Methyl p-Hydroxyphenyllactate

AN INHIBITOR OF CELL GROWTH AND PROLIFERATION AND AN ENDOGENOUS LIGAND FOR NUCLEAR TYPE-II BINDING SITES*

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We previously described and partially characterized endogenous ligands for nuclear type II sites in normal and malignant tissues. Chromatography of these ligands on Sephadex LH-20 revealed that two peaks with binding activity (α and β) could be resolved. The β-peak component was present in all normal tissues that we examined, but not in malignant tissues, and it inhibited the growth of MCF-7 human breast cancer cells in vitro. Conversely, the α-peak component was found to be present in both normal and malignant tissues, and did not inhibit MCF-7 cell growth. The present studies describe the purification and identification of the α-peak and β-peak components in bovine serum and an assessment of the effects of these compounds on normal and malignant cell growth. Gas chromatography-mass spectroscopy analysis of the purified β-peak component demonstrated that the compound was methyl p-hydroxyphenyllactate (MeHPLA). Competition analysis revealed that MeHPLA binds to nuclear type II sites with a high binding affinity, while physiological levels of this compound blocked estradiol stimulation of uterine growth in vivo and inhibited the growth of MCF-7 human breast cancer cells in vitro. The α-peak component was found to be the corresponding acid, p-hydroxyphenyllactic acid (HPLA). This compound interacted with nuclear type II sites with a relatively low affinity and did not block uterotropic response to estradiol or inhibit MCF-7 cell growth. These studies demonstrate that HPLA and MeHPLA are ligands for nuclear type II sites and that MeHPLA may be a very important regulator of normal and malignant cell growth.

Materials and Methods

Animals—Immature (21-day) or adult ovariectomized Sprague-Dawley rats were used for all experiments. The animals were maintained in a pathogen-free environment on a constant light-dark cycle (lights on 6 a.m.–6 p.m.). Food and water were provided ad libitum. Animals were killed by cervical dislocation and the uteri were removed, trimmed of free extraneous tissue, weighted, and placed in 0.9% saline (4°C) prior to tissue homogenization and nuclear pellet preparation. Animals were injected or implanted with estradiol (3) and/or HPLA or MeHPLA as described in the text and figure legends.

Extraction of Type II Ligands from Fetal Bovine Serum—Preliminary experiments revealed that fetal bovine serum (Difco) was an economical source of type II ligands, and identical results were obtained with both rat tissues and fetal bovine serum. For large-scale preparations, 500 ml of fetal bovine serum was diluted with 500 ml of HPLC-grade water and boiled as described previously (1, 2). The boiled serum was centrifuged (1000 x g) for 20 min to remove denatured protein, and the supernatant was adjusted to pH 1.0 with 12 N HCl (high purity, Baker Laboratories). The acidified supernatant was extracted 3 times with equal volumes of ethyl acetate (HPLC grade) in a separatory funnel, and the pooled ethyl acetate extracts were concentrated to approximately 100 ml under vacuum at 50°C.

The abbreviations used are: HPLC, high performance liquid chromatography; HPLA, 3-(4-hydroxyphenyl)lactic acid; MeHPLA, methyl 3-(4-hydroxyphenyl)lactate; GC-MS, gas chromatography-mass spectrometry.
The pooled extracts were washed with an equal volume of HPLC-grade water and then taken to dryness under vacuum. The final washed ethyl acetate extract was solubilized in a minimal volume of methanol (500 µl), dissolved in Tris/EDTA (TE) buffer (10 mM Tris, 1.5 mM EDTA, pH 7.4, at 22 °C), and adjusted to pH 7.4 with sodium hydroxide to a final volume of 5 ml.

Sephadex LH-20 Chromatography of the Bovine Serum Extract—Chromatography on Sephadex LH-20 of bovine serum extracts with type II binding activity was conducted as described previously (1, 2). Briefly, the extract dissolved in TE buffer was introduced onto a Sephadex LH-20 column (3 x 50 cm) equilibrated with the same buffer (160-ml bed volume). TE buffer was employed as the mobile phase (1 ml/min) and 2-ml fractions were collected. An aliquot (50 µl) of each fraction was assayed for type II binding activity (inhibition of [3H]estradiol binding to nuclear type II sites) as described previously (1, 2). The α- and β-peaks (Fig. 1) were separately pooled, adjusted to pH 1.0 with 12 n hydrochloric acid, and extracted 3 times with equal volumes of ethyl acetate. Ethyl acetate extracts were dried under vacuum and redissolved in high purity methanol for subsequent purification by HPLC.

HPLC Purification of Type II Ligands in Bovine Serum Extract—Purification of the α- and β-peaks components obtained by chromatography on Sephadex LH-20 was achieved using a Beckman gradient liquid chromatograph equipped with a 1 x 25-cm Ultrasphere-Octyl (Beckman Instruments) reversed-phase column eluted at a flow rate of 2 ml/min with water:methanol:acetic acid (Solvent A = 90:5:5; Solvent B = 0:60-100%) using two different methylating agents. Initially, HPLA was heated and extracted with ethyl acetate. The ethyl acetate extracts were dried under vacuum and redissolved in methanol for rechromatography on the Ultrasphere-Octyl column. Final purification of both α- and β-peaks components was achieved using isotropic elution. The α-peak material was reinserted onto the column and eluted with water:methanol:acetic acid (90:5:5) at a flow rate of 2 ml/min. During the second chromatographic run (Fig. 2), 0.5-min fractions were collected and 10-µl aliquots were assayed for type II binding activity (Fig. 2). The fractions corresponding to the α- and β-peaks were separately pooled, adjusted to pH 1.0, and extracted with ethyl acetate. The ethyl acetate extracts were dried under vacuum and redissolved in methanol for rechromatography on the Ultrasphere-Octyl column. Final purification of both α- and β-peaks components was achieved using isotropic elution. The α-peak material was reinserted onto the column and eluted with water:methanol:acetic acid (90:5:5) at a flow rate of 2 ml/min. During the second chromatographic run (Fig. 2), 0.5-min fractions were collected and 10-µl aliquots were assayed for type II binding activity (Fig. 2). The fractions corresponding to the α- and β-peaks were separately pooled, adjusted to pH 1.0, and extracted with ethyl acetate. The ethyl acetate extracts were dried under vacuum and redissolved in methanol for rechromatography on the Ultrasphere-Octyl column. Final purification of both α- and β-peaks components was achieved using isotropic elution. The α-peak material was reinserted onto the column and eluted with water:methanol:acetic acid (90:5:5) at a flow rate of 2 ml/min. During the second chromatographic run (Fig. 2), 0.5-min fractions were collected and 10-µl aliquots were assayed for type II binding activity (Fig. 2). The fractions corresponding to the α- and β-peaks were separately pooled, adjusted to pH 1.0, and extracted with ethyl acetate. The ethyl acetate extracts were dried under vacuum and redissolved in high purity methanol for subsequent purification by HPLC.

GC-MS Analysis of Type II Ligands—Fractions from the centers of the purified α- and β-peaks (Fig. 3) were separately pooled and sequentially extracted with ethyl acetate, dried under nitrogen, dissolved in bis(trimethylsilyl)acetamide, and heated at 60 °C for 20 min to form bis(trimethylsilyl) derivatives. An aliquot of each of the two preparations was examined by GC-MS using a Finnigan 1020/OWA instrument equipped with a 10-m DB-5 bonded-phase fused silica capillary column programmed from 100 °C to 300 °C at 10 °C/min. Synthesis of MeHPLA—MeHPLA was prepared from HPLA by using a different methylating agent. Initially, HPLA was heated with dimethylformamide dimethylacetate in methanol for 30 min at 60 °C. However, it was difficult to complete methylation of the carboxylic acid moiety without also methylating the phenolic hydroxyl group. During later syntheses we employed diazomethane prepared from N-methyl-N-nitroso-p-toluene sulfonamide (Alrich). An ethereal solution of diazomethane was added slowly to a methanoic solution of HPLA in an ice bath until a residual yellow color was obtained, indicating that excess diazomethane was present in the reaction vessel. The solvent and excess diazomethane were removed immediately under vacuum using a rotary evaporator. The yield of MeHPLA was in excess of 99.5%.

Spectrophotometric Analysis of Endogenous Type II Ligands and Authentic HPLA and MeHPLA—Ultraviolet spectra of the α- and β-peaks components (Fig. 3) were recorded using a Pye Unicam scanning spectrophotometer. The samples were dissolved in HPLC-grade methanol, and the spectra were recorded against solvent blank scans ranging from 190 to 500 nm. The absorption maximum for all four components (endogenous α- and β-peaks components and HPLA and MeHPLA standards) was determined to be 268-269 nm (Fig. 8) and, on this basis, the samples were diluted to equivalent absorbance units. These fractions were sequentially extracted with ethyl acetate, dried under vacuum, and redissolved in 5 ml of TE buffer, and chromatographed as described in the text. Fractions (2 ml) were collected and aliquots (50 µl) assayed for nuclear type II site binding inhibition (1, 2).

RESULTS
Isolation, Purification, and Identification of the Endogenous Ligands for Nuclear Type II Sites—As reported previously, chromatography of these ligands on Sephadex LH-20 revealed that two peaks with binding activity could be resolved (1, 2). These two peaks (α and β) were obtained in chromatograms of extracts of a variety of rat and mouse tissues, as well as serum samples. Since fetal bovine serum is readily available in quantity for tissue culture, we decided to evaluate it as a potential source of the ligands for qualitative analysis. We did indeed find that the bovine serum extract contained both of the ligands (Fig. 1).

Fractions corresponding to the α- and β-peaks were separately pooled and extracted with ethyl acetate (at pH 1.0), and aliquots were assayed for inhibition of [3H]estradiol binding to nuclear type II sites. The fractions (Fig. 9) were recorded using a Pye Unicam scanning spectrophotometer. The samples were dissolved in HPLC-grade methanol, and the spectra were recorded against solvent blank scans ranging from 190 to 500 nm. The absorption maximum for all four components (endogenous α- and β-peaks components and HPLA and MeHPLA standards) was determined to be 268-269 nm (Fig. 8) and, on this basis, the samples were diluted to equivalent absorbance units. These fractions were sequentially extracted with ethyl acetate, dried under vacuum, and redissolved in 5 ml of TE buffer, and chromatographed as described in the text. Fractions (2 ml) were collected and aliquots (50 µl) assayed for nuclear type II site binding inhibition (1, 2).

Fig. 1. Chromatography of the fetal bovine serum extract on Sephadex LH-20 (160-ml bed volume). The ethyl acetate extract from 500 ml of fetal bovine serum was dried under vacuum, dissolved in 5 ml of TE buffer, and chromatographed as described in the text. Fractions (2 ml) were collected and aliquots (50 µl) assayed for nuclear type II site binding inhibition (1, 2).
and the extracts were dried, dissolved in methanol, and further purified by HPLC on an Ultrasphere-Octyl reversed-phase column. Chromatograms reproduced in Fig. 2 demonstrate that the α- and β-peak components eluted from the HPLC column as single, sharp peaks and that inhibition activity coincided with ultraviolet absorbance at 278 nm. The retention times for the α- and β-peak components on the Ultrasphere-Octyl column were 9.2 and 16.7 min, respectively.

After purification by HPLC, fractions corresponding to the α- and β-peaks were separately pooled, and these components were examined as trimethylsilyl derivatives by GC-MS.

The gas chromatogram obtained for the derivatized α-peak component was dominated by a single peak, the mass spectrum of which is shown in Fig. 3A. This mass spectrum was indistinguishable from that of the trimethylsilyl derivative of p-hydroxyphenyllactic acid (HPLA) in the EPA/NIH Mass Spectral Data Base. The molecular ion of m/z 398 indicated that the bis(trimethylsilyl) ether trimethylsilyl ester derivative had been formed, while the abundant ion of m/z 179 demonstrated that the molecule contained a trimethylsilyl oxybenzyl moiety. Thus, the hydroxy group on the side-chain was α to the carboxylic moiety. To confirm the identity of the α-peak component, we purchased authentic HPLA (Aldrich), derivatized this compound with bis(trimethylsilyl)acetamide, and recorded its mass spectrum. The mass spectrum for the trimethylsilyl derivative of authentic HPLA (Fig. 3B) was found to be indistinguishable from that of the derivative of the α-peak material (Fig. 3A) obtained from bovine serum. The retention index values for the α-peak material and authentic HPLA were both 1902, so we concluded that the α-peak component was HPLA.

A similar analysis was also performed on the β-peak component obtained from fetal bovine serum. The gas chromatogram had two major peaks, the first of which had a retention index value of 1902 and a mass spectrum corresponding to that of the trimethylsilyl derivative of HPLA. The second major peak had a retention index of 2174 and afforded the prominent obtained from fetal bovine serum. The gas chromatogram obtained for the derivatized α-peak material (Fig. 4A) contained a bis(trimethylsilyl)oxybenzyl moiety. However, the molecular ion of m/z 340 indicated that the compound was a bis(trimethylsilyl)methyl derivative of HPLA. The \([\text{M} - \text{COOMe}]^{-}\) ion of m/z 281 provided evidence that the compound was the bis(trimethylsilyl) ether of MeHPLA. The mass spectrum of this compound is not recorded in the EPA/NIH Mass Spectral Data Base, and MeHPLA is not commercially available. Accordingly, we prepared the derivative by sequential methylation and silylation of HPLA using dimethylformamide dimethylacetel and bis(trimethylsilyl)acetamide, respectively, and analyzed the sample by GC-MS. The mass spectrum of the authentic derivative (Fig. 4B) was indistinguishable from that of the trimethylsilyl derivative of the β-peak material (Fig. 4A) obtained from fetal bovine serum. Thus, we concluded that the β-peak component was MeHPLA.

Verification of HPLA and MeHPLA as Ligands for Nuclear Type II Sites—Chromatographic and mass spectral data demonstrated unequivocally that HPLA and MeHPLA were the major constituents of the α- and β-peak fractions, respectively, purified by sequential chromatography on Sephadex LH-20 and HPLC. It was still necessary to determine whether HPLA and MeHPLA were ligands for the type II site and, if so, whether they accounted for the total binding activity of the α- and β-peak fractions.

Confirmation of the identities of the α- and β-peak compounds as HPLA and MeHPLA, respectively, was obtained by careful evaluation of the retention data for the four substances on Sephadex LH-20 and during HPLC. For these experiments we used the α- and β-peak components isolated...
that the retention times for HPLA and the \( \alpha \)-peak component resulted in proportional increases of inhibition activity and UV absorbance on HPLC.

To further confirmation of the identities of the endogenous ligands, we separately collected the fractions corresponding to maximum binding activity on Sephadex LH-20 and Ultrasphere-Octyl, we obtained demonstrated that the positions of elution of the \( \alpha \)-component and of authentic HPLA (Fig. 5, A and B) were indistinguishable, as were the elution profiles of the \( \beta \)-peak component and authentic MeHPLA (Fig. 5, C and D).

For further confirmation of the identities of the endogenous ligands, we separately collected the fractions corresponding to the peaks of binding activity in the chromatograms shown in Fig. 5, A–D, extracted the compounds from the aqueous buffer with ethyl acetate (at \( \text{pH} \ 1.0 \)), and examined them by HPLC on the Ultrasphere-Octyl column. Fractions (0.5 min) were collected, and aliquots were assayed for nuclear type II binding site inhibition. The data obtained demonstrated that the positions of elution of the \( \alpha \)-peak component and of authentic HPLA (Fig. 5, A and B) were indistinguishable, as were the elution profiles of the \( \beta \)-peak component and authentic MeHPLA (Fig. 5, C and D).

Fractions corresponding to maximum binding activity for each of the four chromatograms shown in Fig. 6 were separately pooled, evaporated to dryness, and dissolved in Spectrograde methanol, and ultraviolet absorption spectra were recorded using a Pye-Unicam scanning spectrophotometer. All four spectra (Fig. 7) were very similar, with absorbance maxima at 278 nm.

The specific binding activities of the isolated components were compared with those of the authentic materials to determine whether HPLA and MeHPLA accounted for all of the binding activity exerted by the isolated components. Thus, if there were additional endogenous ligands with identical retention behavior on Sephadex LH-20 and Ultrasphere-Octyl, we would obtain evidence for their existence.

The concentrations of the four solutions used to obtain the spectra shown in Fig. 7 were adjusted so that they each afforded the same absorbance at 278 nm. This concentration was defined as 1 absorbance unit/ml. Aliquots (0.0001–0.005 absorbance unit) were assayed to evaluate their ability to compete for [\( ^3H \)]estradiol binding to nuclear type II sites (Fig. 8). The data demonstrated that the binding inhibition (competition) curves for authentic HPLA and the \( \alpha \)-peak, or authentic MeHPLA and the \( \beta \)-peak, were both qualitatively and quantitatively indistinguishable. Therefore, the binding inhibition in the \( \alpha \)- and \( \beta \)-peak preparations could be attributed entirely to HPLA and MeHPLA and was not due to any

![Fig. 5. Comparison of the elution profiles of the \( \alpha \)- and \( \beta \)-peak components and of authentic HPLA and MeHPLA on Sephadex LH-20. Fractions (1 ml) were collected and assayed as described for Fig. 1.](image-url)
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Additional substances in the extracts. These data demonstrate unequivocally that HPLA and MeHPLA are the previously described ligands for type II binding sites (1, 2).

Finally, we evaluated the binding affinities of authentic HPLA and MeHPLA for nuclear type II sites and the conventional estrogen receptor. The data shown in Fig. 9 demonstrated that, while both compounds competitively inhibited [3H]estradiol binding to nuclear type II sites, MeHPLA was 30-40-fold more effective in this regard than HPLA. Therefore, esterification appears to impart the high binding affinity of MeHPLA for nuclear type II sites. The estimated binding affinity of MeHPLA for nuclear type II sites derived from these data is approximately 5 nM. Conversely, neither HPLA nor MeHPLA was capable of competing for [3H]estradiol binding to the rat uterine estrogen receptor (Fig. 10) which was consistent with our preliminary studies with biological preparations of this material (1, 2). Therefore, as we suggested previously (1, 2) potential modulation and/or antagonism of estrogenic response by MeHPLA or HPLA may occur through an interaction of these compounds with nuclear type II sites rather than through an interaction with the rat uterine estrogen receptor.

Effect of MeHPLA and HPLA on Cell Growth and Proliferation—Two systems were used to evaluate the biological effects of HPLA and MeHPLA. Assays were conducted to determine whether the compounds affected 1) the growth of MCF-7 human breast cancer cells in vitro and 2) the estradiol stimulation of uterine growth in the immature rat.

HPLA failed to inhibit MCF-7 cell growth at concentrations ranging from 1 to 10 μg/ml (Fig. 11). Conversely, MeHPLA treatment resulted in a dose-dependent inhibition of MCF-7 cell growth (Fig. 11), and this effect was subsequently found to be reversible following MeHPLA removal from the tissue culture medium. The failure of the low affinity ligand (HPLA) to inhibit MCF-7 cell growth was consistent with earlier
studies in our laboratory demonstrating that partially purified α-peak type II ligand preparations from rat liver also failed to inhibit MCF-7 cell growth (1, 14). The high affinity ligand (MeHPLA) for type II sites blocked MCF-7 cell proliferation, as observed for partially purified β-peak preparations from rat liver (14). On the basis of these observations, there appears to be a correlation between the binding affinities of MeHPLA and HPLA for nuclear type II sites and inhibition of MCF-7 cell growth.

To determine the "intracellular" concentration of MeHPLA under these experimental conditions, MCF-7 cells were plated and allowed to attach as described above. On day 0, the medium was changed and supplemented with [³H]MeHPLA (68 Ci/mmol; Amersham Radiochemicals). Twenty-four hours later (day 1), the medium and cell monolayers were collected and the radioactivity in these fractions was determined. The data demonstrated that the cell monolayers contained 1.03 ± 0.09% of the [³H]MeHPLA added to the medium for seven experimental tritium determinations. These data suggested that [³H]MeHPLA uptake by MCF-7 cells under these conditions is limited and that the actual intracellular concentrations of the compound in the experiment performed to obtain the data shown in Fig. 11 were likely to be in the 10-100 ng/ml range (i.e. 1% of 1–10 μg/ml). These levels are within the range that we determined to be required for interaction with nuclear type II binding sites (Fig. 9). Although these experiments certainly do not prove cause and effect, they do demonstrate a good correlation between the binding affinity of MeHPLA for nuclear type II sites and cell growth inhibition. This relationship is currently under detailed investigation.

Studies with human breast cancer cells demonstrated that MeHPLA (but not HPLA) was a potent inhibitor of cell growth in vitro. Further experiments were conducted to determine whether HPLA and/or MeHPLA affect estrogen-stimulated cell growth in vivo. Immature female rats (6–8 animals/group) were injected with saline–ethanol (20%) vehicle containing estradiol (0.5 μg) with or without 10 or 100 μg of HPLA or MeHPLA. Animals were killed 24 h following injection, and uterine wet and dry weights were determined as described in the text.

FIG. 9. Competition analysis with authentic HPLA and MeHPLA for [³H]estradiol binding to nuclear type II sites. See text for details.

FIG. 10. Competition analysis with authentic HPLA and MeHPLA for [³H]estradiol binding to the rat uterine estrogen receptor. Uterine cytosol from adult ovariectomized rats was incubated (30 °C for 30 min) with 10 nm of [³H]estradiol with or without the indicated concentrations of HPLA, MeHPLA, or diethylstilbestrol (DES), as described previously (1).

FIG. 11. Effects of authentic HPLA and MeHPLA on MCF-7 human breast cancer cell growth in vitro. Cell growth was evaluated by measuring the number of cells per dish and the total DNA content per dish. See text for details.

FIG. 12. Effects of HPLA and MeHPLA on estrogen-stimulated uterine growth. Immature (21-day-old) Sprague-Dawley rats (6–8 per group) were injected with saline–ethanol (20%) vehicle containing estradiol (0.5 μg) with or without 10 or 100 μg of HPLA or MeHPLA. Animals were killed 24 h following injection, and uterine wet and dry weights were determined as described in the text.

DISCUSSION

Previous studies from our laboratory demonstrated that rat serum and a variety of rat tissues contained an inhibitor of
["H]estradiol binding to nuclear type II sites (1, 2). Preliminary characterization of this inhibitor activity revealed that it could be resolved into two components (which we designated \( \alpha \) and \( \beta \)) on Sephadex LH-20 (1, 2). Interestingly, both components were found to be present in cytosol preparations from normal tissues, but malignant tissues such as rat, mouse, and human mammary tumors contained the \( \alpha \)-peak component but were deficient in the \( \beta \)-peak component (2). This deficiency of the \( \beta \)-peak component in tumors led us to speculate that this inhibitor may be the more important with respect to potential regulation of cell growth (2). To further evaluate this relationship it was necessary to identify the two ligands.

Fetal bovine serum contains both the \( \alpha \)- and \( \beta \)-peak components and is commercially available in quantity. Thus, we were able to isolate sufficient amounts of the \( \alpha \)- and \( \beta \)-peak components from fetal bovine serum for complete qualitative analysis. We now have data which demonstrate that the \( \alpha \)-peak component is HPLA and the \( \beta \)-peak component is MeHPLA:

1) The isolated and authentic compounds have the same retention behavior during chromatography on Sephadex LH-20 (Fig. 5).
2) The isolated and authentic compounds have the same retention behavior during HPLC on Ultrasphere-Octyl (Fig. 6).
3) The isolated and authentic compounds have the same gas chromatographic retention index values.
4) Trimethylsilyl derivatives of the isolated and authentic compounds have the same mass spectra (Figs. 3 and 4).
5) The isolated and authentic compounds have the same ultraviolet absorption spectra (Fig. 7).
6) The isolated and authentic compounds have the same specific binding activities to the type II site (Fig. 8).
7) Neither the isolated nor the authentic compounds bind to the (conventional) rat uterine estrogen receptor (Fig. 10).

In all respect, HPLA was identical to the \( \alpha \)-peak component and MeHPLA was identical to the \( \beta \)-peak component.

The only structural feature that we have not fully investigated is the stereochemistry at C-2 on the side-chain. However, Kamerling and Vliegenthart (27) have shown that the HPLA isomer present in urine of a patient with tyrosyluria is L-3-(4-hydroxyphenyl)lactic acid. The corresponding D-isomer was not detected. Thus, it is probable that HPLA and MeHPLA from fetal bovine serum both exist in the L-form. If this is the case, it is likely that the binding interaction of MeHPLA with nuclear type II sites is of a higher affinity than that shown here (Fig. 9) if only one of the two isomers binds. If nuclear type II sites demonstrate equivalent affinity for both the \( \alpha \)- and \( \beta \)-isomers, our estimated binding affinities are correct. We are planning to resolve the isomers on a chiral HPLC column to address these issues.

The identification of HPLA and MeHPLA as ligands for nuclear type II binding sites has important implications with respect to potential cell growth regulation by these compounds. A number of studies from our laboratory have shown that the stimulation of nuclear type II binding sites in the rat uterus by estrogenic hormones is highly correlated with cellular hypertrophy, hyperplasia, and DNA synthesis (3-5). Therefore, we have suggested that nuclear type II binding sites may be involved in the modulation and/or regulation of cell growth. Further, we proposed that the ligand for nuclear type II sites may also be involved in cell growth regulation through a direct interaction with this binding site. Since studies in our laboratory have demonstrated that estradiol is unlikely to bind to nuclear type II sites in vivo because of its low binding affinity (\( K_d \approx 20 \) nM) for this protein (3-5), we have suggested the function of nuclear type II site is to bind the type II ligand rather than estradiol (1, 2). Nuclear type II site occupancy by the ligand is supported by our earlier experiments which demonstrate nuclear type II site measurement by ["H]estradiol exchange increases as uterine nuclei or cytosol fractions are diluted or stripped with charcoal. Furthermore, we have found that this nuclear dilution effect (i.e. dissociation of MeHPLA from type II sites) is estrogen dependent. Those data support a model in which nuclear type II site occupancy by MeHPLA results in a suppression of uterine growth and cell proliferation in the absence of estrogen. We propose that, following estrogen treatment, the binding affinity of nuclear type II sites for MeHPLA is decreased, perhaps by binding of the estrogen receptor-estradiol complex in the nucleus, such that MeHPLA dissociates from type II sites and cell inhibition is reversed.

On the basis of the data presented in this study with authentic HPLA and MeHPLA, it can be concluded that MeHPLA is the more important of these two ligands with respect to interaction with type II binding sites in vivo. Competition analysis revealed that MeHPLA interacts with nuclear type II sites with a 30-40-fold higher affinity than HPLA and the estimated \( K_d \) for the MeHPLA-type II binding interaction is approximately 5 nM (calculated from data presented in Fig. 9). However, it must be noted that this binding affinity is only approximate since it is estimated on the basis of ["H]estradiol binding data. Direct assessment of the binding of ["H]MeHPLA to nuclear type II sites must be performed to obtain a more accurate value. Nevertheless, it is apparent from the data presented in Fig. 9 that MeHPLA interacts with nuclear type II sites with a much greater binding affinity than HPLA or estradiol (3-5). Therefore, one would predict that MeHPLA may be the more significant compound with respect to potential effects on cell growth regulation. This appears to be the case. When we assessed the effects of the authentic compounds on MCF-7 human breast cancer cell growth, MeHPLA (but not HPLA) was capable of inhibiting cell proliferation at the concentrations tested (Fig. 11) and this effect was reversible following MeHPLA removal from the medium. Although the doses required for cell growth inhibition (1-10 \( \mu \)g/ml) appeared to be somewhat high, cell uptake studies with ["H]MeHPLA revealed that only 1% of the compound was taken up by MCF-7 cells under these experimental conditions. Therefore the calculated "intracellular concentrations" of MeHPLA in this experiment were approximately 10-100 ng/ml, which is in excellent agreement with the nuclear type II binding data (Fig. 9). On the basis of these observations, it is evident that the MeHPLA inhibition of MCF-7 cell growth probably occurs through an interaction with nuclear type II binding sites, although the mechanism remains to be determined. Failure of HPLA to inhibit MCF-7 cell growth (Fig. 11) is consistent with our observations that this compound interacts with nuclear type II sites with a 30-40-fold lower affinity than MeHPLA.

Similarly, HPLA was not as effective as MeHPLA in inhibiting estradiol stimulation of uterine growth in the immature rat. The data in Fig. 12 demonstrate that 10 \( \mu \)g of MeHPLA almost completely blocked estradiol stimulation of true uterine growth (assessed by comparing both wet and dry weight), whereas no significant response was obtained with 10 \( \mu \)g of HPLA. The antagonism observed with 100 \( \mu \)g of HPLA (Fig. 12) is consistent with our binding competition data, suggesting that HPLA will interact with nuclear type II sites at high concentrations. We suspect that complete antagonism of estrogenic response would have been observed with higher doses...
of HPLA. Since 10 µg of MeHPLA blocked uterine growth (Fig. 12) and has a 30-40-fold greater affinity for the type II site than HPLA, one would predict that approximately 300-400 µg of HPLA would be required to exert the same effect as MeHPLA on uterine growth. It is also possible that the inhibition activity obtained for HPLA in vivo is actually due to its partial conversion to MeHPLA. This relationship is currently under detailed study.

Our observation that HPLA and MeHPLA may function to regulate cell growth through an interaction with nuclear type II sites has important implications with respect to regulation of normal and malignant cell growth. At present, we do not know the precise biological origin of HPLA and MeHPLA in rat tissues since HPLA can be formed by metabolism of both bioflavonoids (9) and tyrosine (10-12). The relative contributions of these two pathways to the endogenous HPLA pool has not been clearly delineated. Likewise, methylation of HPLA to form MeHPLA has not been demonstrated, although MeHPLA has been isolated from human brain tissue (13) and we have identified MeHPLA in a variety of rat tissues. Interestingly, we have shown that MeHPLA is deficient in tumor cytosol preparations (2) which contain HPLA. These data suggest that tumors are capable of metabolizing MeHPLA. We suspect that the deficiency of MeHPLA in rat, mouse, and human mammary tumors (1, 2) may result from inactivation via conversion to HPLA. A variety of tumor preparations (15, 16) and MCF-7 human breast cancer cells (17) have been shown to contain significant levels of esterase activity which may be responsible for this conversion.

It is also possible that MeHPLA activity in the rat uterus may be under estrogenic regulation. Hochberg and co-workers have demonstrated that rat, rabbit, and bovine uteri contain significant levels of esterase activity which is likely to be responsible for lipoidal estrogen synthesis (18-22). Likewise, ongoing studies in our laboratory demonstrate that rat uterine cytosol contains an esterase which quantitatively converts MeHPLA to HPLA. Furthermore, this esterase appears to be under estrogen regulation, since MeHPLA hydrolysis is increased 4-fold in cytosol preparations from estradiol-injected rats (24 h following 10 mg of estradiol) as compared to vehicle-injected controls. It is tempting to speculate that uterin- esterases are under estrogen regulation and may be responsible for converting MeHPLA to HPLA, the form of the type II ligand with lower biological activity. Therefore, it is possible that estrogens modulate uterotropic response via regulation of the intracellular MeHPLA pools. A decrease in the concentration of MeHPLA in estrogen target cells is likely to lead to the expression of nuclear type II site function which is directly correlated with estrogenic stimulation of cellular hypertrophy, hyperplasia, and DNA synthesis (3-5). If this model is correct, one would predict that the deficiency of MeHPLA in malignant cells (2) may also be directly related to the uncontrolled rate of proliferation in these cell populations. Whether or not this deficiency results from high levels of tumor esterase activity or from defects in HPLA synthesis remains to be resolved. Our current data support the former hypothesis since we are unable to detect a deficiency of HPLA in tumor cell cytosol preparations (2). Therefore, the uncontrolled proliferation of malignant cells is directly related not only to a permanent stimulation of nuclear type II binding sites (2, 23-26), but also to very low to nonmeasurable levels of MeHPLA (2). Furthermore, the highest levels of type II sites measured in tumor nuclei may be due to a deficiency of MeHPLA, such that more free binding sites are available to be readily detected by [3H]estradiol exchange (2).

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