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[³H]DU41165: A HIGH AFFINITY LIGAND AND NOVEL PHOTOAFFINITY LABELING REAGENT FOR THE PROGESTERONE RECEPTOR

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Summary—17α-Acetoxy-6-fluoro-16-methylene-(9β,10α)pregna-4,6-dien-3,20-dione (DU41165), a retroprogestin (9β,10α) embodying a fluorine-substituted dienone system, has been prepared in high specific activity tritium-labeled form (4 Ci/mmol) and shown to be a high affinity ligand for the progesterone receptor (PgR) and a highly selective photoaffinity labeling reagent for PgR. The radiosynthesis involved conversion of DU41231 (the 17α-hydroxy analog of DU41165) to DU41165 by treatment with tritum-labeled acetic anhydride. The binding affinity of DU41165 for PgR was determined by both a competitive binding assay and a direct binding assay (Scatchard analysis) to be 1.6–2.2-times higher than that of the high affinity synthetic progestin promegestone (R5020). In unlabeled form, DU41165 demonstrates photoinactivation of PgR to the extent of 60% at 60 min. In radiolabeled form [³H]DU41165 demonstrates specific covalent attachment with an efficiency of 5–7%. SDS–polyacrylamide gel electrophoresis of photoattached [³H]DU41165 confirms that there is covalent labeling of both the B subunit (M_r = 118,000), and the A subunit (M_r = 88,000) of PgR in a molar ratio of approximately 1:3.

INTRODUCTION

The best method to date for photoaffinity labeling of the progesterone receptor (PgR) employs promegestone (R5020) as the photoexcitable ligand. While the photoreactive dienone in R5020 results in highly selective labeling of PgR, the photoattachment efficiency is very low (2–5%), a feature which limits the types of studies for which R5020 may be used [1–7]. In addition to R5020, other ligands including RU38486 [8], ORG2058 [9], and Δ⁹-16α-iodo-19-nortestosterone [10] have been prepared as photoaffinity labeling reagents for PgR; however, they all suffer from very low efficiency of photocovalent attachment [6–10].

There is evidence to suggest that fluorine substitution of an unsaturated ketone chromophore might alter the energies of the excited states so that the major photochemical pathway would proceed via the more reactive $n\pi^*$ triplet excited state, rather than the less reactive $\pi\pi^*$ triplet [11, 12]. This might result in an increase in the efficiency of intermolecular coupling reactions (via hydrogen abstraction and radical pair recombination after spin inversion), and it suggests that incorporation of such a system into a progesterone ligand could result in an improvement in the efficiency of covalent attachment to PgR.

In order to investigate these possibilities, we became interested in a series of progestins of the retroprogesterone class (9β,10α) which contain a dienone system substituted with fluorine or chlorine at C-6. (The structures of five of these compounds, as well as R5020 and ORG 2058 are given in Table 1.) These compounds were originally prepared by the Philips–Duphar Company as potential oral contraceptive agents [13–15], and we received samples of them from Duphar International for our investigation.

We evaluated the five retroprogestins for their binding affinity for PgR [16], as well as the glucocorticoid and mineralocorticoid receptors, and their capacity for photoinactivation of PgR [17]. On the basis of its high PgR binding affinity and excellent

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Abbreviations: DU41165: 17α-Acetoxy-6-fluoro-16-methylene-9β,10α-pregna-4,6-diene-3,20-dione; DU41231: 17α-Hydroxy-6-fluoro-16-methylene-9β,10α-pregna-4,6-diene-3,20-dione; DU41156: 17α-Acetoxy-6-chloro-16-methylene-9β,10α-pregna-4,6-diene-3,20-dione; DU41164: 17α-Acetoxy-6-fluoro-1,2β; 16-bis(methylene)-9β,10α-pregna-4,6-diene-3,20-dione; DU41117: 17α-Acetoxy-6-chloro-1,2β; 16-bis(methylene)-9β,10α-pregna-4,6-diene-3,20-dione; RU38486: 17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(1-propynyl)estra-4,9-dien-3-one; RU27987: 21S-hydroxy-17α,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione; RU26752: 3′(3-oxo-7α-propyl-17β-hydroxy-androsta-4-ene-17-yl)-propionic acid lactone; RU28362: 11β,17β-dihydroxy-6-methyl-17α-(1-propynyl)antrosta-1,4,6-trien-3-one. R5020: 17α,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione; ORG2058: 16α-ethyl-21-hydroxy-19-nor-4-pregnene-3,20-dione; Δ⁹-16α-iodo-19-nortestosterone: 16α-iodo-4,9-estradien-17β-ol-3-one.

Table 1. Structure, receptor binding-affinity and photoinactivation efficiency of retroprogestins and other progestions

Compound	Receptor binding affinity (RBA)[a] PR[b] (R5020 = 100)	GR[c] (RU28362 = 100)	MR[d] (RU26752 = 100)	Photoinactivation efficiency[e] at 60 min
DU41165	158%	28%	<0.003	60%
DU41156	162%	20%	<0.003	36%
DU41231	0.89%	2.5%	<0.003	ND
DU41164	74%	5.5%	<0.003	48%
DU41117	48%	12%	<0.003	38%
ORG2058	203%	0.87%	1.62%	7%
R5020	100%	2.04%	1.56%	66%

[a]The receptor binding affinity is determined in a competitive radiometric binding assay, with the tracer and tissue cytosol sources indicated in footnotes b–d. After incubation at 0°C for 18–24 h the excess free ligands were adsorped onto dextran-coated charcoal and removed by centrifugation. These numbers are the average of duplicate determinations. Such duplicate measurements are generally reproducible to within 30% (relative error). For additional details, see Experimental. [b]Cytosol preparations were from estrogen-primed immature rat uterus, with $[^3H]$R5020 as tracer [14]. [c]Cytosol preparations were from saline-perfused liver from 3-day adrenalectomized mature male rats, with $[^3H]$RU28362 as tracer. [d]Cytosol preparations were from saline perfused kidney of 3-day adrenalectomized mature male rats, with $[^3H]$RU26752 as tracer. [e]Photoinactivation efficiency [17] was determined by incubation of progesterone receptor preparations with the unlabeled compound indicated, irradiation for 60 min with >315 nm u.v., and estimation of the receptor sites remaining by exchange with $[^3H]$R5020 for 2 h at 25°C. Suitable corrections were made for non-specific photoreaction, and ORG2058 was used as a photoinert ligand (non-absorptive and non-photoreactive at the wavelength used in these experiments). For further details, see Experimental.

photoinactivation characteristics, we selected DU41165 to prepare in tritium labeled form for further study. $[^3H]$DU41165 has a higher competitive binding affinity than R5020 for PgR and demonstrates photocovalent attachment with somewhat higher efficiency than R5020. Thus, DU41165 may be useful for further evaluation of the binding domain of PgR, and if it can be prepared in ^{18}F-labeled form, it

may be effective as a selective imaging agent for PgR-positive human breast tumors [18].

EXPERIMENTAL

Chemical Procedures

Materials and methods

Acetic anhydride, propionic anhydride, camphorsulfonic acid, and chloroform were obtained from the Aldrich and Fisher Chemical Companies. The anhydrides and chloroform (from CaH_2) were carefully distilled prior to use. [^{3}H]$(CH_3CO)_2O$ (25 mCi, 10 Ci/mmol) was obtained from the Amersham Corporation. The retroprogestins (DU41165, DU41156, DU41231, DU41164, DU41117) were obtained as gifts from the Philips–Duphar N.V. (Amsterdam, The Netherlands).

Normal phase analytical thin-layer chromatography was performed using Merck 0.25 mm silica gel glass-backed plates with F-254 indicator and reverse phase analytical thin-layer chromatography was performed using 0.20 mm octadecyl silane-bonded silica gel glass-backed plates with F-254 indicator (Merck). Visualization was by ultraviolet light. Analytical gas chromatography (GC) was performed on a Hewlett-Packard 5790A chromatograph using a Hewlett-Packard Ultra 1 (crosslinked methyl silicone gum) capillary column ($12\ m \times 0.2\ mm \times 0.33\ \mu m$ film thickness). High-performance liquid chromatography (HPLC) was performed on a Varian 5060 system using a Perkin-Elmer LC-75 Spectrophotometric Detector (variable wavelength set at 254 nm). Radioactive thin-layer chromatography plates were counted using a Radiomatic instruments RTLC Scanner. Liquid scintillation counting was carried out on a Nuclear-Chicago Isocap 300 instrument using xylene-based cocktail containing 0.55% 2,5-diphenyloxazole, 0.01% *p*-bis[2-(5-phenyl oxazoyl)]benzene, and 25% Triton X-114 [15].

Radiosynthesis of [^{3}H]DU41165

DU41231 (1.00 mg, 0.00279 mmol) and camphorsulfonic acid (1.00 mg, 0.00430 mmol) were placed together in a reaction vial constructed with an elongated neck having two preconstricted sites, on arm A of a 2-armed vacuum transfer system. These materials were vacuum dried at 56°C for 2 h, and the apparatus was cooled to room temperature and returned to atmospheric pressure. Propionic anhydride (0.0123 mmol) was added as 10 μl of a stock solution (78.7 μl propionic anhydride in 500 μl $CHCl_3$). [^{3}H]$(CH_3CO)_2O$ (25 mCi, 10 Ci/mmol) in a break-seal tube was attached to arm B of the vacuum transfer system. Arm A was cooled in liquid N_2, and the whole system was evacuated to 10^{-6} torr and then closed off from the vacuum source. The [^{3}H]$(CH_3CO)_2O$ in arm B was released by rupture of the break seal, and was transferred to the reaction vessel (arm A) by cold trapping. The reaction vial on arm A was sealed at the upper constriction site with a gas-oxygen torch and was then warmed and stirred at 50°C for 20 h. The reaction vial was opened at the lower constriction site, and the reaction quenched with H_2O (2 ml) and extracted with CH_2Cl_2 (2 ml). The mixture before purification contained the acetate (7%), the propionate (75%), and several other byproducts as determined by HPLC analysis compared to known standards. The mixture was purified in several batches by normal phase HPLC equipped with a 30-cm Varian SI-5 silica gel analytical column, eluting with 80% hexane, 19% methylene chloride, and 1% isopropyl alcohol at a rate of 1 ml/min. The majority of the radioactivity was found in a peak with a retention time of 16 min, which corresponded to the retention time of unlabeled DU41165. A small amount of radioactivity eluted as a peak with a retention time of 12 min, which corresponded to the retention time of unlabeled propionate. The final radiochemical yield of purified [^{3}H]DU41165 was 3%.

The specific activity of [^{3}H]DU41165 was determined by HPLC analysis. Radioactivity was determined by liquid scintillation counting of an aliquot of the collected radioactive peak, and mass was determined by measurement of peak area and reference to a calibration plot of peak area vs quantity of unlabeled DU41165 injected. Using this method, we found the specific activity of [^{3}H]DU41165 to be 4.0 Ci/mmol.

The radiochemical purity was evaluated by normal phase TLC analysis (ethyl acetate:hexane, 80:20). The Radiomatic Instruments RTLC Scanner indicated a single peak of radioactivity (98%) with an R_f of 0.51, identical to the R_f of an unlabeled standard run on the same plate. The product was stored in several aliquots as 50–150 μCi/ml solutions in CH_2Cl_2.

Biological Procedures

Materials

Radioligands were obtained from the following sources: 16α-ethyl-21-hydroxy-19-nor[6,7-^{3}H]preg-4-en-3,20 dione (ORG 2058), 58 Ci/mmol (Amersham Corp., Arlington Heights, Ill); [17α-methyl-^{3}H]-promegestone (R5020), 86 Ci/mmol, and [6-methyl-^{3}H] 11β,17β-dihydroxy-6-methyl-17α-(1-propynyl)-androsta-1,4,6-trien-3-one, (RU28362), 77 Ci/mmol, (DuPont New England Nuclear, Boston, Mass); [1,2-^{3}H]3′(3-oxo-7α-propyl-17β-hydroxy-androsta-4-ene-17-yl) propionic acid lactone (RU 26752), 60 Ci/mmol, was prepared in our laboratory as described (M. G. Pomper, PhD Thesis, University of Illinois, 1989).

Unlabeled ligands: promegestone and RU 28362 (DuPont New England Nuclear, Boston, Mass), ORG 2058 (kindly supplied by Dr F. Zeelen, Organon Corp., Oss, The Netherlands), RU26752 (kindly supplied by Dr J. P. Raynaud, Roussel-Uclaf, Romainville, France), DU41156, DU41164,

DU41117, DU41231 and DU41165 (kindly supplied by Dr J. Hartog, DuPhar International, Weesp, The Netherlands), estradiol and cortisol (Sigma Chemical Co., St Louis, Mo.).

The following compounds were obtained from the sources indicated: dextran, grade C (Schwarz/Mann, Orangeburg, N.Y.); 2-mercaptoethanol, (ethylene-dinitrilo)-tetraacetic acid, tetrasodium salt (EDTA), acrylamide, N,N'-methylene-bisacrylamide, Photo-Flo 200, N,N'-diallytartardiamide (Eastman Kodak Co., Rochester, N.Y.); Triton X-114 (Chem Central-Indianapolis, Indianapolis, Ind.); bromophenol blue, N,N,N',N'-tetramethyl ethylene diamine (TEMED), sodium azide, 1,4-bis(5-phenyloxazol-2-yl)benzene (Popop), (Aldrich Chemical Co., Milwaukee, Wis.); sodium molybdate (Mallinckrodt Inc., St Louis, Mo.); dodecyl sodium sulfate (SDS) (Matheson, Coleman and Bell, Norwood, Ohio); ammonium peroxydisulfate, glycerin and N,N-dimethyl formamide (DMF) (Fisher Scientific, Fair Lawn, N.J.); periodic acid (G. Frederick Smith Chemical Co., Columbus, Ohio); soybean trypsin inhibitor, leupeptin, phenylmethylsulfonylfluoride (PMSF), activated charcoal, Trizma Base, thioglycerol, ovalbumin (MW 44,600), bovine serum albumin (MW 67,000), phosphorylase B (MW 97,400), myosin (MW 205,000), (Sigma Chemical Co., St Louis, Mo.); Coomassie Brilliant Blue R-250 (Colab Laboratories, Inc., Glenwood, Ill.); and 2,5-diphenyloxazole (PPO) Research Products International Corp., Elk Grove Village, Ill.).

Preparation of cytosol

Progesterone receptor. The progesterone receptor (PgR) levels in the uteri of immature rats were induced by estrogen treatment. Immature female Sprague–Dawley rats (19 day, 60 g) were given three daily subcutaneous injections of 5 mg of estradiol in 0.1 ml sunflower seed oil-ethanol, prepared fresh daily. The cytosol was prepared 24 h after the last injection as previously reported [14]. All cytosol for the progesterone receptor was prepared in PgR buffer (0.01 M Tris–HCl:0.0015 M EDTA:0.02% sodium azide:20 mM Na molybdate:0.012 M mercaptoethanol:20% glycerol, pH 7.4 at 25°C) and stored in liquid nitrogen. In the experiments utilizing protease inhibitors, soybean trypsin inhibitor (5 mg/ml), leupeptin (0.1 mg/ml), and PMSF (1 mM), were added to the buffer just before use. The uteri were collected, homogenized, and assayed in the presence of the inhibitors.

Glucocorticoid and mineralocorticoid receptor. Cytosols for glucocorticoid receptor, (type II sites, liver) and mineralocorticoid receptor (type I sites, kidney) were prepared from the tissues of 300 g male Long–Evans rats which had been adrenalectomized three days previously. The tissues were homogenized in GU buffer (0.01 M Tris–HCl:0.0015 M EDTA:0.02% sodium azide:20 mM Na molybdate:0.012 M thioglycerol, 10% glycerol, pH 7.4 at 25°C). The tissue was homogenized at 1 g tissue/1.5–1.7 ml buffer, centrifuged for 1 h at 140,000 **g**, and the supernatant stored in liquid nitrogen. For assays, the kidney cytosol was diluted 1:2 with buffer (~12 mg/ml) and the liver cytosol was diluted 1:10 with buffer (~6 mg/ml).

Relative binding affinity (RBA)

Assays were a modification of that previously reported for the estrogen receptor [17]. Cytosol was incubated with buffer or several concentrations of unlabeled competitor together with 10 nM ^{3}H-tracer at 0°C for 18–24 h. The unlabeled competitor was prepared in 1:1 dimethylformamide (DMF):buffer to ensure solubility. Standard PgR assays utilized uterine cytosol (~1.5 nM receptor plus 10^{-6} M cortisol to block any glucocorticoid receptor), [^{3}H]R5020 as the tracer, with the competitors dissolved in 1:1 DMF:PgR buffer [14]. In the reciprocal RBA experiments, [^{3}H]ORG 2058 and [^{3}H]DU41165 were also used as tracers.

Glucocorticoid receptor assays utilized liver cytosol (~1 nM Type II sites), [^{3}H]RU28362 as the tracer, with the competitors dissolved in 1:1 DMF:GU buffer. Mineralocorticoid assays were performed with kidney cytosol (~0.2 nM Type I sites plus 10^{-6} M RU28362 to block Type II sites), [^{3}H]RU26752 as the tracer and the competitors dissolved in 1:1 DMF:GU buffer. The charcoal-dextran slurry used to remove unbound ligand was prepared as previously reported [19] and was generally used at 1 part to 10 parts of cytosol solution, at 0°C.

Scatchard assay

Uterine progesterone cytosol was incubated at 0°C for 4 h with various concentrations of ^{3}H-ligand in the absence or presence of a 100-fold excess of unlabeled R5020 or ORG 2058. Aliquots of the incubation solution were counted to determine the concentration of total ^{3}H-steroid. The incubation solutions were then treated with charcoal-dextran and the bound ^{3}H-steroid determined. Data was processed according to the method of Scatchard [20].

Photolysis

Photolysis was carried out at >315 nm (450-watt mercury vapor lamp, Hanovia L679A, surrounded by a solution filter of saturated aqueous copper(II) sulfate) at 2–4°C employing pyrex reaction vessels, as previously described [17].

Inactivation assay

Covalent binding of non-radioactive ligands was estimated by a photolysis-exchange assay similar to that described for the estrogen receptor [17]. For PgR the exchange conditions were 2 h at room temperature. In assay validation experiments, filled PgR sites were completely stable for 2 h at room temperature. Those filled with ORG 2058, exchanged at room temperature with a $t_{1/2}$ of 21–24 min. The photo-

induced covalent attachment of a ligand to the progesterone-binding site is registered as a decrease in exchange capacity. Any glucocorticoid receptor sites in the cytosol preparations were saturated by the addition of 1 μM hydrocortisone in all preparations.

Attachment assay

Covalent binding of labeled ligands was measured directly by a filter disc assay described previously [21]. In photolysis experiments with ^{3}H-ligands, ORG 2058 (a non-photoreactive ligand) was used in a 100-fold excess to provide a "protected" sample where the specific progesterone sites were blocked and only non-specific sites would be assayed. Any glucocorticoid receptor sites in the cytosol preparations were saturated by the addition of 1 μM hydrocortisone to all preparations.

Electrophoresis

Samples for SDS electrophoresis were first treated with charcoal-dextran at 37–40°C, for 15 min to remove both unbound and reversibly bound ligand. They were then denatured in 0.13 M mercaptoethanol, 2% SDS, in a boiling water bath for 2 min. Gels were cast in 12 cm long × 6 mm i.d. glass tubes. The tubes had been coated with a 0.5% aqueous solution of Kodak Photo-Flo 200, and oven dried. The catalyst solution (0.14% ammonium persulfate) was prepared fresh daily. The SDS–polyacrylamide gel electrophoresis system was modified from that of Laemmli[22] substituting N,N′-diallytartardiamide (DATD) for methylene-bisacrylamide to cross-link the separation gel [23]. The separation gel consisted of 7.5% acrylamide, 0.6% DATD, and 0.1% SDS. The gels were run at 2.5 ma/gel, at room temperature. Following electrophoresis, the gels were fixed overnight in 12.5% trichloroacetic acid. Those for radioactivity determinations were frozen on dry ice and sliced into 2.15 mm slices. Each slice was dissolved in 0.5 ml of 2% periodic acid at room temperature and counted in 5 ml of scintillation fluid. The gels of the standard proteins were stained for 1 h at 37°C in 0.25% Coomassie Blue in 5:1:5 methanol:acetic acid:water and destained electrophoretically in 7% acetic acid.

RESULTS

Radiochemical Synthesis

The radiochemical synthesis of [^{3}H]DU41165 was carried out by acetylation of the hydroxy precursor, DU41231 (Scheme 1), using an acylation entrainment procedure. Normally, acetylation reactions utilize an excess of the available and inexpensive acetylation reagent. However, in a radiochemical synthesis utilizing high specific activity, tritium-labeled acetic anhydride, the acetylation reagent will be limiting. In model reactions, we found that the acetylation of DU41231 with less than two equivalents of acetic anhydride was very inefficient; the addition of acid (toluenesulfonic or camphorsulfonic acids), or acyl transfer catalysts (4-dimethylaminopyridine), or agents to generate mixed anhydrides *in situ* (toluenesulfonyl chloride, isobutylchloroformate or isopropenylchloroformate) failed to improve the yield.

We found, however, that by adding an excess of propionic anhydride together with a limiting quantity of acetic anhydride, we could obtain satisfactory yields (up to 21%) of the acetate. Thus, using this entrainment procedure with tritium-labeled acetic anhydride under the conditions shown in Scheme 1, we obtained a radiochemical yield of approx. 7%. The unlabeled propionate, which also forms, is readily

DU41231

- 1.0 mg DU41231 (0.00279 mmol)
- 4.4 eq. propionic anhydride
- 0.9 eq. [^{3}H]acetic anhydride (Amersham, 25 mCi, 10 Ci/mmol)
- 1 mg (1.5 eq.) camphorsulfonic acid
- 10 μl $CHCl_3$
- 20 h, 50 °C

[^{3}H]DU41165 +

Scheme 1

separated from the desired radiolabeled acetate by HPLC; the overall yield of purified [^{3}H]DU41165 was approx. 3%.

The radiochemical purity of the [^{3}H]DU41165 was evaluated by TLC analysis and found to be 98%. The specific activity was determined by HPLC analysis to be 4.0 Ci/mmol, or 80% of the maximum theoretical for anhydride of 10 Ci/mmol.

Receptor Binding Properties of Progestins

Relative binding affinity to the progesterone receptor (PgR, glucocorticoid receptor (GR) and mineralocorticoid receptor (MR)

The relative binding affinity of the five retroprogestins (DU41165, DU41156, DU41231, DU41164, DU41117) for PgR was determined by a competitive radiometric binding assay with [^{3}H]R5020. The data, illustrated in Table 1, are relative to R5020 (RBA = 100%). DU41165 (RBA = 158%), and DU41156 (RBA = 162%) both containing halogen substitution at C-6, a methylene at C-16 and a 17α-acetate have the highest binding affinities of the retroprogestins tested. Although both have higher affinities than R5020, they are still lower affinity than ORG2058. The other retroprogestins DU41164 and DU41117 that have a 1β,2β-methylene group in place of the 16-methylene group, have RBAs that are 2–3-fold lower. DU41231, the 17α-hydroxy analog of DU41165, has very low affinity.

Similar competitive binding assays were used to determine the RBA of these compounds for GR and MR. Only R5020 and ORG2058 have detectable affinity for MR, but all the retroprogestins have substantial affinities for GR (5.5–28% that of RU28362).

Reciprocal relative binding affinity analysis

A reciprocal RBA assay, that is, one using [^{3}H]DU41165 or [^{3}H]ORG2058 rather than [^{3}H]R5020 as tracer, was performed in order to verify that DU41165 was binding to the same sites in the cytosol preparation as R5020 and ORG2058. The competitive binding profile of the three ligands DU41165, R5020, ORG2058 was separately determined, competing in one case with [^{3}H]DU41165 (Fig. 1, panel A), in the second case with [^{3}H]R5020 (Fig. 1, panel B), and finally with [^{3}H]ORG2058 (Fig. 1, panel C). In all cases, the three binding curves carefully parallel one another and the relative binding affinities remain the same, thereby establishing that DU41165 binds to the same sites as R5020 and ORG2058 in the rat uterine cytosol.

Direct binding assay

The binding affinity of [^{3}H]DU41165 for PgR was evaluated through a direct binding assay. The data, presented for [^{3}H]DU41165 (Fig. 2, panel A), and [^{3}H]R5020 (Fig. 3, panel C), include total binding, non-specific binding and the specific binding component. A direct comparison of the specific binding curves (Fig. 2, panel C) indicates the [^{3}H]DU41165 has an affinity for the progesterone receptor 2.2-times higher than that of [^{3}H]R5020. This is in close agreement with the value obtained for unlabeled DU41165 (1.6-times R5020) through the competitive binding assay (Table 1 and Fig. 1).

Photoreactivity of Progestins

Photoinactivation of progesterone receptor

A photoinactivation assay [17] is an indirect method by which the photoreactivity, and hence potential photocovalent reactivity of a non radiolabeled chromophoric ligand, can be established. Receptor ligand complexes are irradiated and the time-course of loss of reversible binding capacity is assayed by an exchange process. Suitable controls for receptor photostability and binding site dependence of inactivation (competition by a photoinert ligand) are included. The photoinactivation of PgR in estrogen-primed uterine cytosol at >315 nm was determined with R5020, ORG2058 and each of the retroprogestins in a separate assay. The results are shown in Table 1 and the time-course of receptor photoinactivation with DU41165 and R5020 is shown in Fig. 3.

Specific photoinactivation is considered to be the difference between the total inactivation (Fig. 3, lower curves, DU41165 or R5020 alone) and the non-specific component of inactivation (upper curves, DU41165 or R5020 + 100-fold excess ORG2058). (Our selection of ORG2058 as a protecting ligand is based on the fact that it is relatively photoinert at this wavelength (>315 nm); its photoinactivation efficiency is only 7% (Table 1).) The photoinactivation time-courses were carried out for 3 h in each case; however, the 1 h specific photoinactivation data shown in Table 1 provide a good point for comparison of the relative effectiveness of each compound. Under these photolysis conditions, R5020, the most thoroughly investigated PgR photoaffinity labeling agent, shows a photoinactivation efficiency of 66%; DU41165 has an efficiency of 60%. The excellent photoinactivation efficiency of DU41165, together with its high relative binding affinity provided the necessary rationale for its preparation in tritium-labeled form in order to study its binding and photocovalent attachment properties.

Efficiency of photocovalent attachment of [^{3}H]DU41165 to the progesterone receptor cytosol preparation

The time-course of photocovalent attachment of [^{3}H]DU41165 to the progesterone receptor was determined over a 4-h period by photolysis at >315 nm at 0°C (Fig. 4). [^{3}H]DU41165 demonstrates photocovalent attachment with an efficiency of approx. 5%. Under the same conditions, [^{3}H]R5020 attaches with an efficiency of approx 4% (Fig. 4, open triangle

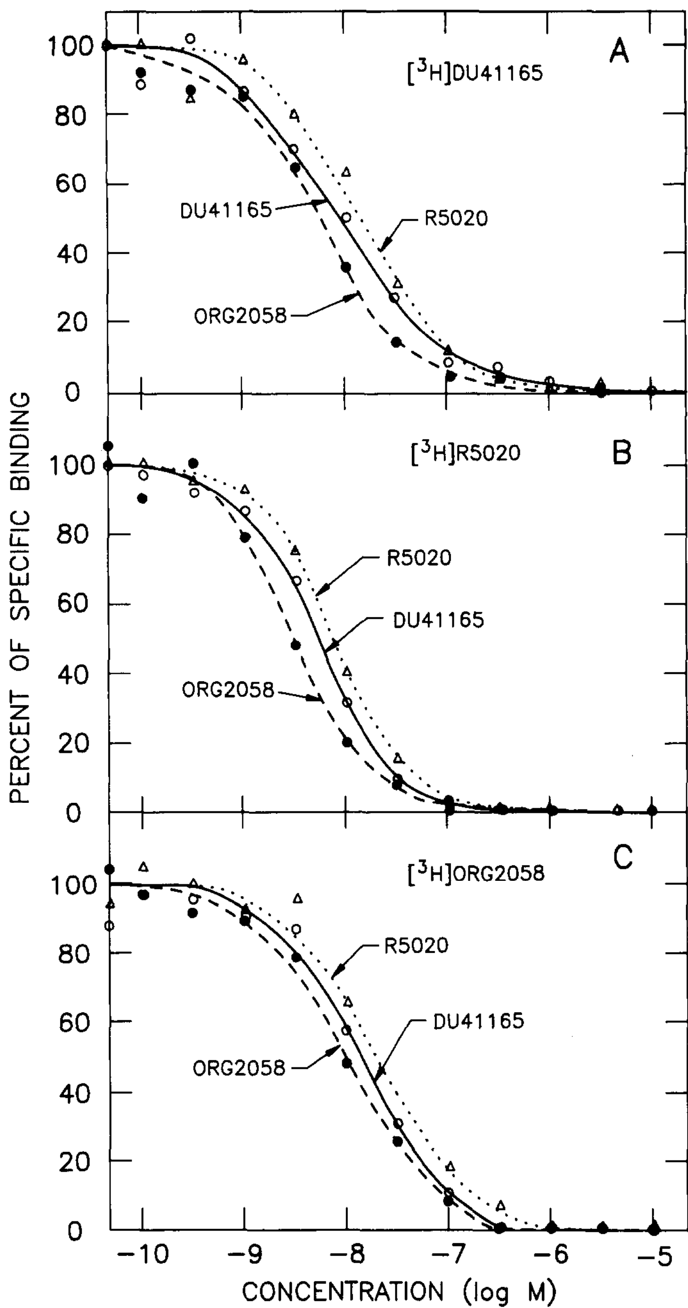

Fig. 1. Reciprocal receptor binding affinity (RBA) assays. Competitive binding assays of DU41165, ORG2058 and R5020 in rat uterine cytosol, using as the tracer, [^{3}H]DU41165 (panel A), [^{3}H]R5020 (panel B), or [^{3}H]ORG2058 (panel C). Assays were performed according to protocols described in the Experimental Section.

point). (In other assays, covalent attachment efficiency of [^{3}H]DU41165 ranged from 4 to 7% (data not shown).) All of the attachment was essentially complete within the first 2 h of photolysis. The attachment efficiency was not improved by photolysis at >290 nm or 350 nm. At >290 nm (pyrex-filtered medium pressure mercury arc light), photoattachment of [^{3}H]DU41165 reached 3% within 30 min, but dropped to zero thereafter, while [^{3}H]R5020 reached 3% by 1 h and remained stable (data not shown); at 350 nm (black light), [^{3}H]DU41165 reached 3% at early times and diminished thereafter, while [^{3}H]R5020 reached 4% at 90 min (data not shown).

Although DU41165 has substantial affinity for GR, the photocovalent labeling of uterine soluble proteins that we have observed does not involve GR, since all cytosol samples were pretreated with hydrocortisone to block potential interaction with GR.

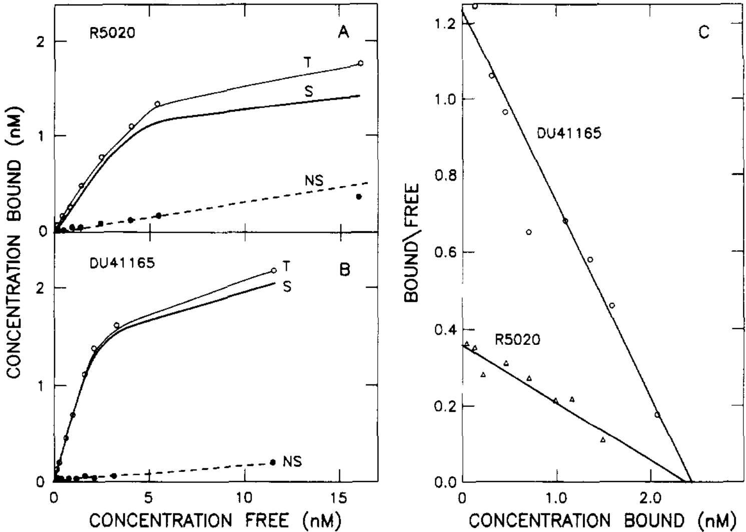

Fig. 2. Binding curves for [³H]R5020 and [³H]DU41165 to PgR. Uterine cytosol was incubated with various concentrations of the two compounds for 4 h at 0–4°C in the absence and presence of a 100-fold excess of unlabeled R5020 and then treated with charcoal-dextran. Scatchard plots of specific binding are shown in panel C, direct binding plots are shown in panels A and B (T: total, NS: non-specific, S: specific).

(Furthermore, in other studies on rat estrogen-primed uterine cytosol, we have found GR levels to be very low.)

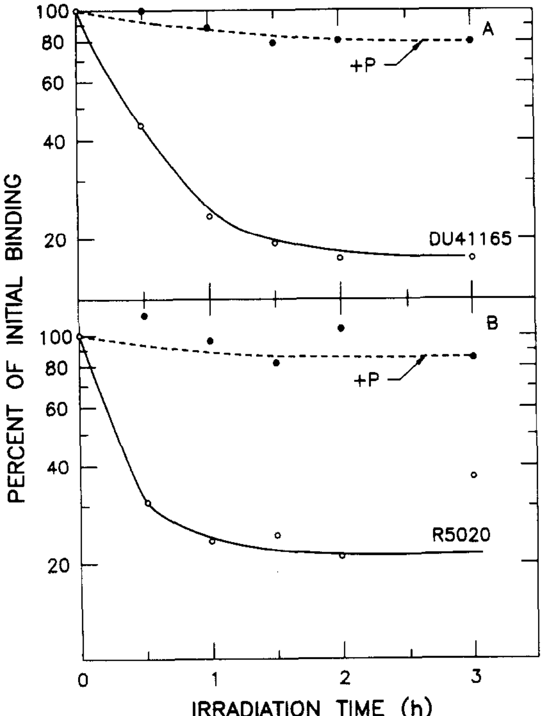

Fig. 3. Photoinactivation of PgR in rat uterine cytosol by DU41165 and R5020. Cytosol was incubated with DU41165 or R5020 in the absence or presence of a 100-fold excess ORG2058 for 1 h at 0°C, then photolyzed at >315 nm for various times. Following charcoal-dextran treatment to remove free ligand, the cytosol was exchanged at room temperature for 2 h against [³H]R5020. Photoinactivation is seen as a loss in exchangeable sites and plotted as a percent of the initial binding before photolysis.

Characterization of the progesterone receptor covalently labeled with [³H]DU41165 and [³H]R5020

PgR covalently labeled with [³H]DU41165 was evaluated for molecular weight by SDS–polyacrylamide gel electrophoretic analysis. PgR was cleanly labeled by [³H]DU41165 (Fig. 5, panel B) in both the B subunit ($M_r = 118{,}121 \pm 10{,}595$) and the A subunit ($M_r = 87{,}951 \pm 5450$) in a molar ratio of approximately 1:3. An assay run in parallel with PgR covalently labeled with [³H]R5020 (Fig. 5, panel A) shows labeling of the B and A subunits of PgR in the same 1:3 molar ratio. The calibration curve of the standard proteins is shown as an insert in Fig. 5, panel A.

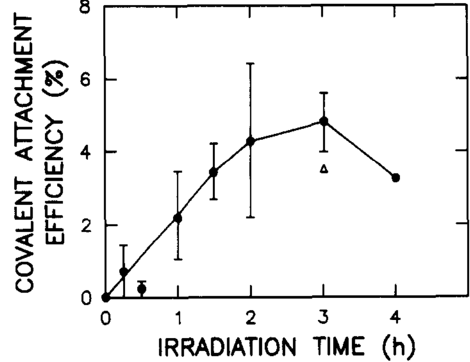

Fig. 4. Time-course of specific photoattachment of [³H]DU41165 ± standard error of means (SEM). Rat uterine cytosol was incubated for 1 h at 0–4°C with [³H]DU41165 with or without a 100-fold excess of unlabeled ORG2058 and then photolyzed at >315 nm. Direct attachment was measured by the ethanol disc assay (see Experimental).

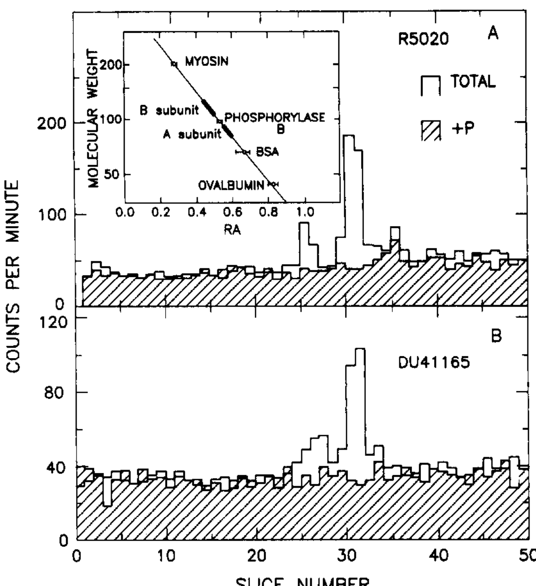

Fig. 5. SDS–polyacylamide gel electrophoresis of photo-attached [^{3}H]DU41165 (panel B) and [^{3}H]R5020 (panel A). There is covalent labeling of both the B subunit (slice 26–27) and of the A subunit (slice 30–31). The calibration curve of the standard proteins is shown as an inset in panel A. The standard proteins are myosin, phosphorylase B, bovine serum albumin and ovalbumin. The points represent the mean of 4 determinations. By linear regression, the correlation coefficient of the line is $r = 0.998$. From this determination the molecular weight of the B subunit is 118,121 ± 10,595 and that of the A subunit is 87,951 ± 5450.

DISCUSSION

We have evaluated a series of five retroprogestins (9β,10α) for their relative binding affinity to the progesterone receptor (PgR) and their photoinactivation efficiency, and we have prepared one of these (DU41165) in high specific activity tritum-labeled form for further binding and covalent attachment studies.

Retroprogestins have inverted stereochemistry at carbons-9 and -10, the A–C-ring junction in the steroid skeleton. This stereoisomer is produced by photolysis of pregna-5,7-dienes—irradiation causes a stereochemical equilibration via a photochemically allowed electrocyclic ring opening to the B-seco-5(10),6,8-triene system, followed by a photochemically allowed recyclization to the retroprogestin. Further synthetic steps are required for the preparation of the five retroprogestins studied here. (For lead references, see [13, 14]).

The retroprogestins were first investigated by the Philips DuPhar Co. in the early 1970s as potential contraceptive agents. Despite their unnatural stereochemistry, their *in vivo* potency was very high, and in initial [24] and subsequent receptor binding studies [15], their affinities for PgR were reported to be high to very high (RBA = 1050–47,000% relative to progesterone or 140–630% relative to R5020). A brief account of the use of one of these compounds, DU41165, as a tritium-labeled ligand for PgR has appeared; however, its radiochemical synthesis was not described, nor were its binding properties examined comprehensively [25].

We were intrigued by these compounds, first because of their stereochemical novelty and their very high reported affinity binding, but also because they embodied a 4,6-dien-3-one chromophore, one related structurally to the 4,9-diene-3-one chromophore in R5020, the most thoroughly developed photoaffinity labeling agent for PgR. In addition, some of the retroprogestins are substituted at carbon-6 with a fluorine or chlorine group. Such a substitution might alter the energies of the excited electronic states of the dienone and change its photochemical reactivity; in particular, there is evidence that in enone systems, substitution at the periphery of the chromophore with fluorine can enhance intermolecular coupling reactions characteristic of the highly reactive $n\pi*$ triplet, rather than the less reactive $\pi\pi*$ triplet state [11, 12]. We were, therefore, intrigued whether the fluorine-substituted dienone system in two of the retroprogestins (DU41165 and DU41164) might exhibit similar behavior and thus provide improved photolabeling reagents for PgR.

Four of the retroprogestins proved to be high affinity ligands for PgR, with affinities comparable to those of the best known synthetic ligands R5020 and ORG2058. The highest affinities we have measured in uterine cytosol from estrogen-primed rats (160% vs R5020), however, are far lower than those reported earlier in rabbit [15, 24] (630% vs R5020). Other than the species difference, the reason for this discrepancy is not apparent. (Large species differences in binding affinity to PgR are known, however; see references cited in [26].) The retroprogestins also have some affinity for the glucocorticoid receptor, although their affinity for the mineralocorticoid receptor is very low. All of the retroprogestins demonstrated substantial specific photoinactivation for PgR; the best was DU41165, and it equaled the activity of R5020 in this assay. Thus, DU41165, being the highest binder and the most potent inactivator was prepared in high specific activity tritium labeled form.

[^{3}H]DU41165 shows high affinity binding for PgR by direct assay, but photocovalent attachment of PgR only proceeds to the extent of 4–7%. Both subunits of PgR are labeled in a manner very similar to that observed with [^{3}H]R5020 when photolabeling is done in parallel. (It is of note that photolabeling of PgR from rat uterus with either DU41165 or R5020 shows the A and B subunits to be in a ratio of 3:1. This contrasts to the *ca.* 1:1 ratio found in chick oviduct, [1, 5, 6] and in human breast cells [3] but is consistent with the subunit ratio observed in R5020 photolabeling studies by others [4].) The efficiency of PgR photolabeling with [^{3}H]DU41165 turns out to be comparable or slightly higher than that with [^{3}H]R5020 (2–5%). Thus, the photochemical behavior of the 6-fluorine substituted 4,6-dien-3-one in

DU41165 does not appear to be markedly different from the 4,9-dien-3-one in R5020.†

It may seem surprising that the efficiency of photoattachment of [^{3}H]DU41165 and [^{3}H]R5020 are only 2–7%, whereas the efficiency of photoinactivation is approx. 60%. These assays, however, do not measure the same thing: *photoattachment* succeeds only when the photoactivated ligand undergoes chemical reactions that result in the formation of a covalent link between the radiolabeled ligand and the receptor protein, whereas *photoinactivation* measures all processes that result in the loss of the capacity of PgR to bind ligand reversibly—this also includes covalent attachment of the ligand, but might also involve photochemical reactions that do not result in such a covalent linkage, such as an oxidation of the receptor resulting from a photochemical hydrogen atom abstraction. Large differentials between photoinactivation efficiency and covalent attachment efficiencies have been observed before, the most notable example being 6-oxo-estradiol; this compound is a potent photoinactivator of estrogen receptor [17], but it fails completely to photolabel receptor [H. N. Meyers and J. A. Katzenellenbogen, unpublished].

The high binding affinity and selectivity of DU41165 for PgR, combined with the very low binding affinity for mineralocorticoid (Type I) receptors, make [^{3}H]DU41165 a valuable ligand for investigation of PgR binding in certain circumstances. The photolabeling properties of DU41165 also make it a useful alternative to R5020 for photoaffinity labeling of PgR. The fluorine substituent on DU41165 makes it an interesting candidate to prepare in F-18 labeled form for possible use as a selective imaging agent for progesterone receptor-positive human breast tumors. Further studies of this nature are underway [18].

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†One should note that the literature cases cited [10, 11] in which fluorine substitution of an enone appeared to alter the electronic state and photochemistry, involve *peripheral* fluorine substitution, that is, a 6,6-difluoro-4-ene-3-one. In contrast, we have studied a 6-fluoro-4,6-dien-3-one system. In the former case, the fluorines are electronically insulated from the chromophore, so their effect is strictly an electron withdrawing inductive effect. In DU41165, by contrast, the fluorine is *part of the chromophore*, so it can engage in resonance electron donation as well. The latter fact could negate the "state switching effect" of fluorine.

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