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A high-affinity subtype-selective fluorescent probe for estrogen receptor β imaging in living cells†

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Zhiye Hu, ‡ Lu Yang, ‡ Wentao Ning, Chu Tang, Qiuyu Meng, Jie Zheng, Chune Dong, and Hai-Bing Zhou*

Estrogen receptor β (ERβ) has recently been identified as a pharmaceutical target in hormone replacement therapy for breast cancers. However, the biological function of ERβ in disease progression remains unclear. A highly ERβ-selective fluorescent probe (FPNM) was discovered exhibiting nanomolar affinity for ERβ with an ERβ/ERα selectivity as high as 80, which allowed specifically labeling of intracellular ERβ. Moreover, distinct ERβ dynamics in various cellular bio-settings such as prostate cancer (DU-145) or triple-negative breast cancer (MDA-MB-231) cells were directly observed for the first time *via* FPNM staining.

The estrogen receptor (ER), mediating the effects of estrogens on their target tissues, plays a fundamental role in physiological functions, such as the control of cell differentiation and the regulation of cell proliferation.[1-4] These effects are mainly mediated via two subtypes of ER: estrogen receptor α (ERα) and estrogen receptor β (ERβ). These two receptors have different distributions in the body and distinct biological functions.[5, 6] ERα was first cloned in 1986 and considered to be the sole ER at that time, however, in 1996, the second estrogen receptor subtype (ERβ) was found and a lot of efforts have been made to study the function of ERβ. ERβ is now considered as a novel pharmaceutical target with therapeutic potential in various diseases and symptoms.[7, 8] For example, studies have shown that selectively target the ERβ may play an important role in cancer treatment.[9] Some available data suggested that selective activation of ERβ represented one of safe therapies for the treatment of Alzheimer's disease.[10] However, the precise expression of this important receptor and its changes in disease processes are not clear.[11] Most importantly, approximately 12-17% of all breast cancers is classified as triple-negative breast cancer (ERα[-]/PR[-]/Her2[-], TNBC), which is difficult to be diagnosed because it has no specific biomarker.[12] The presence of ERβ in triple-negative breast cancer[13] attracted attention because ERβ could be used as a target for diagnostic imaging and radiotherapy of this cancer and it can be detected and quantified by a specific fluorescent ERβ ligand. Such agents could be used to delineate the ERβ positivity of tumors *in vivo* and *in situ* in a noninvasive, comprehensive fashion, even in metastatic tumors and lymph nodes that are inaccessible to surgical or needle biopsy.[14-16] This information can sometimes be used to evaluate tumor aggressiveness and predict the likelihood of responsiveness of TNBC to endocrine therapies.[17] Therefore, there is an urgency to develop probes for ERβ imaging.

To date, fluorescence imaging is widely used to visualize cell biology at cellular and molecular levels.[18] Particularly, small molecule fluorescent probes are attractive for biological studies because of their good cell permeability and minimal interference to living cells.[14] In the past few decades, several fluorescent conjugates for ER with diverse chemical structures have been designed, such as estradiol (E_2) derivatives E_2-Alexa 546, E_2-PBI and EE_2-FI,[19-21] tamoxifen derivative OHT-6C-BODIPY,[19] cyclofenil derivative cyclofenil-SNAPFL,[21] raloxifene derivative Ral-MiDye and some other molecules with different scaffolds.[22] However, all of these conjugates showed weak subtype selectivity because conjugating the fluorescent group to ERβ ligand could decrease the ERβ binding affinity, even attaching a small linker to the ERβ ligand also led to the loss of binding.[22] So it is challenging to develop ERβ-targeted

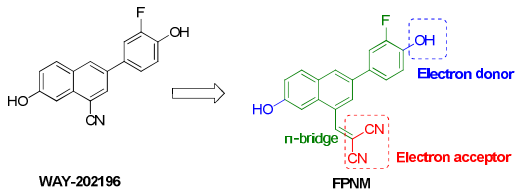

Fig. 1 Design of estrogen receptor β selective fluorescent probe (**FPNM**).

Hubei Province Engineering and Technology Research Center for Fluorinated Pharmaceuticals, Hubei Provincial Key Laboratory of Developmentally Originated Disease, State Key Laboratory of Virology, Wuhan University School of Pharmaceutical Sciences, Wuhan 430071, China. E-mail: zhouhb@whu.edu.cn; Tel: 862768759586

† Electronic Supplementary Information (ESI) available: Detailed experimental procedures, ^{1}H NMR and ^{13}C NMR spectra of compounds are provided. See DOI: 10.1039/x0xx00000x

‡ These two authors contributed equally to this work.

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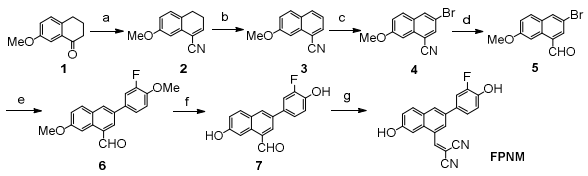
fluorescent probes. Recently, we reported a coumarin-based native fluorescence probe, which had high affinity for ER without conjugating a fluorescent dye, however, expressed low ERβ selectivity.[23] In addition, as we know, ERα and ERβ share high degrees of homology in their binding sites, their binding pockets are nearly the same, with only L384 and M421 in ERα replaced by M336 and I373 in ERβ, respectively.[24] Despite of the recent progress made on developing ERβ-targeted fluorescent probes, the poor selectivity makes it difficult to find fluorescent probes for ERβ imaging. Therefore, how to harness the difference of ERα and ERβ is the key point for the design of ERβ-selective fluorescent probes.

WAY-202196 (Fig. 1) is a well-studied ERβ selective and latent fluorophore ligand,[25] however, its short emission wavelength and low fluorescence quantum limited its wider use. Based on this compound, a long-wavelength fluorescent probe **FPNM** with excellent fluorescence properties was designed for ERβ imaging in live cells, which followed the principle of the intermolecular charge transfer (ICT).[26] Herein, we introduced the dicyanomethylene substituent as the electron acceptor, the phenol hydroxyl groups of lead compound were selected as the electron donor. And the donor-acceptor architectures were bridged by a conjugated π-electron chain. Unlike the reported fluorophore-labeled conjugate probes, the hydroxyl group of phenol and dicyanomethylene group used in the design of the ERβ selective fluorescent probe were also pharmacophores, and always acted as important substituents for ER binding.[27] Moreover, only one "π-electron bridge" was incorporated into the lead compound to significantly reduce the molecular weight, and with conjugation of a double bond, the HOMO-LUMO energy gap of probe becoming smaller, resulting in a significant red shift both in absorption and emission spectra. Here we report the first fluorescence imaging probe, **FPNM**, which shows preferable binding affinity to ERβ over ERα and is suitable for ERβ imaging in living cells.

The synthesis of **FPNM** is presented in Scheme 1. To construct the target molecule, tetralone **1** was converted to the cyano derivative **2**, which was aromatized with Pd/C in *p*-cymene to afford **3**, dibromination of latter followed by selective debromination leading to the key intermediate **4**. Subsequent elaboration of the cyano group to the aldehyde derivative **5**, which coupled under Suzuki conditions led to the phenylnaphthalenes **6**. Finally, treatment of the intermediate



Scheme 1 Synthesis of probe **FPNM**. Reagents and conditions: (a) TMSCN, ZnI_2; (b) 10% Pd/C, p-cymene, reflux; (c) (i) Br_2/AcOH, (ii) $SnCl_2$; (d) DIBAL; (e) $ArB(OH)_2$, $Pd(PPh_3)_4$, K_2CO_3, toluene, 120 °C , 24 h; (f) Pyridine hydrochloride, 190 °C, 2 h; (g) Pyridine hydrochloride, EtOH, 60 °C, 24 h.

Table 1 ERα and ERβ relative binding affinity (RBAs)[a]

Compound	RBA ERα	RBA ERβ	β/α
WAY-202196	1.62 ± 0.32	138.53 ± 6.64	85.51
FPNM	0.214 ± 0.06	17.31 ± 4.4	80.9

[a]Relative binding affinity (RBAs) values are determined by competitive radiometric binding assays and are expressed as $IC_{50}^{estradiol}/IC_{50}^{compound} \times 100$ ± the range or standard deviation (RBA, estradiol = 100%).[28]

with pyridinium hydrochloride could give **7** and the final compound was prepared by condensation of the aldehyde with malononitrile in ethanol. The reaction could be completed within 24 h with high yield (86%). (See ESI† for synthesis details)

We first tested the binding affinities of **FPNM** for both ERα and ERβ by a competitive radiometric receptor-binding assay and the results were reported in Table 1. These affinities were presented as relative binding affinity (RBA) values, where E_2 has an affinity of 100. For reference, the K_D of estradiol is 0.2 nM for ERα and 0.5 nM for ERβ. As expected, **FPNM** showed low binding affinity for ERα, but high affinity for ERβ. The RBA values of **FPNM** were 0.21 and 17.31 for ERα and ERβ, respectively, and the ERβ/ERα selectivity was as high as 80.9. Compared to the parent compound WAY-202196 (RBA values were 1.62 for ERα and 138.53 for ERβ; β/α was 85.51), **FPNM** still retained high binding affinity and good selectivity for ERβ (Table 1).

To gain insight into the binding nature of **FPNM** to ERβ, molecular docking simulations were performed. Molecular modeling showed that the phenol of **FPNM** mimicked the estradiol A-ring,[29] where it interacted with ERβ residues Glu305, Leu 339, Arg 346 and a highly ordered water molecule. In addition, the naphthol hydroxyl projects between helices 3 and 11, and dicyanomethylene group was directed towards helice 8. The core scaffold, consisted with the phenyl and the naphthalene ring, filled the remainder of the primarily hydrophobic pocket, and **FPNM** would behave in a similar affinity and selectivity for ERβ to the parent compound.

Naphthalene is the core of several highly fluorescent entities including our target compound **FPNM**,[30, 31] the absorption and fluorescence properties of **FPNM** were evaluated. As anticipated, with the electron-deficient acceptor as the end

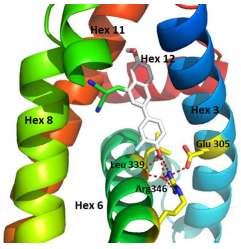


Fig. 2 Model of **FPNM** bound to ERβ. Computer-developed model of **FPNM** bound to ERβ with the conserved H-bond to Glu 305, Leu 339, Arg 346 and an ordered water molecule, and the core scaffold fills the remainder of the primarily hydrophobic pocket.

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Table 2 Photophysical data of **FPNM** in CH_2Cl_2[a]

Compound	λ_{ex} [nm]	λ_{em} [nm][b]	Φ [%][c]	ε[M^{-1} cm^{-1}][d]
WAY-202196	368[a]	414	1.8[b]	ε_{322} = 6200
FPNM	447	593	10.3	ε_{447} = 24010

[a]Photophysical properties at 10.0 μM. [b]Emission maximum excited at the maximum excitation wavelength. [c]Fluorescence quantum yields (Φ_{fl}) were determined using fluorescein as a reference. [d]Molecular extinction coefficient.

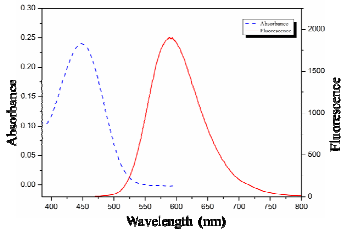

Fig. 3 Excitation and emission spectra of **FPNM**.

group bridged by a π-conjugated system, the probe underwent an excited state charge transfer (CT) accompanied by a larger dipole moment, resulting in a significant red shift on the emission wavelength. As shown in Table 2 and Fig. 3, the absorption maximum was 447 nm with an emission maximum at 593 nm (Stokes shift = 146 nm). The fluorescence performance of **FPNM** was significantly improved compared to the lead compound WAY-20219.

We further explored whether **FPNM** could be used for imaging of ERβ in living cells. Human metastatic prostate cancer cells (DU-145 cells) and triple-negative breast cancers (MBA-MD-231 cells) were chosen as model cell lines. DU-145 cells only express ERβ,[32, 33] after incubation with **FPNM** (10 μM) at 37 °C for 30 min, the ERβ of DU-145 cells exhibited bright fluorescence and fluorescence could be observed mainly in nucleus (Fig. 4c). We next used the E_2 to block the binding of **FPNM** with ERβ. As shown in Fig. 4g, after the treatment of E_2, the fluorescence signals markedly disappeared. Therefore, these results clearly showed that **FPNM** could specifically label intracellular ERβ in subcellular regions. Next, live-cell images were obtained after **FPNM** incubated with MBA-MD-231 cells, which also express ERβ protein.[13] As shown in Fig. 4, fluorescence was observed mainly in nucleus (Fig. 4k) and was sharply decreased by the addition of the competitive reagent (Fig. 4o). These results further confirmed that **FPNM** had the potential to meet the need of monitoring ERβ expression in MBA-MD-231 cells. In addition, due to its cell and organelle permeability, this compound could be used as ERβ protein-selective probe.

Moreover, in an attempt to further prove the ERβ selectivity of **FPNM**, MCF-7 cells were also chosen as another cell line model. MCF-7 is a cell line derived from human breast cancer, which expresses both ERα and ERβ. **EE_2-FI** (Fluorescein Ethynylestradiol Conjugate) was used as positive control, which specifically labeling the ER (ERα and ERβ).[21] As shown in Fig. 5c-d, ER was localized in both the cytoplasm and the

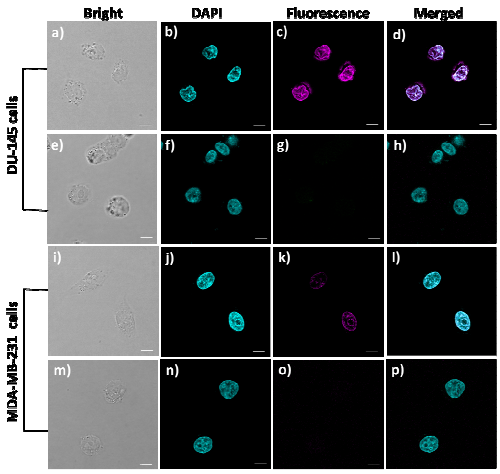

Fig. 4 Confocal images of DU-145 and MDA-MB-231 cells. (a-d) and (i-l) Images of cells treated with **FPNM** (10.0 μM). (e-h) and (m-p) Addition of 10-fold higher parent drug (E_2) completely blocked uptake of **FPNM**. Scale bar: 10 μm.

nucleus.[20, 34] In the presence of **FPNM**, a clear labeling could be observed in MCF-7 cells and it mostly stained in the nucleus (Fig. 5g). This result is similar to Hartman's research by immunofluorescence imaging of ERβ in MCF-7 cells.[13] In addition, the fluorescence signals significantly reduced in the presence of a competitive ER ligand (E_2) (Fig. 5k). As we know, **FPNM** exhibited poor binding affinity for ERα, with an RBA value not exceeding 1 (RBA, estradiol = 100). As a result, fluorescence derived from the complex between the probe and ER was detected mainly in regional area of nucleus (Fig. 5g-h), this observation reflects the typical cellular localization of ERβ,[13] suggesting that **FPNM** showed specifically selective affinity towards ERβ in living cell imaging. Furthermore, co-localization with ERβ antibody study also showed that **FPNM** could specific label the ERβ in MCF-7 cells (Fig. 6c-e), DU-145 and MDA-MB-231 cells (Fig. S1c-e and h-j). Besides, we also conducted co-staining study of **FPNM** with **EE_2-FI**, as shown in

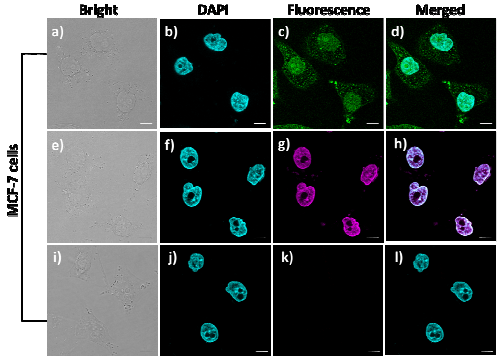

Fig. 5 Confocal images of MCF-7 cells. (a-d) Images of cells treated with **EE_2-FI** (10.0 μM). (e-h) Images of cells treated with **FPNM** (10.0 μM). (i-l) Addition of 10-fold higher parent drug (E_2) completely blocked uptake of **FPNM**. Scale bar: 10 μm.

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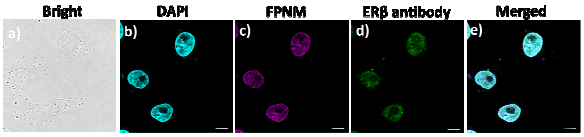

Fig. 6 Co-localization of MCF-7 cells with **FPNM** (10.0 μM) and ERβ antibody. Scale bar: 10 μm.

Fig. S2, the fluorescent images of **FPNM** with **EE_2-Fl** overlapped very well in DU-145 and MDA-MB-231 cells which mainly express ERβ in nucleus (Fig. S2c-e and h-j). In contrast, in MCF-7 cells, which express both ERα and ERβ, and the ERα was expressed both in the cytoplasm and nucleus in this cell line,[20, 34] images in cell cytosolic were also observed in co-staining experiments of **EE_2-Fl** (Fig. S2n). These results indicated that **FPNM** has the ability to selectively stain ERβ in living cells.

In conclusion, we successfully designed and synthesized the ERβ high selective fluorescent probe **FPNM**, which showed preferable binding to ERβ over ERα *in vitro*. To the best of our knowledge, **FPNM** is the first fluorescent probe suitable for visualizing ERβ over ERα preferentially. It would be allowed for evaluating ERβ expression levels and also offering a more direct characterization of tumor lesions to better predict and assess the disease. This high subtype selective probe offers a powerful way to study the function of ERβ in live cells, which may greatly contribute to further investigation on the roles of ERβ in breast cancers, and some other cancers.

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Conflicts of interest

There are no conflicts of interest to declare.

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Distinct ERβ dynamics in various cellular bio-settings were directly visualized for the first time *via* the fluorescent probe **FPNM** staining.