Vol. 135, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 26, 1986 Pages 90-97

ANTIPROGESTIN-RECEPTOR COMPLEXES: DIFFERENCES IN THE INTERACTION OF THE ANTIPROGESTIN RU38,486 AND THE PROGESTIN R5020 WITH THE PROGESTERONE RECEPTOR OF HUMAN BREAST CANCER CELLS

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Received December 18, 1985

SUMMARY. In order to understand the molecular basis for antiprogestin action, we have compared the interaction of the antiprogestin [^{3}H]RU38,486 (RU486) and the progestin [^{3}H]R5020 with the progesterone receptor (PR). In both MCF-7 and T47D human breast cancer cells, we have observed marked differences in the sedimentation properties of the PR on high salt sucrose gradients: while the R5020-receptor complexes sediment at approximately 4 S (4.4±0.1 S), the RU486-receptor sediments as a prominent 6 S species as well as a 4 S species. This binding is abolished by excess unlabelled R5020, RU486 or progesterone, but is unaffected by excess unlabelled hydrocortisone or dexamethasone, indicating that both the 4 S and 6 S species represent the PR and not glucocorticoid receptor. Although the relative distribution of 4 S and 6 S forms is not altered by treatment with DNAse or RNAse, exposure to 10 mM thioglycerol or to 3 M urea results in conversion of the 6 S to the 4 S form, suggesting that disulfide bonds and hydrophobic interactions are important in maintaining the integrity of the 6 S form. These findings suggest that the 6 S antiprogestin complex is formed as a result of the interaction of PR units with each other or with a different protein. This change in receptor association state may be an important aspect of the antiprogestin activity of RU486.

Since progestins play such an important role in the physiological functioning of the reproductive tract, and in regulation of hormone-responsive breast cancer growth, there has been great interest in the development of antiprogestin agents that would selectively antagonize the action of progestins. Such antiprogestins would have considerable potential as postcoital antifertility agents and implantation inhibitors and as anti-cancer agents, especially in treatment of hormone-responsive breast cancer.

Abbreviations: E_2, estradiol-17β; PMSF, phenylmethyl sulfonyl fluoride; PR, progesterone receptor; R5020, 17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione; RU486 (RU38,486), 17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(1-propynyl)-estra-4,9-diene-3-one.

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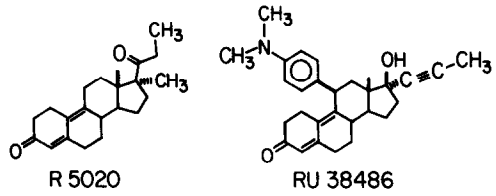

Figure 1: Structure of the progestin R 5020 and of the antiprogestin RU 38486 (RU486).

In addition, they would be particularly useful as experimental tools for elucidating aspects of the mechanism of action of progestins (1-8).

While therapeutically useful synthetic antiestrogens, antiandrogens and mineralocorticoids have been available for some time (1), only quite recently has a promising new synthetic antiprogestin RU38,486 (RU486; see Fig. 1) been developed (2). This compound prepared by the Roussel-UCLAF Company, has high affinity for both PR and glucocorticoid receptor and has potent antiprogestin and antiglucocorticoid activity *in vitro* and *in vivo* (1-7). In particular, RU486 suppresses growth of human breast cancer cells *in vitro* via interaction with the PR (and not via the glucocorticoid receptor; 8-10), and it inhibits progesterone stimulation of several specific progestin induced proteins in T47D and MCF-7 breast cancer cells, while displaying no agonist effect on these proteins.

Although the data are consistent with the antiprogestin activity of RU486 being mediated via the PR of target cells, the mechanism of the antiprogestin activity of this compound is presently unknown. Hence, in this study, our aim was to determine whether RU486 interacts differently with the PR than do typical progestin agonists.

MATERIALS AND METHODS

Chemicals: Radioactive RU38,486 (17 β-hydroxy-11 β-(4-dimethylaminophenyl)-17 α-(1-propynyl)-estra-4,9-dien-3-one [6,7-^{3}H]; 50.6 Ci/mmol) and unlabelled RU38,486 were a gift from Roussel-UCLAF, France. Radioactive R5020 (17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione [17 α-methyl-^{3}H]; 87 Ci/mmole) and unlabelled R5020 were from New England Nuclear. Leupeptin, phenyl methyl sulfonyl fluoride (PMSF), soybean trypsin inhibitor, thioglycerol, DNAse I from bovine pancreas (No. D 4763) and RNAse A from bovine pancreas (No. R 5500, essentially protease free) were obtained from Sigma.

Cell Cultures: MCF-7 and T47D human breast cancer cells were grown in cell culture and passaged exactly as described previously (12,13). At 4 days prior

to use, 10 nM estradiol (E_2) was added to the media of MCF-7 cells in order to increase cellular PR levels (12).

Exposure of Cells to Tritiated Ligand and Preparation of Nuclear Extracts: MCF-7 or T47D cells from a single near confluent T-75 flask were harvested with HBSS containing 1 mM EDTA. The cells were collected by centrifugation and resuspended in growth medium containing 10 nM tritiated ligand ([^{3}H]R5020 or [^{3}H]RU38,486). PR was labelled by incubation of the cell suspension for 30 min at 37°C, after which the cells were washed with 2 ml of 10 mM Tris, 1.5 mM EDTA, 10% glycerol, pH 7.4 at 4°C [TEG, pH 7.4 buffer] and homogenized in TEG buffer containing protease inhibitors (soybean trypsin inhibitor 5 mg/ml, leupeptin 1 mg/ml, PMSF, 1 mg/ml) by 40 strokes of a Dounce homogenizer (B-pestle). The homogenate was centrifuged at 800 x g for 10 min and the pellet thus obtained was resuspended in 200 µl of 10 mM Tris, 1.5 mM EDTA, 10% glycerol, 0.8 M KCl, pH 8.5 at 4°C [TEGK, pH 8.5 buffer] containing protease inhibitors. The suspension was incubated for 1 h at 0-4°C with resuspension every 15 min and then centrifuged at 180,000 x g for 30 min. The supernatant was then layered onto sucrose gradients.

Sucrose Gradient Centrifugation: Gradients (3.6 ml) contained 5-20% sucrose prepared in buffered deuterium oxide. Unless otherwise stated, the KCl concentration was 0.4 M and the buffer used was TEG, pH 7.4. Centrifugation was for 40 h at 357,000 x g (59,000 rpm Beckman SW60Ti rotor) at 0-4°C and two drop fractions were collected and counted in Triton xylene scintillation fluid (14). Sedimentation analyses on gradients of the same composition but containing regular water instead of deuterium oxide (centrifuged for 17 h at 357,000 x g) gave identical profiles. ^{14}C ovalbumin (3.5 S) and ^{14}C gamma globulin (6.6 S) methylated by the method of Rice and Means (15) were utilized as sedimentation markers.

RESULTS

Comparison of [^{3}H]R5020-PR and [^{3}H]RU486-PR Complexes on Sucrose Gradients

When MCF-7 cells were labelled with the high affinity antiprogestin [^{3}H]RU486, or with the high affinity progestin [^{3}H]R5020 (both at 10 nM), analyses of the salt extracted nuclear PR complexes revealed an unusual sedimentation pattern for the antiprogestin-PR complex compared to the progestin-labelled PR complex. Whereas the R5020-labelled PR complexes sedimented as a single 4 S species on high salt sucrose gradients, the antiprogestin-labelled complexes sedimented as both 4 S and 6 S species (Fig. 2-A and 2-B). Studies in the T47D human breast cancer cell line, which has constitutively high levels of PR (8-10), also showed that the antiprogestin-labelled receptor complexes sedimented as both a 4 S and 6 S species (Fig. 2-C and 2-D). In T47D cells, the more rapidly sedimenting 6 S form represented ca. 50% of the total PR complexes. Although Fig. 2 shows data from sucrose density gradients containing 0.4 M KCl, nearly identical profiles were

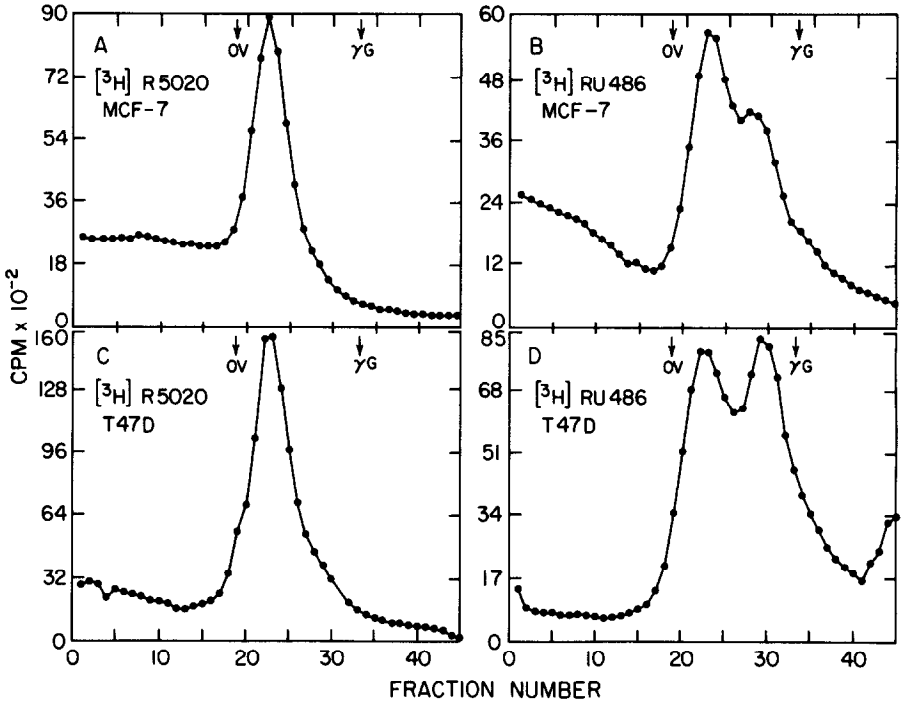

Figure 2: Panels A and B. MCF-7 cells that had been treated with 10 nM estradiol (E_2) for 4 days to increase cellular PR levels, were incubated in growth medium with 10 nM E_2 plus 10 nM [^{3}H]RU486 or 10 nM [^{3}H]R5020 for 30 min at 37°C. (These concentrations result in nuclear localization/"fixation" of over 95% of PR in the nucleus.) Cells were homogenized and the nuclear salt extract prepared. Nuclear salt (0.6 M KCl) extracts, representing over 85% of total nuclear PR, were layered on 5-20% sucrose gradients prepared in TEG pH 7.4 buffer with 0.4 M KCl. Panels C and D. T47D cells were incubated in growth medium plus 10 nM [^{3}H]RU486 or 10 nM [^{3}H]R5020 for 30 min at 37°C. Cells were homogenized and the nuclear salt (0.6 M KCl) extract was prepared and layered onto 5-20% sucrose gradients prepared in TEG buffer with 0.4 M KCl. Gradients were centrifuged for 40 h at 357,000 x g at 0-4°C and contained the internal ^{14}C-labelled markers ovalbumin (3.6 S) and γ -globulin (6.6 S), whose positions are designated by arrows.

obtained when the nuclear salt extracted samples were centrifuged through sucrose gradients containing either 0.2 M or 0.8 M KCl.

Competitive Binding Analyses Indicating that the 4 S and 6 S ^{3}H-RU486-Labelled Species Represent PR

Competition studies (Fig. 3) demonstrated that both 4 S and 6 S forms represent PR and not glucocorticoid receptor to which RU486 and R5020 can also bind. As shown in Fig. 3, a 100-fold excess of progesterone or R5020 or RU486 gave complete competition for [^{3}H]RU486 binding, whereas a 100-fold excess of hydrocortisone or a 100-fold excess of dexamethasone (not shown) exhibited no competition.

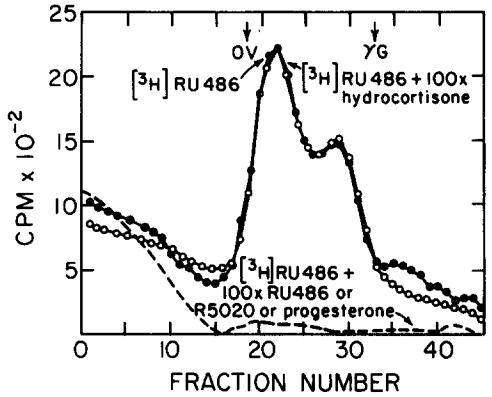

Figure 3: Competition analyses indicating that [^{3}H]RU486 labelled species represent progesterone receptors (PR) and not glucocorticoid receptor. MCF-7 cells were exposed to 10 nM estradiol for 4 days to increase cellular PR levels. Cells were then incubated in growth medium plus 10 nM E_2 in the presence of 1000 nM unlabelled progesterone, R5020, RU486, or hydrocortisone for 1 h prior to the addition of 10 nM [^{3}H]RU486 for 30 min at 37°C. Cells were homogenized and nuclear salt extracts were centrifuged on sucrose gradients exactly as described in the legend to Fig. 2. Separate gradient patterns have been overlayed for clarity.

Effects of RNAse, DNAse, Sulfhydryl Reagents and Urea Disaggregation on the 6 S ^{3}H-RU486 Receptor Form

We found that the relative distribution of 4 S and 6 S forms was not altered by treatment with RNAse A or DNAse I even up to quite high concentrations (5 or 50 µg/ml), suggesting that RNA and DNA are not components of the 4 S or 6 S species (data not shown). In contrast, exposure of extracts to thioglycerol or 3 M urea resulted in conversion of the 6 S complex to the 4 S species, so that a single 4 S form with [^{3}H]RU486 was seen on sucrose gradients after these treatments (Fig. 4).

DISCUSSION

Although RU486 represents the most effective antiprogestin available to date, the molecular basis underlying its antiprogestin activity is not known. Given that RU486 binds to PR with high affinity (12,6,8-10), similar to an agonist such as R5020, one might expect its antagonistic action to be a result of its inability to effect conformational and/or other changes in the receptor protein necessary for effective interaction of the receptor with chromatin. Hence, our observation regarding the effect of ligand binding on sedimentation behavior of PR is particularly intriguing.

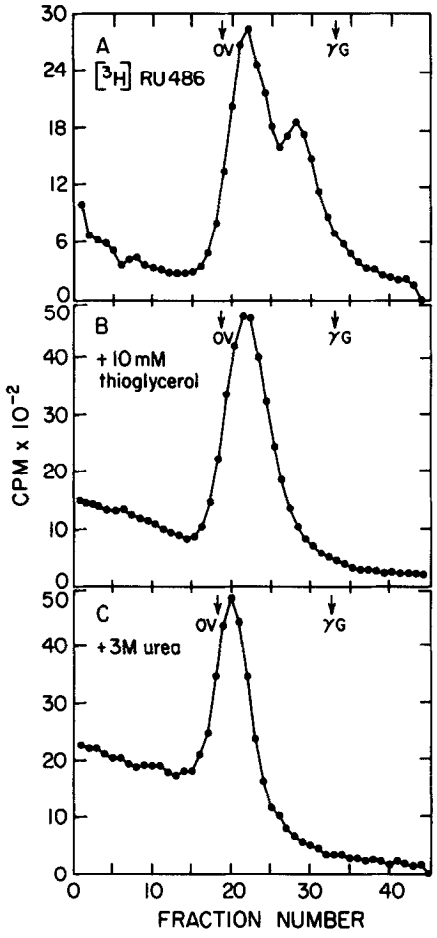

Figure 4: MCF-7 cells that had been treated with 10 nM E_2 for 4 days were incubated with 10 nM [^{3}H]RU486 for 30 min at 37°C. The cell suspension was divided into 3 aliquots. For the control (A), cells were homogenized and nuclear salt extracts were prepared as described in Materials and Methods. For processing of cell sample B, 10 mM monothioglycerol was added to buffers used in cell homogenization and nuclear salt extraction. For cell sample C, nuclear salt extraction was performed in the presence of 3 M urea. High speed supernatants from samples A and C were layered onto 5-20% sucrose gradients containing 0.4 M KCl and prepared in TEG pH 7.4, whereas in the case of sample B, 10 mM thioglycerol was added to sucrose solutions. Gradients were centrifuged for 40 h at 357,000 x g.

Our studies show that nuclear PR complexed with the progestin agonist R5020 sediments as a 4 S species while antiprogestin-PR complexes sediment as both 4 S and 6 S species. Interestingly, these interactions can be obliterated by exposure to a protein disaggregating agent (urea), or a reductant (thioglycerol), suggesting that the 6 S form represents a protein dimer or oligomer held together by hydrophobic and by disulfide bonds. Indeed, similar sorts of studies have indicated the role of disulfides in the

subunit structure of the insulin receptor (16) as well as in the state of association of the estrogen receptor (17). We find that the integrity of the 4 S and 6 S salt extracted nuclear PR complexes with RU486 is not affected by either DNAse or RNAse, indicating that these forms do not contain receptor with associated nucleic acid. Thus, these PR species appear to be different from certain 5-6 S forms of cytosol receptors for androgens (18) and glucocorticoids (19) that, are associated with RNA.

The finding that antiprogestin-labelled complexes exist in a heavier form which can be converted to the smaller 4 S form by disaggregation with urea is similar to our observations of physical differences in estrogen receptors when complexed with antiestrogen versus estradiol (14,20,21). It is, therefore, tempting to hypothesize that the antagonistic action of some antihormones may result from their ability to engage the receptor in a biologically non-productive interaction which we detect as a larger sedimenting species.

Of particular relevance to our observations here in breast cancer cells, are studies on the chick oviduct cytosol PR done in other laboratories during the 1970's. In these studies (22-24) cytosolic PR appeared as a 6 S form (A-B or B-B dimer) on gel filtration and sucrose gradients at low ionic strengths, but as a 4 S form (separate A and B units, with molecular weights of ca. 80,000 and 110,000, respectively) in the presence of 0.3 M KCl. Furthermore, the 6 S complex showed little DNA-binding activity, suggesting that the DNA binding site on the 4 S receptor unit was masked in the 6 S complex.

It is possible that the 6 S form of PR that we have observed here is also deficient in its potential for chromatin interaction and, therefore, that antiprogestins may act by forming complexes with PR that are conformationally altered so that this larger, biologically less active form is stabilized. These issues will be addressed in future investigations.

ACKNOWLEDGEMENTS

We are very grateful to Drs. J-P. Raynaud, T. Ojasoo and R. Deraedt of the Roussel-UCLAF Company for generously providing us with tritiated RU486 for these studies. This work was supported by grants from the NIH to BSK.

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