RU 486 was used instead of DXM for the correction for non-specific binding. No excess of cortisol was used.

RESULTS

The u.v.-absorption spectra of the synthetic metabolites and their behavior in our HPLC system were analyzed. All the synthetic metabolites shared a common u.v.-absorption maximum at 304 nm. Each also had a characteristic u.v.-absorption maximum: RU 42633 at 250 nm, RU 42848 at 240 nm and RU 42698 at 258 nm. Their retention times in our HPLC system were 4 min 36 s, 3 min 56 s and 2 min 49 s, respectively.

Plasma concentrations (mean + SEM) of RU 486 and of its monodemethylated (RU 42633), di-

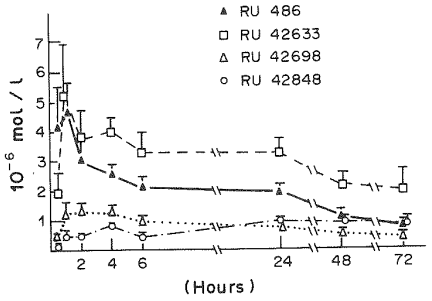


Fig. 2. Plasma concentrations (mean + SEM) of RU 486, RU 42633, RU 42848 and RU 42698, after oral ingestion of 100 mg of RU 486 by five female volunteers.

peak concentrations within 1–2 h suggesting rapid first pass metabolism of RU 486. Plasma concentrations of the didemethylated metabolite (RU 42848) increased slowly between 6 and 24 h, maximum concentrations were measured 24 h after ingestion of RU 486. After initial redistribution of 6 h the plasma concentrations of RU 486 and three of the metabolites assayed plateaued for 24 h or more. Concentrations of the monodemethylated metabolite exceeded those of the parent RU 486. Plasma concentrations of the didemethylated and the alcoholic metabolite were lower than those of RU 486 but still notable. Importantly, both RU 486 and the three metabolites were still present in micromolar concentrations at 72 h.

The binding of RU 486 and its metabolites to human progesterone receptor *in vitro* was studied

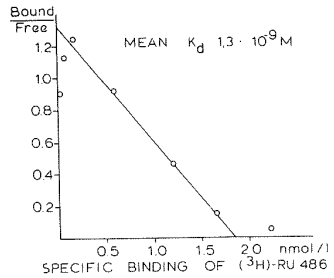


Fig. 3. [^{3}H]RU 486 Scatchard plot analysis of human myometrial progesterone receptor. Mean K_d 1.3×10^{-9} M.

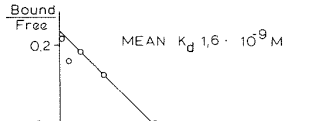


Table 1. The relative binding affinities of the steroids investigated for human myometrial and endometrial progesterone receptor

Compound*	Relative† affinity %
ORG-2058	373
RU 486	100
Progesterone	43
RU 42633	21
RU 42698	15
RU 42848	9

*For systematic names see Experimental.
†Relative to RU 486 (= 100%).

using both human endometrial and myometrical cytosol. The relative binding affinities were identical and therefore combined. The K_d (mean of three separate experiments) of the binding of RU 486 to the human myometrial progesterone receptor was 1.3×10^{-9} M (Fig. 3). The relative binding affinity of RU 486 to the human progesterone receptor was higher than that of progesterone but lower than that of the potent synthetic progestin ORG-2058. All the metabolites of RU 486 studied had a lower affinity to the progesterone receptor than progesterone itself. The relative binding affinities of ORG-2058, progesterone and the three metabolites of RU 486 to the progesterone receptor are given in Table 1.

The binding of RU 486 and its metabolites to the human glucocorticoid receptor *in vitro* was studied using human placental cytosol. Figure 4 shows a representative Scatchard-plot of the interaction between the placental glucocorticoid receptor and tritiated DXM. The mean K_d in four experiments was 1.6×10^{-9} M. Competition studies revealed that all three major metabolites of RU 486, along with the parent compound, had higher affinities for the glucocorticoid receptor than the potent glucocorticoids dexamethasone and cortisol. Table 2 gives the relative affinities of the steroids tested for the human placental glucocorticoid receptor (mean values of 5 separate experiments).

DISCUSSION

Synthetic steroid derivatives may have biologically active metabolites. Radioimmunoassays often lack

Table 2. The relative binding affinities of the steroids investigated for human glucocorticoid receptor

	Relative affinity

the specificity to discriminate between the parent compounds and their metabolites. Furthermore, a metabolite cross-reacting in the radioimmunoassay may lack biological activity.

Earlier studies on plasma RU 486 concentrations were carried out using direct radioimmunoassay [16, 17]. We have developed methods to specifically measure plasma concentrations of RU 486 and its three most proximal metabolic products using Chromosorb®-column chromatography and HPLC. The HPLC method described previously [9] had to be improved since it did not separate the monodemethylated metabolite from the alcoholic metabolite. Our results show that after ingestion of 100 mg of RU 486 by human female volunteers, at least three metabolites of RU 486, the monodemethylated (RU 42633), didemethylated (RU 42848) and alcoholic non-demethylated (RU 42698) forms, are circulating in micromolar concentrations, i.e. close to that of the parent compound for 72 h. When measured by a specific Chromosorb®-HPLC-method the plasma concentrations of RU 486 did not differ significantly when the single oral dose of RU 486 was increased from 100 to 800 mg [10]. This suggests rapid distribution of RU 486 into the tissues, and rapid first-pass metabolism of RU 486. Oral administration of [^{3}H]RU 486 resulted in remarkable extravascular diffusion in rats as reported by Deraedt *et al.*[9]. Studies employing specific HPLC method will reveal whether there is a change in the ratios between RU 486 and its metabolites after the administration of different oral and parenteral doses of RU 486. In general the receptor binding ability of a steroid gives an indication, although not proof, of its biological activity. Deraedt *et al.* determined the relative binding affinities of RU 486, RU 42633, RU 42848 and RU 42698 to cytosolic progesterone and glucocorticoid receptors. Oral administration of RU 486, RU 42633, RU 42848 or RU 42698 in rats resulted in abortion or inhibited the thymolytic effect of dexamethasone thus demonstrating their antiprogestational and antiglucocorticoidal nature, respectively [9]. Their results indicate that the alcoholic metabolite might have a higher biological activity in relation to receptor binding as compared with the monodemethylated metabolite. The relative binding affinities of RU 486 and its three metabolites to the human glucocorticoid and progesterone receptors were determined, using dexamethasone and ORG-2058, respectively, as reference steroids. Before accepting the previously characterized progesterone [11] and glucocorticoid receptor [12] systems as models, receptors [15]. The lower binding affinity of progesterone to the human progesterone receptor, as compared to RU 486 (43%, Table 1), is in accord with the value of 67%, which was reported previously by Gravanis *et al.*[8].

The hydrophobic molecular structure of RU 486 reveals features suggesting high affinity binding to progesterone receptor [18]. The antiprogestagenic properties of RU 486 are thought to be due to the dimethylaminophenyl side chain at carbon 11 [6]. Demethylation of this side chain decreases its hydrophobicity, and also decreases the binding affinity of mono- and didemethylated metabolites to 21 and 9%, respectively (Table 1). Hydroxylation of the side chain at carbon 17 decreases the binding affinity of the compound from 100% (RU 486) to 15% [RU 42698) (Table 1).

Based on the relative receptor binding affinities of the metabolites (Table 1) and their plasma concentrations (Fig. 2), it is possible to estimate the contribution of the metabolite pool in the antiprogestational action of RU 486. The theoretical contribution of the prevailing metabolite pool to the antiprogestational activity of RU 486 after ingestion of 100 mg of RU 486 amounts to about 23% at 1 h but as high as 33% at 24 h.

Comparatively little is known about the relative affinity of RU 486 for human glucocorticoid receptors. However, in comparison with published clinical and experimental studies [2, 4, 7], the high affinities of RU 486 and of its metabolites to the human glucocorticoid receptor (Table 2) are not surprising. However, it must be kept in mind that competition studies performed at 0–+4°C in cell-free conditions do not necessarily correctly reflect the situation at +37°C and in the whole organism [19]. The theoretical contribution of the metabolites of RU 486 to the antiglucocorticoidal action of RU 486 was calculated. This was based on the relative receptor binding affinities (Table 2) and plasma concentrations (Fig. 2) of the metabolites. These results suggests that 1 and 24 h after the intake of 100 mg of RU 486, the three metabolites would represent 47 and 61%, respectively, of the total antiglucocorticoid activity of RU 486.

Despite the high affinity binding of RU 486 and its metabolites to the human glucocorticoid receptor *in vitro*, previous clinical experience suggests that large single doses of RU 486 ($\geqslant$400 mg) are needed to promote antiglucocorticoid effects *in vivo* [2, 20]. Chronic treatment with 25–200 mg/day of RU 486, doses sufficient to produce uterine bleeding in 80%

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