Synthesis of an Estrogen Receptor β -Selective Radioligand: 5-[¹⁸F]Fluoro-(2*R**,3*S**)-2,3-bis(4-hydroxyphenyl)pentanenitrile and Comparison of in Vivo Distribution with 16 α -[¹⁸F]Fluoro-17 β -estradiol

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Received February 8, 2005

Estrogen receptor β (ER β), a less active ER subtype that appears to have a restraining effect on the more active ERa, could be a factor that determines the level of estrogen action in certain estrogen target tissues. ER β is found in breast cancer, and its levels relative to ER α decline with disease progression. Thus, the independent quantification of ER α and ER β levels in breast cancer by imaging might be predictive of responses to different hormone therapies. To develop an imaging agent for ER β , we synthesized a fluoroethyl analogue of DPN (2,3-bis(4hydroxyphenyl)propanonitrile), a known ER β -selective ligand. This analogue, FEDPN (5-fluoro- $(2R^*, 3S^*)$ -2,3-bis(4-hydroxyphenyl)pentanenitrile), has an 8.3-fold absolute affinity preference for ER_β. [¹⁸F]Fluoride-labeled FEDPN was prepared from a toluenesulfonate precursor, which provided [18F]FEDPN with a specific activity greater than 3100 Ci/mmol after HPLC purification. Biodistribution studies in immature female rats using estradiol as a blocking agent revealed specific uptake of $[^{18}F]$ FEDPN in the uterus and ovaries. Experiments using ER α and $ER\beta$ -knockout mice demonstrated the expected $ER\alpha$ -subtype dependence in the tissue uptake of the known 16α -[¹⁸F]fluoro- 17β -estradiol ([¹⁸F]FES), which has a 6.3-fold preference for ERa. The tissue uptake of [¹⁸F]FEDPN in the ER knockout mice showed some evidence of mediation by $ER\beta$, but the levels of specific uptake of this agent were relatively modest. Based on our results, imaging of ER α can be done effectively with [¹⁸F]FES, but imaging of ER β will likely require agents with more optimized $\text{ER}\beta$ binding affinity and selectivity than [¹⁸F]FEDNP.

Introduction

The estrogen receptor (ER), a ligand-regulated transcription factor, is the principal mediator of genomic responses to estrogens, and it plays an important regulatory role in reproductive and other tissues.¹ Tumors derived from estrogen target tissues, such as breast tumors, often retain receptors for estrogens and in many cases are sensitive to antiestrogens, such as tamoxifen and raloxifene, and assays of ER levels in tumors are used to assess the prospects for a favorable clinical response to hormone therapy.^{2,3} Previously, we demonstrated that ER-positive breast tumors can be imaged by positron emission tomography (PET) using F-18-labeled estrogens,^{4–7} and that these images can be used to provide an early assessment of response to hormone therapy.^{8,9}

Even though it was originally believed that there was only one ER, a second ER gene was reported in 1996.¹⁰ This gene is called ER β to distinguish it from the longknown ER, which is now called ER α . ER α and ER β subtypes have rather different tissue distributions.^{11–13} Tissues that have high ER β levels include prostate, testes, ovaries, gastrointestinal tract, lung, bladder, hematopoetic and central nervous systems, and certain regions of the brain, whereas ER α predominates in the uterus, breast, kidney, liver, and heart. Many tissues contain both ER α and ER β , such as breast, epididymis, thyroid, adrenal, bone, and certain other regions of the brain.¹⁴

The response of ER α and ER β to estrogens of different structure can vary considerably. In addition, because the gene transcriptional activity of ER β is generally less than that of ER α , ER β is thought to act as a proliferative "brake" on ER α .¹⁵ Thus, the relative levels of ER α vs ER β in a particular tissue are likely to determine how much activity an estrogen will have in that tissue and how it will respond to ligands of different structures.

Although normal breast tissue contains both ER α and ER β , in breast tumors, ER β levels decline relative to ER α as this disease progresses and the cancer becomes more malignant.^{16–20} Thus, an independent determination of ER α and ER β levels in breast tumors might provide a useful molecular assessment of the stage of the disease and its sensitivity to various endocrine therapies, information that could help in the selection of the best alternative therapies for an individual breast cancer patient. It would be particularly convenient if this quantification of ER α and ER β levels could be done noninvasively by PET imaging.

We have recently found that 2,3-bis(4-hydroxyphenyl)propionitrile (DPN) is an agonist for both ER subtypes, but has a 70-fold higher relative binding affinity (RBA, that is, ER β vs ER α binding relative to the affinity of estradiol) and 78-fold higher potency (based

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Table 1. Relative Binding Affinities (RBA)^a of DPN Derivatives for the ERa and ER β^{21}



^{*a*} Determined by a competitive radiometric biding assay with [³H]estradiol using full-length human ER α and ER β . Values are reported as the mean \pm SD. ^{*b*} These ER α /ER β ratios are corrected for the fact that the tracer, estradiol, binds to the two ER subtypes with different affinities (K_d (ER α) = 0.2 nM; K_d (ER β) = 0.5 nM).

on EC₅₀ values) in transcription assays with ER β .²¹ To further investigate the ER β affinity-selective charac-



teristic of DPN, we prepared a series of DPN analogues (Table 1).²¹ Because of their very high ER β affinity preference, DPN and its analogues appeared to be good starting points for development of radiopharmaceuticals for imaging only ER β . Fluorine-18 is an excellent radionuclide for in vivo imaging with PET.²²

Herein, we report the synthesis of 5-[¹⁸F]fluoro-(2 R^* ,3 S^*)-2,3-bis(4-hydroxyphenyl)pentanenitrile ([¹⁸F]-FEDPN), its labeling with F-18 and an assessment of its selectivity for in vivo imaging of ER β . Tissue distribution studies of [¹⁸F]FEDPN were performed in rats as well as in mice in which either ER α or ER β had been knocked out, and its uptake in these mice is compared with that of the known (and ER α preferential) imaging agent 16-[¹⁸F]fluoro-17 β -estradiol ([¹⁸F]FES). In these animal models, the patterns and time course of tissue distribution of both [¹⁸F]FEDNP and of [¹⁸F]-FES were found to reflect, to some degree, their affinity preferences for ER β and ER α , respectively. The ER α selective uptake of [¹⁸F]FES, however, is more pronounced than the ER β -selective uptake of [¹⁸F]FEDNP.

Results

Synthesis of an Unlabeled Standard of FEDPN. From a series of DPN analogues (Table 1) with good $\text{ER}\beta/\text{ER}\alpha$ selectivity, $(2R^*,3S^*)$ -5-fluoro-2,3-bis(4-hydroxyphenyl)pentanenitrile (5) was chosen as a target compound for $\text{ER}\beta$ imaging. It is a fluorine analogue of $(2R^*,3S^*)$ -2,3-bis(4-hydroxyphenyl)pentanenitrile (R = Et in Table 1), which has a relative binding affinity (RBA) value of 17 ± 5 for ER α and 75 ± 6 for ER β (estradiol = 100 for both ER α and ER β).

Scheme 1^a



 a Reagents and conditions: (a) NaOMe, EtOH; (b) CH₂=CHMgBr, CuI, THF; (c) i) BH₃·THF, ii) NaOH, H₂O₂; (d) DAST, CH₂Cl₂; (e) BBr₃, CH₂Cl₂, -76 °C.

It is of note that the fluorine analogue of DPN itself, 3-fluoro-2,3-bis(4-hydroxyphenyl)propanenitrile, was not selected because it was expected to undergo rapid defluorination under physiological conditions by a facile elimination mechanism.^{23–25} The cyanide group in the fourth DPN analogue could be labeled by C-11 (R = CN in Table 1); however, the half-life of C-11 (20 min) was considered to be too short for breast cancer imaging.

In our first approach, the methoxy-protected phenol compounds 4-(methoxy)phenylacetonitrile and p-anisaldehyde were used as starting materials to prepare the target compound, (2R*,3S*)-5-fluoro-2,3-bis(4-hydroxyphenyl)pentanenitrile (Scheme 1). α -Cyanostilbene 1a was prepared by the condensation of the arylaldehyde and arylacetonitrile in quantitative yield by modification of a previous method.²¹ The product that precipitated was collected on a glass frit and washed by ethanol. The filtrate was concentrated under vacuum in order to remove water produced during condensation reaction, dry EtOH was added up to an original solvent volume when necessary, and the same amount of NaOMe was added into the mixture as new base. The second precipitate was collected and washed by ethanol. In this manner, the yield was quantitative, which is much higher than that previously reported (72%).

The vinyl group was introduced at β -position with high diastereoselectivity by the conjugate addition of the Grignard reagent in the presence of CuI. Pure erythro diastereomer was obtained by repeated recrystallization (1 h) from refluxing ethyl acetate/hexane (1:1). Stereochemical assignment was based on spectroscopic analogy to the β -ethyl analogue²¹ (R = Et in Table 1). Hydroboration of the vinyl group with BH₃·THF followed by oxidation gave the primary alcohol, $(2R^*, 3S^*)$ -5-hydroxy-2,3-bis(4-methoxyphenyl)pentanenitrile (3a), in moderate yield. The alcohol group of compound 3a was then replaced by fluoride directly using (diethylamino)
sulfur trifluoride (DAST) to give $(2R^{\ast}\!,\!3S^{\ast}\!)\text{-}5\text{-}$ fluoro-2,3-bis(4-methoxyphenyl)pentanenitrile (4a). However, attempts to deprotect the methyl ether to give unlabeled FEDPN (5) failed to do so without also

Scheme 2^a



 a Reagents and conditions: (a) NaOMe, EtOH, reflux; (b) CH₂=CHMgBr, CuI, THF; (c) i) BH₃·THF, ii) NaOH, H₂O₂; (d) DAST, CH₂Cl₂; (e) H₂, Pd/C.

cleaving the C–F bond (Reagents including BBr_{3} ,^{26,27} BF_{3} , $AlCl_{3}$,²⁸ pyridine·HCl,^{29,30} and $CH_{3}SO_{3}H^{31}$ were tried under various reaction conditions.) Thus, we decided to change to a more easily cleaved protecting group.

In our second synthesis (Scheme 2), a benzyl group was chosen to protect the phenol because it can easily be removed by hydrogenation. The condensation reaction between 4-(benzyloxy)benzaldehyde and 4-(benzyloxy)phenylacetonitrile was carried out in refluxing ethanol because of low initial solubility at room temperature. The yield was increased to nearly quantitative by continuous removal of water and addition of more base. The vinyl group was introduced at β -position using vinyl Grignard reagent with copper catalysis, and pure erythro-2b was obtained by repeated recrystallization (1 h) from refluxing ethanol. Hydroboration followed by oxidation and fluorination of primary alcohol were achieved by employing similar reaction conditions as those in Scheme 1 to give $(2R^*, 3S^*)$ -2,3-bis(4-(benzyloxy)phenyl)-5-fluoropentanenitrile (4b). Deprotection of the benzyl groups was successfully achieved by a typical hydrogenolysis reaction over Pd/C catalyst to give $(2R^*, 3S^*)$ -5-fluoro-2,3-bis(4-hydroxyphenyl)pentanenitrile (5). Ethyl acetate was used as a solvent because of the very poor solubility of compound **4b** in alcohol. The presence of fluorine was clearly seen from ¹H and ¹³C NMR data through the coupling of fluorine with the proton and carbon-13 signals. Compound 5 was further characterized by high-resolution mass spectrometry.

Even though phenol benzyl ether could be cleaved under mild conditions, the deprotection took 4 h. The benzyl group is, therefore, not optimal as a phenol protecting group for labeling FEDPN with F-18, because of the relatively short half-life of F-18 ($t_{1/2} = 110$ min). Of various other phenol protecting groups such as methoxymethyl, methanesulfonate, and *tert*-butyldimethylsilyl, the methoxyethoxymethyl (MEM) group was selected because it is stable under base conditions, yet can be removed very rapidly under relatively mild acid conditions.³² Scheme 3^a



 a Reagents and conditions: (a) BBr₃, CH₂Cl₂, 0 °C; (b) MEMCl, $iPr_2Net, CH_2Cl_2;$ (c) i) BH₃·THF, ii) NaOH, H₂O₂; (d) TsCl, py, CH₂Cl₂; (e) DAST, CH₂Cl₂.

The methyl ethers in compound **2a** were cleaved by overnight treatment of BBr₃, affording $(2R^*, 3S^*)$ -2,3bis(4-hydroxyphenyl)pent-4-enenitrile (6) in quantitative yield (Scheme 3). The free phenol groups in 6 then reprotected with MEM groups, using MEM chloride and N,N-diisopropylethylamine, to give $(2R^*, 3S^*)$ -2,3bis(4-((2-methoxy)methoxy)phenyl)pent-4-enenitrile (7). Hydroboration with BH_3 ·THF followed by treatment with aqueous NaOH/H₂O₂ converted the alkene in this compound to the terminal alcohol. The hydroxy group in 8 was converted to the tosylate group by treatment with *p*-toluenesulfonyl chloride and pyridine to give $(3R^*, 4S^*)$ -3,4-bis(4-((2-methoxy)methoxy)phenyl)-4-cyanobutyl 4-methylbenzenesulfonate (9), which is ready for labeling with $[^{18}F]$ fluoride ion. The hydroxy group in compound 8 was converted to fluorine using DAST to give (2R*,3S*)-2,3-bis(4-((2methoxyethoxy)methoxy)phenyl)-5-fluoropentanenitrile (10), which is used as standard for $[^{18}F]$ 10.

Binding Affinities of FES and FEDPN. Binding affinities of FES and FEDPN were determined by a competitive radiometric binding assay with [³H]estradiol as tracer, using purified full-length human ER α and ER β , as previously described.^{33,34} Affinities from these competitive binding assays are expressed as relative binding affinity (RBA) values, that is, relative to the affinity of estradiol, which is 100% by definition (Table 2).

One should note that affinity calculations based on relative binding affinity (RBA) values (i.e., compared to estradiol) do not reflect the fact that the tracer, estradiol, binds to ER α and ER β with somewhat different affinities (K_d : 0.2 nM for ER α and 0.5 nM for ER β). Therefore, in both Tables 1 and 2 we express the binding affinity ratios both in terms of relative affinities (i.e., based on RBA values, left) and absolute affinities (right), the latter being corrected for the 2.5-fold difference in estradiol affinity for ER α vs ER β .

The binding affinity of FES is relatively high for both ER α and ER β subtypes, being in the nanomolar range.

Table 2. Relative Binding Affinities $(RBA)^a$ of FES and FEDPN

| | | RBA (%) ^a | | absolute |
|---------------------------|--------------------------------------------------------------------|---------------------------------------------------------------------|------------------------|---------------------------------------|
| ligand | ERα | $\mathrm{ER}eta$ | $ER\alpha/ER\beta$ | $ER\alpha/ER\beta$ ratio ^b |
| estradiol FES FEDPN | $\begin{array}{c} 100 \\ 54.9 \pm 15 \\ 0.42 \pm 0.09 \end{array}$ | $\begin{array}{c} 100 \\ 22.0 \pm 1.6 \\ 8.74 \pm 1.87 \end{array}$ | [1.00] 2.5 0.048 | 2.5^b 6.3 0.12 |

^{*a*} Values are reported as the mean range. ^{*b*} These ER α /ER β ratios are corrected for the fact that the tracer, estradiol, binds to the two ER subtypes with different affinities (K_d (ER α) = 0.2 nM; K_d (ER β) = 0.5 nM).

Scheme 4^a





 a Reagents and conditions: (a) $^{18}{\rm F}^-,$ K_2CO3, K222, MW; (b) 3M HCl, 110 °C, 5 min.

FES showed 2.5 times higher RBA selectivity for ER α over ER β (55 vs 22), thus giving it a 6.3-fold absolute affinity preference for ER α .

The affinity of FEDPN for ER α is less than 0.5% that of estradiol, while its affinity for ER β is around 10% of estradiol, giving an ER β affinity selectivity of 21 based on RBA comparison or 8.3 in terms of absolute affinities. Thus, the fluorine substituent decreased the binding affinity considerably compared to the non-fluorine substituted analogue, β -ethylated DPN (R = Et in Table 1), which has an RBA of 75 for ER β and 17 for ER α , corresponding to an ER β RBA preference of only 4.²¹ Thus, although the RBA of FEDPN is about 10-fold less on ER β compared to that of β -ethylated DPN, its affinity for ER α is even more reduced, giving it an RBA selectivity for ER β of 21, which is actually higher than that of the ethyl-substituted analogue.

In general, an increase in steric bulk at the β position next to the nitrile in DPN analogues (Table 1) tends to raise ligand binding affinity for both receptors while decreasing ER β /ER α selectivity ratio. However, addition of a fluorine substituent, considered to be between hydrogen and methyl in size,³² onto the β ethyl group causes a reverse effect, lowering RBA for both ER subtypes but raising ER β selectivity. This is likely the result of introducing a more polar function near the center of the ligand.

Because of its reasonable ER β /ER α selectivity (21fold based on relative affinities, 8.3, based on absolute affinities), we labeled FEDPN with fluorine-18, with the objective of developing a PET imaging agent that would be specific for ER β .

Radiochemical Synthesis of [¹⁸**F**]**FEDPN.** Scheme 4 illustrates the microwave-mediated two-step radiosynthesis of [¹⁸F]FEDPN. Fluorine-18 obtained from proton bombardment of oxygen-18 enriched water was treated by ion-exchange resin (BioRad AG-1 \times 8) to produce metal ion-free fluoride, and the ¹⁸O-water was recycled. This material was converted to [¹⁸F]KF/ Kryptofix-222 by the addition of Kryptofix-222 and careful azeotropic drying with acetonitrile. The activity was then resolubilized in MeCN and transferred to a Pyrex tube containing diastereomerically pure tosylate precursor **9**.

The nucleophilic substitution of the tosyl group by [¹⁸F]fluoride ion was optimized by varying the fluoride source, the amount of substrate and the microwave irradiation time. The incorporation yield was determined by radio-TLC where the R_f value of [¹⁸F]**10** matched well with that of the unlabeled compound **10** (0.49 vs 0.50). The substrate **9** was very sensitive to base and degraded quickly with heating during fluorination. Heating in a specially designed microwave cavity for 35 s resulted in the highest labeling yield, whereas longer heating or the repeated cycles of irradiation and cooling decreased incorporation. Microwave dielectric heating is known to give cleaner reaction mixtures because of decreased reactant decomposition and shorter reaction time compared to conventional heating.³⁵

The fluoride source was critical to effective labeling. Maximal incorporation yield was less than 4% when non-ion exchange-treated water was used as the ¹⁸F ion source. The tiny amount of metal ions in nontreated water appeared to accelerate decomposition of the substrate before displacement of tosylate by fluoride ion. When nontreated water was used, more than 70% of the toluenesulfonate precursor was degraded right after the incorporation reaction. (as determined by HPLC) When less than 2 mg of substrate was used, incorporation yield also decreased significantly compared to using more than 3 mg of substrate (7.2 ± 3.0% vs 23.0 ± 6.9%), presumably also due to decomposition of substrate.

After the replacement of tosylate by fluorine-18 ion, the MEM protecting groups were deprotected within 3 min by treatment with 3 M HCl at 110 °C to give final product [¹⁸F]FEDPN. The $R_{\rm f}$ value (0.58) of [¹⁸F]FEDPN determined by radio-TLC matched exactly that of unlabeled **5**. The radiochemical yield decreased by about 30% (determined by radio-TLC) following deprotection, indicating cleavage of the C-F bond during the acid hydrolysis. Only the peaks for [¹⁸F]FEDPN and free fluoride were seen on radio-TLC.

Solid-phase purification to remove free fluoride was performed by using an OASIS HLB cartridge, as described previously.³⁶ Purification of the final tracer was accomplished by reverse phase HPLC. The desired fractions were collected by monitoring the radioactivity trace, since the UV absorption of the final product [¹⁸F]-FEDPN was below the detection limit. The collected fractions were evaporated under reduced pressure and then reconstituted in 10-20% ethanol/saline mixture for further studies.

The total radiosynthesis time including HPLC purification took less than 110 min, and it proceeded with a radiochemical yield of $3.4 \pm 3.0\%$ (n = 4, decay corrected). The overall radiochemical yield was low, but is in keeping with multistep radiofluorination reactions.³⁷ The radiochemical purity determined by reverse phase analytical HPLC was greater than 99%. Based on the detection limit measurement of cold FEDPN mass by the UV detector, the specific activity was

Table 3. Biodistribution of $[1^{8}F]$ FEDPN in Immature Female Sprague–Dawley Rats^{*a*}

| organ | 30 min | 1 h | 1 h block | 2 h |
|-------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| blood lung liver spleen | $\begin{array}{c} 0.64 \pm 0.31 \\ 0.71 \pm 0.20 \\ 1.42 \pm 0.29 \\ 0.31 \pm 0.10 \end{array}$ | $\begin{array}{c} 0.55 \pm 0.20 \\ 0.44 \pm 0.10 \\ 1.51 \pm 0.15 \\ 0.16 \pm 0.04 \end{array}$ | $\begin{array}{c} 0.30 \pm 0.10 \\ 0.27 \pm 0.10 \\ 0.76 \pm 0.17 \\ 0.10 \pm 0.05 \end{array}$ | $\begin{array}{c} 0.24 \pm 0.07 \\ 0.19 \pm 0.07 \\ 0.62 \pm 0.14 \\ 0.06 \pm 0.01 \end{array}$ |
| kidney muscle fat heart bone uterus ovaries thymus | $\begin{array}{c} 2.00 \pm 0.60 \\ 0.57 \pm 0.14 \\ 0.57 \pm 0.22 \\ 0.49 \pm 0.14 \\ 2.12 \pm 0.49 \\ 0.95 \pm 0.23 \\ 0.93 \pm 0.33 \\ 0.52 \pm 0.09 \end{array}$ | $\begin{array}{c} 1.75 \pm 0.37 \\ 0.22 \pm 0.00 \\ 0.37 \pm 0.16 \\ 0.29 \pm 0.07 \\ 2.22 \pm 0.26 \\ 0.74 \pm 0.29 \\ 0.67 \pm 0.18 \\ 0.29 \pm 0.11 \end{array}$ | $\begin{array}{c} 1.01 \pm 0.38 \\ 0.20 \pm 0.08 \\ 0.21 \pm 0.14 \\ 0.19 \pm 0.08 \\ 1.82 \pm 0.44 \\ 0.34 \pm 0.15 \\ 0.26 \pm 0.11 \\ 0.16 \pm 0.07 \end{array}$ | $\begin{array}{c} 0.97 \pm 0.21 \\ 0.09 \pm 0.01 \\ 0.10 \pm 0.06 \\ 0.11 \pm 0.02 \\ 1.88 \pm 0.11 \\ 0.28 \pm 0.11 \\ 0.21 \pm 0.06 \\ 0.08 \pm 0.01 \end{array}$ |

 a Data are expressed as the %ID/g \pm SD with five animals per data point.

calculated to be >3100 Ci/mmol (by UV absorption) at the end of synthesis.

Biodistribution Studies Using Immature Female Sprague–Dawley Rats. Immature female rats are useful for screening estrogen receptor ligands because they have low endogenous estrogens and yet have ERrich target tissues (uterus and ovaries).³² By using immature female rats (<25 days old), the effect of endogenous estradiol can be essentially obviated because the estrus cycle in rat begins around 30 days after birth.³⁸ Radiochemically pure [¹⁸F]FEDPN ([¹⁸F]5), reconstituted in 20% ethanol/saline, was injected into immature female Sprague–Dawley rats. Due to the low solubility of estradiol in aqueous solution, 20% ethanol/ saline (150 μ L) was used to dissolve estradiol (8 μ g) for the blocking study.

Table 3 presents the uptake of [¹⁸F]FEDPN in selected tissues of immature female Sprague–Dawley rats at 30 min, 1 h (control and block by E_2) and 2 h (n = 5 at each time point). Highest uptake was found in bone at all time points including in estradiol blocked animals, indicative of the uptake of free [¹⁸F]fluoride ions produced by the in vivo metabolism of the radio–labeled compound.^{37,39} Bone activity remained within the standard deviations at the three time points, indicating that degradation of [¹⁸F]FEDPN occurred within 30 min. The percentage of injected dose per gram (%ID/g) in the kidney was higher than that in liver at all three time points.

Except for these three tissues, the highest %ID/g was found in the estrogen receptor-rich organs such as ovaries and uterus. The uptake of [¹⁸F]FEDPN in the uterus at 30 min was comparable with that of ovaries at 30 min (0.95 vs 0.93), and slightly higher than that of ovaries at 1 and 2 h. The uterus/muscle ratio was 1.7 at 30 min and increased to more than 3 at 1 and 2 h time points. The ovary/muscle ratio was 1.6 at 30 min, 3.1 at 1 h and 2.3 at 2 h. The uterus/blood ratio was highest at 30 min (1.5) and decreased to 1.4 at 1 h and 1.2 at 2 h, while the ovaries/blood ratio was 1.5 at 30 min and 1.2 at 1 h, but dropped below 1 at 2 h postinjection. Even though the uterus and ovary uptake is not high, ratios with muscle and blood are suggestive of the fact that [¹⁸F]FEDPN uptake in these tissues might be the result of its binding to the estrogen receptor.

More direct evidence of the estrogen receptor-mediated uptake of [¹⁸F]FEDPN comes from comparisons with uptake in estradiol-blocked rats (Figure 1). Estradiol blocked the uptake of [¹⁸F]FEDPN in the estrogen receptor-rich organs; uterus and ovary were blocked by 54% and 61%, respectively, which is much greater than the blockage in ER-negative tissues, such as the 9% and 18% decrease in muscle and bone, respectively. However, clearance organs such as liver and kidney also showed lower uptake in the blocking study compared to control (0.76 vs 1.51 in liver; 1.01 vs 1.75 in kidney). Although the blocking effects are modest, the results of this study are consistent with the uptake of [¹⁸F]FEDPN in estrogen-rich organs being mediated by specific receptor binding; the modest level of these effects is consistent with the limited binding affinity of this compound for the ERs.

Biodistribution Studies Using Estrogen Receptor Knockout Mice. To determine whether the tissue distribution of [¹⁸F]FEDPN reflects its preferential binding affinity for the ER β subtype, a second biodistribution study was carried out using two different sets of mice. In these studies, uptake of [¹⁸F]FES, an established ER imaging agent, was compared to uptake of [¹⁸F]FEDPN, the ER β preferential ligand.

The estrogen receptor- knock-out (α ERKO) mouse model was created by disrupting the ER α gene by gene



Figure 1. Comparison of tissue biodistribution data of [18F]FEDPN in control rats vs estradiol-blocked rats at 1 h postinjection.

| Table 4. | Biodistribution | of [18F]FES ir | n αERKO and | βERKO Mice ^a |
|----------|-----------------|---------------------------------------|-------------|-------------------------|
| | | · · · · · · · · · · · · · · · · · · · | | |

| | αΕRΚΟ | | | β ERKO | | | |
|---------|-----------------|-----------------|-----------------|----------------------------------------|-----------------|-----------------|--------------------|
| organ | 30 min | 1 h | 2 h | $\overline{30 \text{ min diestrus}^b}$ | $30 \min^c$ | $1 h^c$ | $2 \ \mathrm{h}^c$ |
| blood | 0.65 ± 0.15 | 0.21 ± 0.02 | 0.12 ± 0.01 | 0.73 ± 0.15 | 0.67 ± 0.16 | 0.30 ± 0.01 | 0.16 ± 0.04 |
| lung | 2.19 ± 0.52 | 0.39 ± 0.08 | 0.17 ± 0.02 | 3.57 ± 0.57 | 3.74 ± 0.40 | 1.48 ± 0.22 | 0.54 ± 0.08 |
| liver | 7.47 ± 1.32 | 5.24 ± 0.19 | 3.67 ± 0.85 | 7.91 ± 0.87 | 9.45 ± 2.49 | 5.85 ± 1.62 | 4.54 ± 1.49 |
| spleen | 0.59 ± 0.14 | 0.19 ± 0.02 | 0.18 ± 0.10 | 1.29 ± 0.14 | 1.40 ± 0.19 | 0.91 ± 0.21 | 0.37 ± 0.04 |
| kidney | 2.46 ± 0.46 | 1.07 ± 0.12 | 0.72 ± 0.07 | 4.44 ± 0.67 | 3.83 ± 0.61 | 2.14 ± 0.37 | 1.05 ± 0.23 |
| muscle | 0.77 ± 0.27 | 0.18 ± 0.05 | 0.11 ± 0.04 | 1.89 ± 0.41 | 1.70 ± 0.15 | 1.29 ± 0.27 | 0.54 ± 0.07 |
| fat | 1.51 ± 0.36 | 0.14 ± 0.03 | 0.09 ± 0.01 | 2.31 ± 0.39 | 2.01 ± 0.15 | 1.07 ± 0.25 | 0.41 ± 0.05 |
| heart | 0.62 ± 0.15 | 0.15 ± 0.03 | 0.10 ± 0.01 | 0.95 ± 0.09 | 1.04 ± 0.20 | 0.46 ± 0.04 | 0.20 ± 0.04 |
| brain | 0.41 ± 0.05 | 0.07 ± 0.04 | 0.03 ± 0.01 | 0.81 ± 0.26 | 0.71 ± 0.10 | 0.35 ± 0.03 | 0.14 ± 0.02 |
| bone | 0.44 ± 0.05 | 0.24 ± 0.06 | 0.23 ± 0.12 | 1.26 ± 0.24 | 1.28 ± 0.17 | 1.08 ± 0.16 | 0.53 ± 0.10 |
| uterus | 2.40 ± 0.49 | 0.82 ± 0.25 | 0.36 ± 0.03 | 18.86 ± 0.11 | 8.34 ± 2.91 | 9.81 ± 3.42 | 9.23 ± 3.04 |
| ovaries | 2.52 ± 0.42 | 0.49 ± 0.04 | 0.18 ± 0.01 | 6.60 ± 2.68 | 6.54 ± 0.58 | 5.61 ± 1.29 | 3.40 ± 0.79 |
| thymus | 0.68 ± 0.18 | 0.21 ± 0.02 | 0.10 ± 0.01 | 1.65 ± 0.37 | 1.38 ± 0.19 | 0.92 ± 0.14 | 0.38 ± 0.07 |

^{*a*} Data are expressed as the $\text{MID/g} \pm \text{SD}$ with three animals per data point. ^{*b*} Mice were in nonestrus stage (n = 2). ^{*c*} Mice were in estrus (n = 3 per each time point).

| Table 5. Uptake Ratios of β ERKO/ α ERKO of [¹⁸ F]FES and [¹⁸ F]FEDPN in Uterus and O | varies |
|-----------------------------------------------------------------------------------------------------------------------------------|--------|
|-----------------------------------------------------------------------------------------------------------------------------------|--------|

| | βER | β ERKO/ α ERKO in uterus ^c | | | β ERKO/ α ERKO in ovaries ^c | | | |
|---------------------------------------------------------------------|-----------------------------------------------------------------------|----------------------------------------------------------------------|----------------------------------------------------------------------|-----------------------------------------------------------------------|----------------------------------------------------------------------|-----------------------------------------------------------------|--|--|
| ligand | 30 min | 1 h | 2 h | 30 min | 1 h | 2 h | | |
| ${[^{18}F]FES^{a}} {[^{18}F]FEDPN^{b}} {[^{18}F]FES/[^{18}F]FEDPN}$ | $\begin{array}{c} 3.48 \pm 0.57 \\ 1.67 \pm 0.29 \\ 2.08 \end{array}$ | $\begin{array}{c} 12.0 \pm 1.4 \\ 1.46 \pm 0.32 \\ 8.22 \end{array}$ | $\begin{array}{c} 25.4 \pm 1.8 \\ 1.29 \pm 1.03 \\ 19.7 \end{array}$ | $\begin{array}{c} 2.60 \pm 0.26 \\ 1.32 \pm 0.25 \\ 1.97 \end{array}$ | $\begin{array}{c} 11.6 \pm 1.8 \\ 0.79 \pm 0.13 \\ 14.7 \end{array}$ | $\begin{array}{c} 18.6\pm 3.3\\ 0.61\pm 0.28\\ 30.5\end{array}$ | | |

^{*a*} α α ERKO mice were in diestrus but β ERKO mice were in estrus. ^{*b*} All mice were in diestrus. ^{*c*} The ratios of β ERKO/ α ERKO of [¹⁸F]FES and [¹⁸F]FEDPN in uterus and ovaries are expressed as the mean \pm SD.

targeting. The ER β gene remains functional in these animals;⁴⁰ however, female α ERKO mice have hypoplastic uteri and hyperemic ovaries with no apparent corpora lutea, and they are infertile.⁴⁰ Later, the ER β knock-out (β ERKO) mouse model was generated using a similar gene targeting method. The ER α gene remains functional in these animals,⁴¹ and the uteri of female β ERKO mice appear to be anatomically normal compared to α ERKO.⁴²

In analyses below, we have frequently used as a measure of the ER subtype selectivity of the uptake, the ratio of uptake of a compound in the two ER knockout animal models: When the α ERKO/ β ERKO ratio > 1, it highlights preferential interaction with ER β (because the α ERKO animals contain only ER β , whereas the β ERKO animals contain only ER α); by contrast, when the inverse ratio β ERKO/ α ERKO > 1, it highlights preferential interaction with ER α .

Tissue Distribution of [¹⁸F]FES in ERKO Mice. As a first step, we used [¹⁸F]FES to see how alteration of the levels of the ER subtypes in these knockout mice would affect in vivo uptake of an ER ligand whose distribution pattern in other species, including humans, had been well characterized.^{38,43-46} As expected from its 6.3-fold ERα absolute affinity selectivity, [¹⁸F]FES uptake in βERKO animals was higher than that in αERKO mice in all selected organs (Table 4). The highest %ID/g was observed in the uterus of diestrus βERKO mice at 30 min postinjection (18.9 ± 0.1%ID/ g).

As described in the Experimental Section in detail, only two β ERKO mice were in diestrus for the biodistribution study at 30 min postinjection. All other biodistribution studies with β ERKO mice were done during estrus because of experimental complications, primarily false pregnancy presumably induced by repeated swab testing. For all organs except the uterus, uptake of [¹⁸F]-FES was similar at 30 min in both estrus and diestrus β ERKO mice. The %ID/g in the uterus of β ERKO mice during estrus was roughly half that of during diestrus, though still very high (8.34 ± 2.91 vs 18.9 ± 0.1). This is similar to our earlier studies of the effect of estrus on radioestrogen biodistribution in rats.⁴⁷ In case of 16α-[¹²⁵I]iodoestradiol, uterus/blood and uterus/nontarget ratios at 1 h postinjection were about 3-times higher during diestrus than during estrus.⁴⁷

All biodistribution studies in α ERKO mice, which lack the ERa subtype to which FES binds preferentially, were done in diestrus. Thus, not surprisingly, because they lack ERa, uterine uptake in α ERKO mice at 30 min is 7.9-fold lower than that in β ERKO mice in diestrus (2.40 \pm 0.49 vs 18.9 \pm 0.1). While the uterine uptake of [¹⁸F]FES in β ERKO mice in estrus was high and increased at 1 h (8.3 at 30 min; 9.8 at 1 h), that in α ERKO mice was lower, peaking at 30 min and washing out by 2 h.

The β ERKO/ α ERKO ratio of uterine uptake of [¹⁸F]-FES increased from 3.5 ± 0.6 at 30 min to 12.0 ± 1.4 at 1 h and 25.4 ± 1.8 at 2 h, consistent with ER α being the dominant factor determining [¹⁸F]FES uptake in this organ (Table 5). Similarly, ovary uptake of [¹⁸F]-FES in β ERKO mice (in estrus) peaked at 30 min (6.5 ± 0.6%ID/g) and then slowly decreased (5.6 ± 1.3 at 1 h; 3.4 ± 0.8 at 2 h), whereas in α ERKO mice, ovary uptake rapidly decreased, resulting in a higher β ERKO/ α ERKO ratio at later time points (2.6 ± 0.3 at 30 min; 11.6 ± 1.8 at 1 h; 18.6 ± 3.3 at 2 h).

Interestingly, there was no significant difference in ovary uptake of [¹⁸F]FES in estrus and diestrus β ERKO mice (6.5 ± 0.6 vs 6.6 ± 2.7%ID/g). All other organs but one also showed comparable activity uptake both in diestrus and in estrus; only uterus uptake of [¹⁸F]FES seemed to be highly dependent on the stage of the estrous cycle. The strong dependence of uterus uptake on estrous cycling could be attributed to variations in endogenous ER ligand concentrations, mostly estradiol.

Table 6. Serum Estradiol Levels in BALB/c in Diestrus and $\beta {\rm ERKO}$ in Estrus

| mouse | concentrated of $estradiol^a (pg/mL)$ |
|--------------------------------------------|-------------------------------------------------------------|
| BALB/c (diestrus) β ERKO (estrus) | $\begin{array}{c} 28.7 \pm 5.7 \\ 43.8 \pm 3.1 \end{array}$ |

 a Values are reported as the mean \pm SD with three samples per mouse.

We measured the circulating level of estradiol in the serum of control BALC/c mice and β ERKO mice in estrus (Table 6, n = 3 each). Serum was collected from all mice, and samples were sent to an outside laboratory (AniLytics, Inc., Gaithersburg, MD) for determination of estradiol (E2) levels. 17β -Estradiol is not a species specific hormone; therefore, the radioimmunoassay was performed using commercially available reagents for testing human serum (MP Biomedicals). As the assay is run, both free and protein-bound estradiol are measured in each serum sample.

Estradiol levels in β ERKO mice in estrus were 1.5 times higher than in BALB/c mice in diestrus (43.8 vs 28.7 pg/mL). The estradiol level in BALB/c mice (28.7 pg/mL) is comparable with that of α ERKO mice (29.5 pg/mL) and that of β ERKO mice (30.5 pg/mL).^{48,49} The serum estradiol level of β ERKO mice in estrus was much higher than the reported value in adult β ERKO females (43.8 vs 24.2 pg/mL).⁴⁸ Higher endogenous estradiol levels during estrus might effectively block the limited number of ER binding sites in uterus, accounting for decreases in uterine uptake during estrus compared to during nonestrus.

In organs other than uterus, ovaries and liver, the highest β ERKO/ α ERKO uptake ratio of [¹⁸F]FES was at 1 h postinjection. In the cases of muscle and fat, these ratios were above 7.2 at 1 h and 4.8 at 2 h. Spleen, brain, bone, and thymus also showed β ERKO/ α ERKO ratio of greater than 4 at 1 h. These sizable ratios for nominally estrogen nontarget tissues might reflect the fact that they contain small, but significant amounts of ER, as do, in fact, most tissues.^{12,48} Blood, liver, and kidney uptakes in β ERKO mice, however, were slightly higher than corresponding values in α ERKO mice at all time points. Compared to biodistribution of [¹⁸F]FES in immature female Sprague–Dawley rats,⁴⁴ both sets of ERKO mice showed higher blood retention at all time points, but faster clearance through liver and kidney.

Tissue Distribution of [¹⁸F]**FEDPN in ERKO Mice.** Comparisons of organ uptake of [¹⁸F]FEDPN in αERKO and βERKO mice are summarized in Table 7. FEDPN has a RBA of 8.7 with ERβ and 0.42 with ERα subtype, giving it an ERβ selectivity of 21 based on RBA values and an 8.3-fold ERβ selectivity based on absolute affinities (Table 2). Even though the RBA of FEDPN for ERβ is less than 10% of that of estradiol, because of its greatly reduced binding affinity for ERα (less than 0.5% than estradiol), different tissue distribution patterns are expected in αERKO versus βERKO mice.

In contrast to the ER α -preferential ligand [¹⁸F]FES, with which all uptake levels in β ERKO mice were higher than those in α ERKO mice, many organs showed higher uptake of [¹⁸F]FEDPN in α ERKO rather than β ERKO mice, particularly at later time points, consistent with the presence of ER β in the α ERKO animals and the preferential affinity of [¹⁸F]FEDPN for ER β . At 2 h postinjection, all organs except liver, brain, and uterus showed higher uptake in α ERKO. However, the α ERKO/ β ERKO uptake ratio was less than 2 in most organs except thymus, which could be attributed to the relatively low binding affinities of FEDPN for both ER subtypes.

The most striking difference in organ uptake between ^{[18}F]FES and ^{[18}F]FEDPN was found in uterus. In case of [¹⁸F]FES, the uterine β ERKO/ α ERKO uptake ratio increased from 3.5 ± 0.6 at 30 min to 12.0 ± 1.4 at 1 h and 25.4 \pm 1.8 at 2 h, even though the β ERKO mice were in estrus. Considering that uterine uptake of [¹⁸F]-FES in β ERKO mice during diestrus is more than twice that during estrus (18.9 vs 8.3%ID/g), the uterine β ERKO/ α ERKO uptake ratio in uterus might also be doubled during diestrus. By contrast, the β ERKO/ α ERKO uterine uptake ratio of [¹⁸F]FEDPN was always less than 2, and furthermore the ratio was decreased over time from 1.7 \pm 0.3 at 30 min to 1.5 \pm 0.3 at 1 h and then 1.3 ± 1.0 at 2 h (Table 5). Compared to those of [¹⁸F]FES, these uptake ratios for [¹⁸F]FEDPN are consistent with what is expected for this $ER\beta$ -selective ligand, although the level of preferential uptake is quite modest, reflecting the lower affinity of [¹⁸F]FEDPN for $ER\beta$.

When the biodistribution of [¹⁸F]FEDPN in immature female Sprague–Dawley rats (Table 3) is compared with that in both sets of ERKO mice (Table 7), similar activity uptake and clearance patterns are found.

Comparison of β ERKO/ α ERKO Uterine and Ovary Uptake Ratio of [¹⁸F]FES and [¹⁸F]FEDPN.

Table 7. Biodistribution of $[^{18}F]$ FEDPN in α ERKO and β ERKO Mice^a

| | | αERKO | | | β ERKO | |
|---------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| organ | 30 min | 1 h | 2 h | 30 min | 1 h | 2 h |
| blood | 0.73 ± 0.15 | 0.42 ± 0.11 | 0.23 ± 0.01 | 0.79 ± 0.02 | 1.12 ± 1.14 | 0.18 ± 0.04 |
| lung | 4.32 ± 0.80 | 2.04 ± 0.72 | 0.75 ± 0.28 | 5.38 ± 0.36 | 1.71 ± 0.33 | 0.44 ± 0.08 |
| liver | 3.70 ± 0.73 | 3.80 ± 2.26 | 1.93 ± 0.19 | 4.55 ± 0.68 | 3.79 ± 2.74 | 2.04 ± 0.56 |
| spleen | 0.99 ± 0.32 | 0.61 ± 0.68 | 0.12 ± 0.02 | 0.87 ± 0.17 | 0.40 ± 0.23 | 0.12 ± 0.05 |
| kidney | 7.22 ± 2.97 | 2.97 ± 0.83 | 1.94 ± 0.86 | 4.83 ± 0.54 | 2.42 ± 1.33 | 1.39 ± 0.12 |
| muscle | 1.21 ± 0.26 | 0.59 ± 0.39 | 0.36 ± 0.35 | 1.45 ± 0.37 | 0.75 ± 0.50 | 0.27 ± 0.23 |
| fat | 0.57 ± 0.08 | 0.27 ± 0.17 | 0.09 ± 0.01 | 0.89 ± 0.42 | 0.33 ± 0.16 | 0.09 ± 0.06 |
| heart | 1.57 ± 0.38 | 0.74 ± 0.41 | 0.25 ± 0.02 | 1.60 ± 0.05 | 0.43 ± 0.03 | 0.21 ± 0.03 |
| brain | 0.96 ± 0.11 | 0.51 ± 0.08 | 0.28 ± 0.05 | 0.93 ± 0.16 | 0.47 ± 0.04 | 0.30 ± 0.08 |
| bone | 3.47 ± 0.50 | 4.13 ± 0.95 | 5.45 ± 1.86 | 3.35 ± 0.69 | 4.92 ± 0.77 | 5.15 ± 0.43 |
| uterus | 1.11 ± 0.50 | 0.37 ± 0.21 | 0.24 ± 0.11 | 1.85 ± 0.15 | 0.54 ± 0.13 | 0.31 ± 0.19 |
| ovaries | 1.06 ± 0.09 | 0.52 ± 0.10 | 0.28 ± 0.04 | 1.40 ± 0.54 | 0.41 ± 0.14 | 0.17 ± 0.09 |
| thymus | 1.27 ± 0.52 | 0.68 ± 0.51 | 0.28 ± 0.08 | 1.20 ± 0.03 | 0.25 ± 0.07 | 0.11 ± 0.04 |

^a Data are expressed as the %ID/g SD with three animals per data point.

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Overall, there is a very clear distinction between the pattern, level, and time course of tissue uptake of the two radiolabeled compounds that seems to be consistent with their different binding preferences for the two ER subtypes. If our animal models were hypothesized to reflect these in vitro binding affinities precisely, then the β ERKO/ α ERKO ratio of [¹⁸F]FES in uterus should be 52 times (6.3 \times 8.3) higher than that of [¹⁸F]FEDPN. Actually, the β ERKO/ α ERKO ratio of [¹⁸F]FES uptake in the uterus at 2 h was calculated to be 20 times higher than that of [¹⁸F]FEDPN (25.4 vs 1.3) (Table 5). In addition, considering, as we noted above, that this uptake ratio might more than double in animals during diestrus compared with those in estrus, the number, 46 (20×2.3) , would actually be even closer to the theoretical value, 53.

We hasten to note that this simple calculation is made for illustrative purposes only, and one should not anticipate being able to predict the β ERKO/ α ERKO uterine uptake ratio based solely on in vitro binding affinity results, because this does not take into consideration many important physiological factors, including the different anatomy and histology of aERKO and β ERKO animals, the different endogenous hormone levels in the two ERKO mice, and different patterns of ER expression in tissue. For example, reproductive tract in β ERKO mice is normal, whereas the uteri in α ERKO mice are underdeveloped and the ovaries are enlarged due to hemorrhagic cysts.⁵⁰ Furthermore, aERKO female mice have elevated estradiol and testosterone levels, whereas the β ERKO ovaries produce normal serum levels of estradiol and testosterone.^{48,50} Also, Korach et al. recently reported that $ER\beta$ mRNA is barely detectable in the rodent uterus, including those from aERKO mice.48,51,52 In our biodistribution study with [18F]FES, the uptake ratios of uterus-to-blood and uterus-to-muscle were over 3 in αERKO mice at all time points, indicating that this ligand can show $ER\beta$ receptor-mediated selective uptake in the uterus, even though the level of ER β protein in the uterus is low and the receptor binding is likely blocked, at least to some extent, by high endogenous estradiol levels.⁵² Interestingly, uterine uptake of $[^{18}F]FES$ in $\alpha ERKO$ mice was about double that of [¹⁸F]FEDPN at all time points. which is comparable with the 2.5-fold higher RBA of $[^{18}F]FES$ for ER β compared to that of $[^{18}F]FEDPN$ (22.0 vs 8.7; Table 2). The fact that $[^{18}F]FES$ is ER α preferential is irrelevant in animals in which this ER subtype has been knocked out.

In contrast to the uterus, both ER α and ER β are clearly expressed in ovary in rodents.^{12,48} Highest expression of ER β mRNA was observed in the ovary and prostate of rats.¹² Therefore, the uptake ratio in ovaries between two knockout models might reflect ligand binding selectivity better than that in uterus. Even though the α ERKO/ β ERKO uptake ratios of [¹⁸F]-FEDPN in uterus were less than 0.8 at all three time points and remained relatively constant, *ovary* uptake ratios of α ERKO/ β ERKO increased over time from 0.8 at 30 min to 1.3 at 1 h and then 1.6 at 2 h (Table 5), consistent with ER β -dominated uptake in this organ. By comparison, the ovarian β ERKO/ α ERKO uptake ratio for [¹⁸F]FES was 30 at 2 h postinjection, which is slightly lower than 46 in uterus and 52 from the in vitro RBA-based calculation.

Discussion

In this study, we have sought to develop a fluorine-18-labeled radioligand that would be effective in imaging ER β by PET. Thus, we have synthesized and examined the tissue distribution of an $ER\beta$ -selective ligand, [¹⁸F]FEDPN, in various animal models, in some cases comparing this distribution with that of [¹⁸F]FES, a well-studied radiopharmaceutical for imaging ER. The unlabeled standard of FEDPN and its precursor were synthesized by using benzyl and MEM groups to protect the phenols, respectively, and [18F]FEDPN was successfully labeled with [¹⁸F]fluoride ion in high specific activity. FEDPN has moderate in vitro $ER\beta$ binding selectivity, but its absolute binding affinity values are considerably lower than those of the non-fluorine substituted analogues of DPN. In immature female Sprague-Dawley rats there was weak but meaningful specific uptake of FEDPN in the uterus and ovaries that was blocked by coadministration with estradiol.

 α ERKO and β ERKO mice were found to be good animal models to assess the ER subtype selectivity of radioligand uptake in target tissues. [18F]FES, which has a higher affinity for both of the ER subtypes and a 6.3-fold preference for ER α , showed very high β ERKO/ α ERKO uptake ratios for uterus and ovaries at 2 h (25.4 and 18.6, respectively), clear evidence for the involvement of ER α . By contrast, the uterine β ERKO/ α ERKO uptake ratios with [¹⁸F]FEDPN was always less than 2 and decreased over time, wherease the ovary $\alpha ERKO/$ β ERKO uptake ratios with [¹⁸F]FEDPN increased over time, reaching a maximum of 1.6 at 2 h. While the uptake preferences of [¹⁸F]FEDPN are not sufficiently pronounced to be useful for PET imaging of ER β , they suggest that the distribution of [¹⁸F]FEDPN is being regulated by an ER β -mediated process. A strong dependence of uterus uptake of [18F]FES upon estrus cycle in mice has also been demonstrated. Thus, overall, the time course and target tissue distribution patterns of both [¹⁸F]FES and [¹⁸F]FEDPN in the two ERKO mice systems are consistent with their absolute and preferential affinities for ER α and ER β , respectively, as well as the relative levels of these two ER subtypes in the ERKO animals.

From this work, we can make a number of other conclusions: Even though its ER α absolute binding selectivity is only 6.3, [¹⁸F]FES is a very effective agent for imaging ER α ; its affinity for this ER subtype is very high, and in most tissues, ER α is by far the predominant ER. Still, it might be possible to improve the selectivity of ER α imaging by using some FES analogues that have a somewhat greater ER α binding preference. For example, while the closest FES analogue, 17 β -ethynyl-FES,⁵³ has a similar ER α /ER β absolute binding selectivity ratio of 6.0, another 17-ethynyl-substituted FES compound, 17 α -ethynyl-FES (FEES),⁵³ has somewhat higher ER α absolute selectivity (ER α /ER β 11).⁵⁴

Even though [¹⁸F]FEDPN, the principal subject of this study, showed some significant binding selectivity for the ER β subtype in in vivo animal studies, its absolute

binding affinity for the estrogen receptors is not high enough for it to be effective in imaging studies. Therefore, radioligands with higher affinity and greater selectivity for ER β are needed. In this regard, it is of note that Malamas and co-workers have recently reported new diphenolic azoles as highly selective $ER\beta$ agonists.55 The more potent and selective analogues of these series have binding affinities for $ER\beta$ that are comparable to that of the natural ligand 17β -estradiol, vet are >100-fold selective over ER α . The 7-positionsubstituted benzoxazoles were the most selective ligands of both azole series, with ERB-041 being >200-fold selective for $\text{ER}\beta$. ER ligands based on this compound series might prove to be effective agents for imaging $ER\beta$ in vivo. Compounds with comparable $ER\beta$ affinity and selectivity in the indazole system were also reported by us recently.⁵⁶

While we have used the ER α and ER β knockout mice in a relatively straightforward manner to help us characterize the respective roles of the ER β and ER α subtypes in the uptake process of the ¹⁸F-ligands, it is of note that more sophisticated experimental designs should also be considered. In our biodistribution studies, the animals were not ovariectomized, because the ovaries were used as critical target organs. These are adult animals, however, and as we have noted, their high levels of circulating estradiol are likely to compete for receptor binding with the ¹⁸F-ligands. While an uptake blocking study using estradiol as a blocking agent might be informative in this respect, an improved experimental design, suggested by a reviewer, might be to pretreat the animals with an aromatase inhibitor such as letrozole, or a steroidogenic inhibitor such as aminoglutethimide. In this way, circulating levels of estradiol could be lowered without removing the ovaries. An alternative model for $ER\beta$ uptake might be the prostate of the castrated male rat, which is relatively rich in ER β ; circulating estradiol levels are also low. These alterative in protocols, as well as the use of ERsubtype selective ligands in blocking the uptake of estrogen radiotracers in wild-type animals will be considered in our future studies.

Experimental Section

General Methods and Materials. 4-(Benzyloxy)phenylacetonitrile was purchased from Oakwood Products, Inc (West Columbia, SC) and all other solvents and reagents were obtained from Aldrich and used as received without any further purification. An unlabeled standard for 16α -[¹⁸F]fluoroestradiol- 17β was purchased from ABX advanced biochemical compounds (Radeberg, Germany). Water was distilled and then deionized ($18 \text{ M}\Omega/\text{cm}^2$) by passing through a Milli-Q water filtration system (Millipore Corp., Bedford, MA). ¹H and ¹³C NMR spectra were measured using a Varian Gemini 300 instrument, and chemical shifts are reported in ppm on the δ scale relative to TMS or solvent peak. Proton chemical shifts are annotated as follows: ppm (multiplicity, coupling constant (Hz), integral). Mass spectra were obtained from Washington University Mass Spectrometry Resource.

 $\rm H_2^{18}O$ was purchased from Rotem Industries (Israel). ¹⁸F-Fluoride was produced in Washington University by the ¹⁸O-(p,n)¹⁸F reaction through proton irradiation of enriched (95%) ¹⁸O water using either the JSW BC16/8 cyclotron (The Japan Steel Works Ltd, Tokyo, Japan) or the CS15 cyclotron (The Cyclotron Corp., Berkeley, CA). Materials were heated using a custom-designed microwave cavity, model 420BX (Micro-Now Instruments, Skokie, IL).⁵⁷ Screw-cap test tubes used for microwave heating were purchased from Fisher Scientific (Pyrex No. 9825). Oasis cartridges were purchased from Waters Corporation, (HLB-6 cc, Part No.186000115). For the TLC analyses, EM Science Silica Gel 60 F_{254} TLC plates were purchased from Fisher Scientific (Pittsburgh, PA). Radio-TLC was accomplished using a Bioscan 200 imaging scanner (Bioscan, Inc., Washington, DC). Radioactivity was counted with a Beckman Gamma 8000 counter containing a NaI crystal (Beckman Instruments, Inc., Irvine, CA). High performance liquid chromatography (HPLC) was performed with a SpectraSYSTEM P2000 liquid chromatography, equipped with an ultraviolet detector operating at 254 nm and a well-scintillation NaI (TI) detector and associated electronics, and a fraction collector.

The purification of the final product was achieved using a semipreparative HPLC Chromanetics C18 column (Partisil ODS-3, 100×10 mm, 5 μ m, MeCN:EtOH:H₂O (2:20:78), 3 mL/ min). For the quality control, the radiochemical purity of the [¹⁸F]FEDPN was assayed by analytical radioHPLC (Alltech, Econosil C18 column, 250 × 4.6 mm, 10 μ m; 0.1 M formate: MeCN (60:40) at a flow rate of 1.5 mL/min and UV at 254 nm) in the presence of the unlabeled compound as standard. TLC was used in determining the compounds during various synthesis steps (silica, ethyl acetate:hexane = 3:1 v/v). Radiochemical yields are decay corrected to the beginning of synthesis time (BOS).

Immature female Sprague–Dawley rats were purchased from Charles River Laboratories, and α ERKO and β ERKO mice were obtained from Taconic Inc. (Germantown, NY). Estrogen receptor knockout mice (α/β ERKO) were staged according to the estrous cycle by microscopic examination of daily prepared vaginal smears⁴⁷ and were subjected to biodistribution study during diestrus. The circulating estradiol level in serum was measured in Ani Lytics, Inc. (Gaithersburg, MD). All animal experiments were conducted in compliance with the Guidelines for the Care and Use of Research Animals established by Washington University's Animal Studies Committee.

Cold Synthesis. (*Z*)-2,3-Bis(4-methoxyphenyl)acrylonitrile (**1a**) was prepared by a modification of a previously described method.²¹

(2R*,3S*)-2,3-Bis(4-methoxyphenyl)pent-4-enenitrile (2a). To a mixture of (Z)-2,3-bis(4-methoxyphenyl)acrylonitrile (2770 mg, 10.4 mmol) and CuI (198 mg, 1.04 mmol) in dried THF (30 mL) was added 1 M vinylmagnesium bromide (21 mL) dropwise at -76 °C. The flask was allowed to warm to roomtemperature overnight with stirring under Ar atmosphere. The mixture was quenched by saturated NH₄Cl solution. After filtering off the precipitate, the filtrate was diluted with 40 mL of CH₂Cl₂, washed by brine, dried over Na₂SO₄, and concentrated under vacuum. The residue was recrystallized for 1 h from refluxing ethyl acetate/hexane (1:1) to give white solid (erythro:threo = 9:1). Repeated recrystallization from refluxing ethyl acetate/hexane (1:1) gave pure erythro-2a. (1671 mg, 55%): ¹H NMR (CDCl₃, 300 MHz) δ 3.58 (t, J = 8.0Hz, 1H), 3.68 (s, 6H), 3.94 (d, J = 9.0 Hz, 1H), 5.12 (m, 2H), 6.14 (m, 1H), 6.74 (m, 4H), 6.99 (m, 4H); ¹³C NMR (CDCl₃, 75 MHz) δ 43.5 (CHCN), 53.9 (CHCH=CH₂), 55.39 and 55.45 (OCH₃), 114.1 and 114.2 (Ph ring CH), 117.9 (CHCH=CH₂), 120.1 (CN), 126.0, 129.1 and 129.4 (Ph ring CH), 130.9, 137.1 (CHCH=CH₂), 159.1 and 159.5 (Ph ring C). HRMS (FAB): m/z $300.1566 ([M + Li]^+, C_{19}H_{19}NO_2Li, calcd 300.1576).$

(2*R**,3*S**)-5-Hydroxy-2,3-bis(4-methoxyphenyl)pentanenitrile (3a). To a clear THF solution (15 mL) of (2*R**,3*S**)-2,3-bis(4-methoxyphenyl)pent-4-enenitrile (439 mg, 1.50 mmol) was added 1 M BH₃·THF (1.7 mL) dropwise at -40 °C. The mixture was allowed to warm to room temperature over 1 h and was kept stirring for an additional 1.5 h under an Ar atmosphere. After quenching excess BH₃ with water (2 mL), 0.5 M NaOH (3 mL) and 35% H₂O₂ (300 µL) were added to the mixture at 0 °C. The flask was stirred for 1 h at 0 °C. The mixture was diluted with water (20 mL), the avtracted by ethyl acetate (20 mL). After layer separation, the aqueous layer was further extracted by same amount of ethyl acetate. The

Estrogen Receptor β -Selective Radioligand

combined organic layers were washed by NaHCO₃ and brine, dried over Na₂SO₄, and concentrated to give clear oil. The crude product was chromatographed over silica using ether as eluent to give white solid. (313 mg, 67%): ¹H NMR (CDCl₃, 300 MHz) δ 1.99 (m, 2H), 3.15 (m, 1H), 3.34–3.58 (m, 2H), 3.758 (s, 3H), 3.761 (s, 3H), 4.01 (d, *J* = 6.3 Hz, 1H), 6.79 (m, 4H), 6.98 (m, 4H); ¹³C NMR (CDCl₃, 75 MHz) δ 35.8 (CCH₂CH₂OH), 43.8 (CCN), 46.4 (CCH₂CH₂OH), 55.2 and 55.3 (OCH₃), 60.1 (CH₂CH₂OH), 113.9 and 114.1 (Ph ring *C*H), 120.2 (CN), 126.4, 129.4 and 129.6 (Ph ring *C*H), 130.7, 159.0 and 159.3 (Ph ring *C*). HRMS (FAB): *m/z* 318.1688 ([M + Li]⁺, C₁₉H₂₁NO₃Li, calcd 318.1681).

 $(2R^*, 3S^*)$ -5-Fluoro-2,3-bis(4-methoxyphenyl)pentanenitrile (4a). To a dry CH_2Cl_2 solution (8 mL) of (2 R^* , 3 S^*)-5hydroxy-2,3-bis(4-methoxyphenyl)pentanenitrile (285 mg, 0.92 mmol) was added (diethylamino)sulfur trifluoride (DAST, 600 μ L, 4.58 mmol) dropwise at -76 °C. The mixture was stirred under Ar atmosphere and allowed to warm to room-temperature overnight. After quenching with saturated NaHCO₃ at 0 °C, the solution was diluted with water (15 mL), then extracted CH_2Cl_2 (2 \times 15 mL), dried over MgSO₄, and concentrated in vacuo. The crude product was purified by flash chromatography (silica, ethyl acetate/hexane (1:1)) to give a pale yellow solid. (187 mg, 65%): ¹H NMR (CDCl₃, 300 MHz) δ 1.98–2.32 (m, 2H), 3.19 (m, 1H), 3.80 (s, 6H), 4.02 (d, J = $6.9~\mathrm{Hz},~1\mathrm{H}),~4.10{-}5.31~\mathrm{(m,~2H)},~6.20~\mathrm{(m,~4H)},~7.01~\mathrm{(m,~4H)};$ ¹³C NMR (CDCl₃, 75 MHz) δ 34.1 (d, J_{CF} = 20.0 Hz, CH_2CH_2F), 43.8 (CHCN), 46.1 (d, $J_{CF} = 4.1$ Hz, CHCH₂CH₂F), 55.40 and 55.49 (OCH₃), 81.6 (d, $J_{CF} = 164.7$ Hz, CH₂CH₂F), 114.2 and 114.3 (Ph ring CH), 120.0 (CN), 126.2, 129.5 and 129.7 (Ph ring CH), 130.0, 159.3, 159.6. HRMS (FAB): m/z 320.1635 ([M + Li]⁺, C₁₉H₂₀FNO₂Li, calcd 320.1638).

(Z)-2,3-Bis(4-(benzyloxy)phenyl)acrylonitrile (1b). The mixture of 4-(benzyloxy)benzaldehyde (5257 mg, 24.8 mmol), 4-(benzyloxy)phenylacetonitrile (5530 mg, 24.8 mmol), and NaOMe (156 mg, 2.89 mmol) in dry ethanol (100 mL) was refluxed for 2 h and cooled to room temperature to produce a precipitate of white crystals. After removing the solid by filtration, to the filtrate was added more NaOMe (503 mg, 9.31 mmol), and the mixture was refluxed for 2 h and cooled to room temperature to precipitate more crystals. After second crop, the filtrate was concentrated and the residue was redissolved in CH₂Cl₂, washed by brine, dried over MgSO₄, and concentrated. The residue was dissolved in EtOH (80 mL) again, then NaOMe (579 mg, 10.7 mmol) was added. The mixture was refluxed for 3 h, then cooled to room temperature to give white crystals. All crops were washed with a small amount of ethanol and dried in air. (10.02 g, 97%): ¹H NMR (CDC₁₃, 300 MHz) δ 5.12 (s, 2H), 5.14 (s, 2H), 7.04 (m, 4H), 7.36 (s, 1H), 7.44 (m, 10H), 7.59 (m, 2H), 7.86 (m, 2H); ¹³C NMR (CDCl₃, 75 MHz) δ 70.4 (OCH₂Ph), 108.7 (CH=CCN), 115.4 and 115.5 (Ph ring CH), 118.8 (CN), 127.2, 127.3, 127.7, 128.35, 128.41, 128.9, 131.1, 136.6, 136.7, 140.2 (CH=CCN), 159.5, 160.6. HRMS (FAB): m/z 417.1726 ([M]⁺, C₂₉H₂₃NO₂, calcd 417.1729).

(2R*,3S*)-2,3-Bis(4-(benzyloxy)phenyl)pent-4-enenitrile (2b). To a dry THF solution (35 mL) of (Z)-2,3-bis(4-(benzyloxy)phenyl)acrylonitrile (3433 mg, 8.22 mmol) and CuI (157 mg, 0.82 mmol) was added 1 M vinylmagnesium bromide (10 mL) dropwise at -25 °C. The flask was allowed to warm to room temperature over 1 h and stirred overnight under Ar gas atmosphere. The reaction was quenched by saturated NH₄-Cl, and the mixture was stirred for 2 h at 0 °C. After filtering off the precipitate, the filtrate was concentrated, redissolved in ethyl acetate, then washed by NaHCO₃ solution, dried over MgSO₄, and concentrated. The crude product was recrystallized for 1 h from refluxing ethanol to give a white solid. Repeated recrystallization from refluxing ethanol gave pure erythro-2b (1695 mg, 46%): ¹H NMR (CDCl₃, 300 MHz) δ 3.64 (t, J = 7.7 Hz, 1H), 3.99 (d, J = 7.2 Hz, 1H), 5.04 (s, 4H), 5.12(d, $J=17~{\rm Hz},\,1{\rm H}),\,5.26$ (d, $J=10~{\rm Hz},\,1{\rm H}),\,6.20$ (m, 1H), 6.89 (m, 4H), 7.05 (m, 4H), 7.37 (m, 10H); ¹³C NMR (CDCl₃, 75 MHz) & 43.9 (CHCN), 54.6 (CHCH=CH₂), 70.2 (OCH₂Ph), 115.15 and 115.19 (Ph ring CH), 118.9 (CHCH=CH2), 120.2 (CN), 126.6, 127.7, 128.20, 128.26, 128.8, 129.2, 129.5, 132.0, 136.3 (CHCH=CH₂), 136.9 and 137.1 (OCH₂C), 158.2 and 158.6 (COCH₂). HRMS (FAB): m/z 452.2184 ([M + Li]⁺, C₃₁H₂₇NO₂Li, calcd 452.2202).

(2R*,3S*)-2,3-Bis(4-(benzyloxy)phenyl)-5-hydroxypentanenitrile (3b). To a dry THF solution (20 mL) of $(2R^*, 3S^*)$ -2,3-bis(4-(benzyloxy)phenyl)pent-4-enenitrile (503 mg, 1.13 mmol) was added 1 M BH3·THF (1.6 mL) dropwise at 0 °C. The flask was allowed to warm to room temperature slowly and stirred for 5 h under Ar gas atmosphere. The mixture was cooled to 0 °C again and quenched by water (2 mL). 3 M NaOH (4 mL) and 35% H₂O₂ (4 mL) were added, and the resulting reaction mixture was stirred for 30 min more in ice bath. The solution was diluted with water (15 mL) and extracted by ethyl acetate (2 \times 20 mL). The combined organic layer was washed by saline, dried over MgSO₄, and concentrated. The crude product was chromatographed over silica using ethyl acetate/ hexane (1:1) to give pure product. (228 mg, 44%): ¹H NMR (CDCl₃, 300 MHz) & 2.02-2.26 (m, 2H), 3.19-3.41 (m, 2H), 3.56 (m, 1H), 3.95 (d, J = 7.2 Hz, 1H), 5.040 (s, 2H), 5.044 (s, 2H), 6.91 (m, 4H), 7.07 (m, 4H), 7.42 (m, 10H); $^{13}\mathrm{C}$ NMR (CDCl₃, 75 MHz) & 34.6 (CHCH₂CH₂OH), 44.2 (CHCN), 46.6 (CH₂CH₂OH), 60.2 (CH₂CH₂OH), 70.1 (OCH₂Ph), 115.1 (Ph ring CH), 120.4 (CN), 126.6, 127.6, 128.08 and 128.14 (Ph ring CH), 128.7, 129.35 and 129.40 (Ph ring CH), 131.5, 136.8 and 137.0 (OCH₂C), 158.2 and 158.5.

(2R*,3S*)-2,3-Bis(4-(benzyloxy)phenyl)-5-fluoropentanenitrile (4b). To a solution of $(2R^*, 3S^*)$ -2,3-bis(4-(benzyloxy)phenyl)-5-hydroxypentanenitrile (157 mg, 0.34 mmol) in dry CH₂Cl₂ (6 mL) was added (diethylamino)sulfur trifluoride (DAST, 222 μ L, 1.69 mmol) dropwise at -76 °C. The mixture was stirred under Ar gas and allowed to warm to roomtemperature overnight. The flask was cooled to 0 °C again and quenched by saturated NaHCO₃. The solution was diluted by water (15 mL), then extracted by CH₂Cl₂, dried over MgSO₄, and concentrated. The crude product was recrystallized in refluxing ethanol to give pale yellow crystal. (83 mg, 53%): ¹H NMR (CDCl₃, 300 MHz) δ 2.02-2.50 (m, 2H), 3.24 (m, 1H), 3.97 (d, J = 6.6 Hz, 1H), 4.06-4.51 (m, 2H), 5.06 (s, 4H), 6.93 (m, 4H), 7.09 (m, 4H), 7.42 (m, 10H); $^{13}\mathrm{C}$ NMR (CDCl_3, 75 MHz) δ 32.5 (d, J_{CF} = 20.5 Hz, CH_2CH_2F), 44.1 (CHCN), 46.1 (d, $J_{\rm CF}$ = 4.5 Hz, CHCH₂CH₂F), 70.2 (OCH₂Ph), 81.4 (d, $J_{\rm CF}$ = 165.1 Hz, CH₂CH₂F), 115.3 (Ph ring CH), 120.1 (CN), 126.4, 127.6, 128.17, 128.22, 128.7, 129.4, 130.9, 136.8 and 137.0 (OCH₂C), 158.4 and 158.7 (COCH₂).

(2R*,3S*)-5-Fluoro-2,3-bis(4-hydroxyphenyl)pentanenitrile (5). To a clearly dissolved solution of $(2R^*, 3S^*)$ -2,3bis(4-(benzyloxy)phenyl)-5-fluoropentanenitrile (83 mg, 0.18 mmol) in ethyl acetate (15 mL) was added 10% Pd/C (106 mg), and the mixture was stirred under H₂ gas for 4 h. The Pd/C catalyst was filtered off through a Celite plug and washed with small amount of ethyl acetate and MeCN. After concentration, the residue was purified by flash chromatography (silica, ethyl acetate/hexane (1:1) to give a clear oil. (39 mg, 77%): ¹H NMR $(acetone-d_6, 300 \text{ MHz}) \delta 2.04 - 2.48 \text{ (m, 2H)}, 3.22 \text{ (m, 1H)}, 4.04 - 2.48 \text{ (m, 2H)}, 3.22 \text{ (m, 1H)}, 4.04 - 2.48 \text{ (m, 2H)}, 3.22 \text{ (m, 1H)}, 4.04 - 2.48 \text{ (m, 2H)}, 3.22 \text{ (m, 1H)}, 4.04 - 2.48 \text{ (m, 2H)}, 3.22 \text{ (m, 1H)}, 4.04 - 2.48 \text{ (m, 2H)}, 3.22 \text{ (m, 1H)}, 4.04 - 2.48 \text{ (m, 2H)}, 3.22 \text{ (m, 2H)}, 3.22 \text{ (m, 2H)}, 3.24 - 2.48 \text{ (m, 2H)}, 3.22 \text{ (m, 2H)}, 3.24 - 2.48 \text{ (m, 2H)}, 3.24 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 + 2.48 +$ $4.48 \text{ (m, 2H)}, 4.19 \text{ (d, } J = 8.1 \text{ Hz}, 1 \text{H}), 6.75 \text{ (m, 4H)}, 7.04 \text{$ 4H), 8.41 (b s, 2H); $^{13}\mathrm{C}$ NMR (acetone- $d_{6},$ 75 MHz) δ 34.0 (d, $J_{\rm CF} = 19.4$ Hz, CH_2CH_2F), 44.0 (CCN), 46.6 (d, $J_{\rm CF} = 5.7$ Hz, CCH_2CH_2F), 82.3 (d, $J_{CF} = 162.8$ Hz, CH_2CH_2F), 116.2 and 116.3 (Ph ring CH), 121.4 (CN), 126.7, 130.4 (Ph ring CH), 130.6, 157.5 and 158.0 (Ph ring C). HRMS (ESI): m/z 286.1232 $([M + H]^+, C_{17}H_{17}FNO_2, calcd 286.1243)$. $R_f = 0.58$ (silica, ethyl acetate: hexane = 3:1).

(2*R**,3*S**)-2,3-Bis(4-hydroxyphenyl)pent-4-enenitrile (6). To a solution of $(2R^*,3S^*)$ -2,3-bis(4-methoxyphenyl)pent-4enenitrile (473 mg, 1.61 mmol) in anhydrous CH₂Cl₂ was added 1 M BBr₃ (7 mL) in portions at 0 °C. The solution was allowed to warm to room temperature slowly and stirred overnight. The reaction was quenched by water (2 mL) at 0 °C. The mixture was diluted by ethyl acetate (30 mL), then washed by brine solution, dried over MgSO₄, and concentrated completely under vacuum to give a pale yellow solid. (422 mg, 99%): ¹H NMR (acetone-d₃, 300 MHz) δ 3.76 (t, J = 8.3 Hz, 1H), 4.33 (d, J = 8.4 Hz, 1H), 4.98 (m, 2H), 6.01 (m, 1H), 6.80 (m, 4H), 7.11 (m, 4H), 8.30 (s, 1H), 8.46 (s, 1H); ¹³C NMR (acetone–d₃, 75 MHz) δ 43.3 (CHCN), 54.8 (CHCH=CH₂), 116.15 and 116.29 (Ph ring CH), 117.3 (CHCH=CH₂), 121.2 (CN), 126.8, 130.4 and 130.6 (Ph ring CH), 131.8, 139.3 (CHCH=CH₂), 157.6 and 158.1 (COH). HRMS (FAB): m/z 272.1270 ([M + Li]⁺, C₁₇H₁₅NO₂Li, calcd 272.1263).

 $(2R^*, 3S^*)$ -2,3-Bis(4-((2-methoxyethoxy)methoxy)phenyl)pent-4-enenitrile (7). To a solution of $(2R^*, 3S^*)$ -2,3-bis-(4-hydroxyphenyl)pent-4-enenitrile (422 mg, 1.59 mmol) in mixture solvent of anhydrous CH₂Cl₂ (8 mL) and THF (4 mL) were added 2-methoxyethoxymethyl chloride (MEM chloride, 829 μ l, 7.26 mmol) and N,N-diisopropylethylamine (2.1 mL, 12.1 mmol) consecutively. The flask was stirred under Ar gas atmosphere for 2.5 days. The solution was diluted with ethyl acetate (25 mL), then washed by water, 0.5 M HCl, NaHCO₃, and brine, dried by MgSO₄, and concentrated under vacuum. The crude product was purified by flash column chromatography (silica, ethyl acetate/hexane (1:1)) to give a clear oil. (442) mg, 60%): ¹H NMR (CDCl₃, 300 MHz) δ 3.348 (s, 3H), 3.352 (s, 3H), 3.54 (m, 4H), 3.66 (m, 1H), 3.78 (m, 4H), 4.05 (d, J =6.9 Hz, 1H), 5.11 (m, 2H), 5.23 (s, 4H), 5.98 (m, 1H), 6.99 (m, 8H); ¹³C NMR (CDCl₃, 75 MHz) δ 43.3 (CHCN), 53.8 (CHCH= CH₂), 59.0 (OCH₃), 67.7 and 67.8 (CH₂CH₂OCH₃), 71.6 (CH₂-CH₂OCH₃), 93.46 and 93.50 (PhOCH₂O), 116.3 and 116.4 (Ph ring CH), 117.9 (CHCH=CH₂), 119.9 (CN), 127.1, 129.5 and 129.6 (Ph ring CH), 136.9 (CHCH=CH₂), 156.7, 157.1.

 $(2R^*, 3S^*)$ -2,3-Bis(4-((2-methoxyethoxy)methoxy)phenyl)-5-hydroxypentanenitrile (8). To a solution of $(2R^*, 3S^*)$ -2,3-bis(4-((2-methoxyethoxy)methoxy)phenyl)pent-4-enenitrile (442 mg, 1.0 mmol) in dry THF was added 1M BH₃·THF (1.2 mL) dropwise at 0 °C. The mixture was allowed to warm to room temperature slowly and stirred under argon gas for 6 h. After quenching with water (2 mL), to the flask were added 3 M NaOH (4 mL) and 35% H₂O₂ (4 mL) at 0 °C. The resulting mixture was stirred for 1 h in ice bath. The solution was diluted by ethyl acetate (40 mL), then washed by water and brine and dried over MgSO₄. The residue, after evaporation, was chromatographed over silica using ethyl acetate/hexane (3:1) as eluent to give a clear oil. (248 mg, 54%): 1H NMR (CDCl₃, 300 MHz) & 1.96 (m, 2H), 3.16 (m, 1H), 3.35 (s, 6H), 3.33-3.57 (m, 2H), 3.54 (m, 4H), 3.79 (m, 4H), 3.99 (d, J = 7.2 Hz, 1H), 5.22 (s, 4H), 6.97 (m, 8H); ¹³C NMR (CDCl₃, 75 MHz) δ 35.8 (CHCH₂CH₂OH), 43.9 (CHCN), 46.5 (CH₂CH₂OH), 59.1 (OCH₃), 60.2 (CH₂CH₂OH), 67.79 and 67.83 (CH₂OCH₃), 71.69 and 71.72 (CH₂CH₂OCH₃), 93.5 and 93.6 (OCH₂O), 116.38 and 116.54 (Ph ring CH), 120.1 (CN), 127.7, 129.44 and 129.58 (Ph ring CH), 132.1, 156.8, 157.1. HRMS (FAB): m/z 466.2428 ([M + Li]⁺, C₂₅H₃₃NO₇Li, calcd 466.2417).

 $(3R,\!4S)\!\cdot\!3,\!4\text{-}Bis(4\text{-}((2\text{-}methoxy)methoxy)phenyl)\text{-}$ 4-cyanobutyl 4-methylbenzenesulfonate (9). To a solution $of(2R^*, 3S^*)$ -2,3-bis(4-((2-methoxyethoxy)methoxy)phenyl)-5hydroxypentanenitrile (248 mg, 0.54 mmol) in $CH_2Cl_2\,(10\ mL)$ were added p-toluenesulfonyl chloride (113 mg, 0.59 mmol) and pyridine (2 mL, 24.7 mmol) consecutively. The reaction mixture was stirred under Ar atmosphere at room temperature for 26 h. The solution was diluted with ethyl acetate (30 mL), then washed by water, 10% citric acid $(3 \times 20 \text{ mL})$, 0.1 M HCl, and brine, dried over MgSO₄, and concentrated. The crude product was purified by flash column chromatography (silica, ethyl acetate/hexane (3:1)) to give a clear oil. (122 mg, 37%): 1H NMR (CDCl₃, 300 MHz) & 2.02–2.13 (m, 2H), 2.44 (s, 3H), 3.07 (m, 1H), 3.370 (s, 3H), 3.374 (s, 3H), 3.56 (m, 4H), 3.71 (m, 1H), 3.82 (m, 4H), 3.91 (d, J = 7.2 Hz, 1H), 3.99 (m, 1H), 5.23 (s, 2H), 5.25 (s, 2H), 6.87 (b s, 4H), 6.96 (s, 4H), 7.29 (d, J = 8.4 Hz, 2H), 7.66 (d, J = 8.4 Hz, 2H); ¹³C NMR (CDCl₃, 75 MHz) & 21.8 (PhCH₃), 32.5 (CHCH₂CH2O), 43.6 (CHCN), 46.0 (CH₂CH₂OS), 59.2 (OCH₃), 67.8 (CH₂CH₂OS), 67.9 (CH₂CH₂-OCH₃), 71.74 and 71.77 (CH₂CH₂OCH₃), 93.61 and 93.58 (OCH₂O), 116.5 and 116.7 (Ph ring CH), 119.7 (CN), 127.1, 128.0, 129.4, 129.5, 130.0, 130.6, 132.9 (CCH₃), 145.1 (OSC), 157.1, 157.3. HRMS (FAB): m/z 620.2492 ([M + Li]⁺, C₃₂H₃₉-NO₉SLi, calcd 620.2506).

 $(2R^*,3S^*)$ -2,3-Bis(4-((2-methoxyethoxy)methoxy)phenyl)-5-fluoropentanenitrile (10). To a solution of $(2R^*,3S^*)$ -2,3-bis(4-((2-methoxyethoxy)methoxy)phenyl)-5-hydroxypentanenitrile (54 mg, 0.12 mmol) in dry CH₂Cl₂ (10 mL) was added (diethylamino)sulfur trifluoride (DAST, 200 µL, 1.53 mmol) dropwise at -94 °C. The mixture was stirred under Ar gas and allowed to warm to room-temperature overnight. The flask was cooled to 0 °C again and quenched by saturated NaHCO₃. The solution was diluted with ethyl acetate (30 mL), then washed by brine solution $(2 \times 20 \text{ mL})$, dried over MgSO₄, and concentrated. The crude product was chromatographed over silica using ethyl acetate/hexane (3:1) as eluent to give a pale yellow oil. (28 mg, 52%): ¹H NMR (CDCl₃, 300 MHz) δ 1.98-2.24 (m, 2H), 3.19 (m, 1H), 3.374 (s, 3H), 3.379 (s, 3H), 3.56 (m, 4H), 3.82 (m, 4H), 4.0 (d, J = 7.2 Hz, 1H), 4.07-4.51(m, 2H), 5.26 (s, 4H), 7.00 (m, 8H); ¹³C NMR (CDCl₃, 75 MHz) δ 34.1 (d, $J_{\rm CF}$ = 20.5 Hz, CH₂CH₂F), 43.8 (CHCN), 46.1 (d, $J_{\rm CF}$ = 4.5 Hz, CHCH₂CH₂F), 59.2 (CH₂OCH₃), 67.90 and 67.97 (CH₂CH₂OCH₃), 71.77 and 71.80 (CH₂CH₂OCH₃), 81.5 (d, J_{CF} = 164.0 Hz, CH₂CH₂F), 93.61 and 93.67 (OCH₂O), 116.5 and 116.7 (Ph ring CH), 119.9 (CN), 127.4, 129.5 and 129.7 (Ph ring CH), 131.3, 157.1, 157.3. $R_f = 0.50$ (silica, ethyl acetate: hexane = 3:1). HRMS (FAB): m/z 468.2372 ([M + Li]⁺, C₂₅H₃₂-FNO₆Li, calcd 468.2374).

Estrogen Receptor Binding Affinity. The relative binding affinity was obtained by a competitive radiometric binding assay using 10 nM [³H]estradiol as tracer and purified full– length human ER α and ER β , following previously published procedures.^{33,34} Incubations were done at 0 °C for 18–24 h, and hydroxyapatite was used to absorb the purified receptor– ligand complexes. The binding affinities are given relative to estradiol [RBA E₂ = 100]. The RBA values are reported as the average of two determinations \pm the range.

Radiochemical Synthesis. $16\alpha \ [^{18}F]$ Fluoroestradiol- $17\beta \ ([^{18}F]FES)$ was prepared according to literature methods. 44,58,59

 $(2R^*, 3S^*)$ -5-[¹⁸F]Fluoro-2,3-bis(4-hydroxyphenyl)pentanenitrile ([18F]FEDPN). [18F]Fluoride, trapped on a resin (BioRad AG-1X8, 100–200 mesh), was eluted with ca. 500 μ L of 0.02 M K₂CO₃ prior to use. A resin-treated water containing the [18F]fluoride anion (100-250 mCi) was transferred to a Vacutainer (Becton Dickinson, Franklin Lakes, NJ) containing Kryptofix [2.2.2] (1.1 mg per 100 μ L of treated water). The water was removed by azeotropic distillation at 110 °C under a gentle stream of nitrogen with successive addition of three (0.5 mL) aliquots of acetonitrile. During the final evaporation the Vacutainer was removed from the oil bath when the final volume reached about 100 μ L. Evaporation to dryness of the residue was conducted at room temperature. The resolubilized $[^{18}\mathrm{F}]\mathrm{fluoride}$ anion was dissolved in 400 $\mu\mathrm{L}$ of anhydrous acetonitrile and transferred to a 10-mL Pyrex brand tube with screw cap containing 2.3-3.5 mg of (3R,4S)-3,4-bis(4-((2methoxyethoxy)methoxy)phenyl)-4-cyanobutyl 4-methylbenzenesulfonate (9). A 3 mm glass bead was also added to the tube for a more homogeneous heat distribution during the heating and the tube capped firmly. The mixture was vortexed and reacted under microwave irradiations on medium power (60 W) for 35 s. After cooling, 100 μ L of 3 M HCl was added and the mixture heated for 3 min in an oil bath at 110 °C.

The crude reaction mixture was diluted with water to about 6 mL. Solid-phase purification was performed using a Waters (HLB-6 cc) OASIS cartridge previously rinsed with 6 mL of methanol and 2×6 mL of MilliQ water. After the radioactive sample had been applied, the cartridge was rinsed with an additional 2×6 mL water to eliminate unreacted fluoride and salts, then the radiolabeled product was eluted with ethanol $(2 \times 3 \text{ mL})$. When the evaporation of ethanol was nearly complete, the residue was taken up in a mixture of 1.6 mL of 20% ethanol in water and then purified by a semipreparative HPLC (Chromanetics, Partisil ODS-3, 100×10 mm, 5 µm, MeCN:EtOH:H₂O (2:20:78), 3 mL/min). The radioactive peak corresponding to the product was detected by the radioactivity monitor 15-19 min after injection and was collected for further study. The total reaction and purification time was less than 110 min; decay-corrected radiochemical yield was between 1.2% and 8.5% (n = 4). Radio-TLC showed that the Rf of the intermediate compound, (2R*,3S*)-2,3-bis(4-((2-methoxyethoxy)-

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methoxy)phenyl)-5-[18F]fluoropentanenitrile ([18F]10), was 0.49 and the R_f of [¹⁸F]FEDPN was 0.58.

Radiochemical Purity and Specific Activity. The radiochemical purity of [18F]FEDPN was determined by analytical HPLC column (Alltech, Econosil C18 column, 250 \times 4.6 mm, 10 µm; 0.1 M formate:MeCN (60:40) at a flow rate of 1.5 mL/min) and found to be greater than 99%. By comparison of the integrated sample UV signal with a calibrated compound 5 mass/UV absorbance curve, the specific activity was determined to be >3100 Ci/mmol at the end of synthesis. No cold mass was detected in any UV chromatograms, and the detection limit of the UV detector was 0.5 ppm in our system. The identity of the tracer was confirmed by the coelution of ^{[18}F]FEDPN with nonradioactive standard **5** on the analytical HPLC system. Retention time for [18F]FEDPN was 6.9 min.

Biodistribution Studies. In the first biodistribution study, immature female Sprague-Dawley rats (44-56 g, 21-24 days old, n = 5 per time point) were used for evaluation of uptake due to their low level of endogenous estrogens and estrogen receptor rich uterine tissue.³² The ¹⁸F-labeled FEDPN was concentrated in vacuo, redissolved in ethanol, and diluted with isotonic saline to give final 20% ethanol/saline solution. Anesthetized rats under isoflurane/O2 were injected in the tail vein with 20 μ Ci of the labeled compound in a volume of 150 μ L. One set of animals was also coinjected with 8 μ g of estradiol (E_2) to block uptake mediated by the estrogen receptor. At selected time points postinjection (30 min, 1 h, and 2 h), rats were sacrificed. Blood and organs were removed, blotted, weighed, and counted in a Gamma 8000 well counter along with a standard dilution of the injectate. Uptake was calculated as percent injected dose (%ID) per gram and per organ. Blood weight is assumed to be 7% body weight (BW), muscle 41% BW, and bone 10.9 BW.

In the second biodistribution study, female estrogen receptor- knock-out (α ERKO) and estrogen receptor- β knock-out (β ERKO) mice (ca. 22 g, 6-8 weeks old, n = 3 each per time point) were used to determine whether the labeled estrogen ligand shows any subtype selectivity. The ERKO mice were allowed to recover from the stress of shipping for 48 h, and the estrous cycle was checked by swabbing vagina at 72 h postarrival. Attempts were made to stage these animals to a nonestrus condition for the biodistribution study. The mice were immediately placed on a special chow which was estrogen free in order to decrease their circulating estrogens. In the first case, nine each of α ERKO and β ERKO mice in a nonestrus stage were each injected with 11 μ Ci of [¹⁸F]FEDPN (130 μ L of 10% ethanol/saline). The remaining mice were kept in the same cage, in the hope that they would begin cycling together; however, we ended up with all of aERKO mice cycling together. Nine of the α ERKO mice and two of the β ERKO mice that were cycling with them were injected with 13 μ Ci of [¹⁸F]-FES (100 μ L of 10% ethanol/saline) and sacrificed (α ERKO – 30 min, 1 and 2 h (n = 3 each); β ERKO - 30 min (n = 2)).

Unfortunately, the repeated vaginal swabbing in some mice used to stage the estrous cycle seemed to induce false pregnancy. Even though these mice were not swabbed for an additional 9 days, they did not come out of pseudopregnancy. As a result, the biodistribution study of [18F]FES was completed by employing β ERKO mice in estrus (n = 3 each at 30 min, 1 h, 2 h). Three serum samples of β ERKO (during estrus) and normal BALC/c mice were sent out for the measurement of circulating estradiol level.

Acknowledgment. This work was supported by the United States Department of Energy (DEFG02-84ER-60218) and the National Institutes of Health (PHS 5R37 CA25836). We thank Nicole M. Fettig and Lynne A. Jones for their technical support in the biodistribution study. The authors also thank Nerissa Torrey for preparation of [¹⁸F]FES and Kathryn Carlson for the binding affinity measurements. Mass spectrometry was provided by the Washington University Mass Spectrometry Resource, an NIH Research Resource (Grant No. P41RR00954).

Supporting Information Available: Additional experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM050121F