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Fluorescent Ligand for Human Progesterone Receptor Imaging in Live Cells

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Supporting Information

ABSTRACT: We employed molecular modeling to design and then synthesize fluorescent ligands for the human progesterone receptor. Boron dipyrromethene (BODIPY) or tetramethylrhodamine were conjugated to the progesterone receptor antagonist RU486 (Mifepristone) through an extended hydrophilic linker. The fluorescent ligands demonstrated comparable bioactivity to the parent antagonist in live cells and triggered nuclear translocation of the receptor in a specific manner. The BODIPY labeled ligand was applied to investigate the dependency of progesterone receptor nuclear translocation on partner proteins and to show that functional heat shock protein 90 but not immunophilin FKBP52 activity is essential. A tissue distribution study indicated that the fluorescent ligand preferentially accumulates in tissues that express high levels of the receptor in vivo. The design and properties of the BODIPY-labeled RU486 make it a potential candidate for in vivo imaging of PR by positron emission tomography through incorporation of 18F into the BODIPY core.

The progesterone receptor (PR) is a ligand-activated steroid receptor that belongs to the nuclear receptor superfamily of transcription factors.1,2 PR is expressed at low levels in most physiological systems but peaks in the female reproductive system and in the central nervous system.3,4 Thus, it plays a central role in reproductive events and sexual behavior. PR dysfunction has been indicated in multiple disorders including reproductive conditions,5 neurological syndromes,6 and cancer (breast,7 ovarian,7 endometrial8). As such, considerable effort has been focused on understanding PR functions and their underlying mechanisms in normal and pathological conditions. The human PR is encoded by a single gene that is expressed as two isoforms, PR-A and PR-B, which share most of the functional elements but have distinct functions. While PR-A remains predominantly in the nucleus, PR-B resides mostly in the cytosol as part of a multiprotein complex, which modulates its activity. According to current understanding, upon ligand binding PR-B dissociates from at least part of the complex, dimerizes, and translocates to the nucleus, where it recruits coregulating proteins and binds specific DNA sequences to exert its transcriptional effect. Recently, fusions of fluorescent protein tags to PR and its regulators have enabled their imaging with high spatial and temporal resolution, significantly improving understanding of dynamic processes such as localization, cell cycle dependence, and recycling.9–11 However, this approach requires genetic manipulation, expression of non-native PR, and often the use of cells that do not express PR endogenously. Complementary to receptor labeling, fluorescent ligands offer advantages such as receptor imaging in endogenously expressing cells, quantification of ligand–receptor interactions, and measurement of receptor–ligand complex diffusion rates.12 While biologically functional fluorescent ligands for many G protein-coupled receptors,13 retinoic acid receptor,14 and estrogen receptor15 have been reported, efforts to develop fluorescent ligands for PR were either unsuccessful16 or have not been applied to receptor imaging.17,18 The only functional fluorescent PR-ligand in mammalian cells was reported almost a decade ago, when fluorescein labeled RU486 (Mifepristone), a PR antagonist, was demonstrated to concentrate in the nuclei of PR expressing cells.19 However, it required prolonged incubation time and cells had to be fixed prior to imaging. Recently, an elegant procedure for fluorine displacement in boron-dipyrromethene (BODIPY) dyes has been described20 which was later used to introduce a 18F radioisotope into a BODIPY scaffold to generate a dual fluorescence/positron emission tomography (PET) imaging reagent.21 Other chemistries for rapid incorporation of a PET isotope into a BODIPY scaffold to generate a functional fluorophore exist, e.g., a near-infrared-absorbing cyanine dye with a pendant fluaborate,22 but the size of that dye and its polar substituents would probably prevent membrane permeation. With this in mind, we sought to develop a PR...
fluorescent ligand based on a BODIPY dye that could be used for fluorescent imaging of PR in vitro and potentially be translated into a PET tracer for PR imaging in vivo, without modifying the original structure.

RU486 is a synthetic 19-nor steroid that acts as a competitive antagonist to PR (Figure 1). It has high affinity for PR ($\sim$5 times lower $K_d$ compared to the natural agonist progesterone $^{23}$), and upon binding to PR, it preserves many of the processes initiated by progesterone binding, i.e., dissociation of PR from the multiprotein complex, dimerization, translocation to the nucleus, and DNA binding. The main functional activity as determined by ratio of IC$_{50}$(RU486) to IC$_{50}$ of tested compound. * Measured using shake flask method. In parentheses: from DrugBank [DB00834]. $^{a}$Units in nm.
S3d). Taken together, these results show that RU486-BODIPY and RU486-TAMRA can bind PR as high affinity antagonists with spectroscopic properties suitable for fluorescence imaging.

Next, we evaluated the fluorescent ligands for imaging endogenously expressed PR in live cells. In T47D cells incubated with 5 nM RU486-BODIPY, fluorescence was almost entirely confined to the nuclei and excluded from the nucleoli (Figure 2a). Low levels of fluorescence were also detectable in the cytoplasm. Interestingly, the nuclear distribution of the fluorescent ligand was retained even 24 h after a brief incubation (SI Figure S4). This nuclear accumulation can be reversed by applying unlabeled RU486 (SI Figure S5), thus representing specific binding of RU486-BODIPY to PR and not with GR agonist. T47D cells were coincubated with 5 nM RU486-BODIPY and 100 nM progesterone (PR agonist) or dexamethasone (GR selective). While excess progesterone completely inhibited accumulation of fluorescence in the nuclei, dexamethasone had no observable effect (Figure 2b), demonstrating the specificity of the fluorescent ligand to PR in this experimental setting. In addition, this result establishes that RU486-BODIPY binds PR through the ligand binding domain (LBD) and not through allosteric sites.

RU486-TAMRA showed similar accumulation patterns as RU486-BODIPY, concentrating in the nuclei of T47D cells but not in MDA-MB-231 cells. Nuclear localization was similarly specific to PR and persisted for at least 24 h (SI Figure S8a–c). In contrast to RU486-BODIPY’s tendency to accumulate in membranes in the absence of PR, RU486-TAMRA was easily washed out, maintaining a high ratio of nuclear-to-cytoplasmic fluorescence even at high concentrations (SI Figure S9), probably due to its higher hydrophilicity. In addition, fluorescence was accumulated in the nucleus at a much faster rate than RU486-BODIPY (∼18 min, SI Figure S10). However, a higher concentration was required to observe its effect (SI Figure S9).

Altogether, these results demonstrate that the fluorescent ligands specifically bind human PR in T47D cells, causing PR to translocate to the nucleus and slow down the receptor’s recycling process, thus mimicking the biological effects of unlabeled RU486.

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After establishing that the fluorescent ligands retain many of the biological properties of RU486, we applied RU486-BODIPY to study the dependency of PR nuclear translocation process on proteins involved in its complex. In vitro assembly studies established the identity of the proteins required for a functional PR complex as well as the order and stoichiometry of their assembly.29 Heat shock protein 90 (HSP90) is a molecular chaperone involved in many cellular processes and is a key component in PR complexes. In addition to stabilizing PR in the cytoplasm, HSP90 potentiates PR’s hormone-dependent response by binding to its LBD, causing it to adopt an open conformation that allows the hormone to enter and bind.30 This process is ATP-dependent31 and was demonstrated in binding assays to be inhibited by geldanamycin,32 a specific inhibitor of HSP90 ATPase domain. Therefore, we first tested whether 17-AAG (17-N-allylamino-17-demethoxygeldanamycin), a less toxic synthetic derivative of geldanamycin, interferes with RU486-BODIPY nuclear accumulation. Indeed, in T47D cells treated with 17-AAG for one hour prior to RU486-BODIPY application, nuclear fluorescence accumulation was inhibited in a dose-dependent manner (Figure 3a,b). The half-maximal effective concentration (EC_{50}) of 17-AAG for PR nuclear translocation inhibition in this experimental setting was eventually surpassing the nuclear signal (SI Figure S7). When MDA-MB-231, an epithelial breast cancer cell line that does not express PR, was similarly treated with RU486-BODIPY, fluorescence was completely excluded from the nuclei but was observed in the cytoplasm (Figure 2a). The cytoplasmic retention of RU486-BODIPY in the absence of its target binding site (i.e., PR) represents nonspecific binding which is probably a result of the molecule’s hydrophobicity (log P = 3.5). Another possible consequence of the hydrophobicity of RU486-BODIPY is the extended time required for PR nuclear translocation process to complete (∼1 h). Antiprogestins, such as RU486, have been found to bind to both the PR and the glucocorticoid receptor (GR) with high affinity. We therefore tested the specificity of RU486-BODIPY nuclear accumulation in T47D cells by competing it with 20-fold excess of either progesterone (PR selective) or dexamethasone (GR selective). While excess progesterone completely inhibited accumulation of fluorescence in the nuclei, dexamethasone had no observable effect (Figure 2b), demonstrating the specificity of the fluorescent ligand to PR in this experimental setting. In addition, this result establishes that RU486-BODIPY binds PR through the ligand binding domain (LBD) and not through allosteric sites.
76 ± 11 nM, much lower than its reported IC_{50} in T47D cells (3.82 ± 0.97 μM upon 24 h treatment). Inhibition of histone deacetylases (HDAC) by broad spectrum inhibitors such as vorinostat (SAHA) and panobinostat (LBH-589), which leads to HSP90 hyper-acetylation and dysfunction, also resulted in a marked decrease of nuclear fluorescence accumulation (Figure 3c and SI Figure S11). Together, these results reinforce the importance of a functional HSP90 to PR translocation and demonstrate that RU486-BODIPY is effective in sensing perturbations to this process. HSP70 is another chaperone involved in the early steps of PR complex assembly. The highest uptake of RU486-BODIPY was observed in the liver, suggestive of its role in vivo, preferentially in tissues that naturally express high levels of progesterone receptor. The fluorescent ligands for the human progesterone receptor. The fluorophore BODIPY was recently reported speciﬁcally to the progesterone receptor in vivo; hence its effect on PR cannot be solely attributed to HSP70 inhibition. FKBPs (FK506-binding protein 4) is an immunophilin thought to act in steroid-receptor complexes, including PR, as an adapter to the motor protein dynein to facilitate receptor shuttling along cytoskeletal tracks. Inhibition of FKBPs (peptidylprolyl isomerase) by FK506 has been demonstrated to block PR hormone-dependent transcription activation. Therefore, we tested the effect of FKBPs-potent inhibitors on PR trafficking. Pretreatment of T47D cells with FK506 (10 μM, 12 h) had no effect on RU486-BODIPY nuclear accumulation. In addition, inhibition of dynein by erythro-9-amino-β-hexyl-α-methyl-9H-purine-9-ethanol (EHNA, 500 μM, 1 h) also failed to affect PR translocation. In conclusion, we have designed and synthesized two fluorescent ligands for the human progesterone receptor. The ligands show antagonistic potency comparable to their parent translocation does not exclusively rely on active cytoskeletal transport and highlight the need to clarify the role of active movement machinery in PR complex shuttling. In addition, they also suggest that the requirement for FKBPs activity in PR transcriptional activity is downstream of the nuclear translocation process.

Finally, we tested whether RU486-BODIPY will accumulate preferentially in tissues that naturally express high levels of progesterone receptor in vivo. We first evaluated the detectable dose by intravenous injection of either 1 or 10 nmol RU-486-BODIPY into FBV/N female mice (one mouse each) and analyzing tissue uptake by HPLC/MS/MS 4 h postinjection. While the lower dose was hardly detectable in any tissue (data not shown), at the higher dose, RU486-BODIPY was detected in most analyzed tissues. Therefore, we used the high-dose conditions (10 nmol) to similarly treat and analyze three more mice (Figure 3d). At 4 h post injection, no probe was detected in the blood or the brain. Although RU486 has a very long half-life in human, in rodents it is considerably reduced (30 vs 1 h, respectively). The highest uptake of RU486-BODIPY was observed in the liver, suggestive of its role in metabolism and excretion of the probe, and also in accordance with RU486 biodistribution in rodents. Importantly, RU486 BODIPY accumulation in uterus was consistently and significantly higher than in muscle (∼3.5-fold on average). The ovaries also showed a consistently higher uptake than muscle (∼1.2-fold), but this difference was not statistically significant.

In conclusion, we have designed and synthesized two fluorescent ligands for the human progesterone receptor. The ligands show antagonistic potency comparable to their parent

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**Figure 3.** Effect of PR-multiprotein-complex modulators on RU486-BODIPY mediated PR nuclear translocation. (a) Dose–response curve of PR nuclear translocation in T47D cells treated with HSP90 inhibitor 17-AAG. Cells were incubated with 17-AAG at indicated concentrations for 1 h before treated with 5 nM RU486-BODIPY. Each data point represents mean nuclear (●) or cytoplasmic (▲) fluorescence intensity of 30 cells. Error bars represent ± SD. (b) Effect of 17-AAG on PR nuclear translocation at 0.1 and 10 μM. Scale bar 20 μm. (c) Cellular distribution of 5 nM RU486-BODIPY in T47D cells pretreated with: 0.1% DMSO for 12 h (control), 10 μM 17-AAG or 500 μM EHNA for 1 h, 10 μM FK506 for 2 h, 10 μM vorinostat, panobinostat, VER-155008, or methylene blue for 12 h. Each bar represents the ratio of mean nucleus-to-cytoplasm fluorescence of 30 cells. Error bars represent ± SD. * p < 0.01 (two-tail t-test). (d) Tissue distribution of RU486-BODIPY in FVB/N female mice 4 h post LV. injection (n = 4). Error bars represent ± SD. * p < 0.05 (one-tail t-test).

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RU486 in live cells and have spectroscopic properties suitable for fluorescence imaging. Both ligands triggered PR nuclear translocation in a receptor-dependent and specific manner in endogenously expressing cells. RU486-BODIPY was used to study the effect of PR complex components inhibition on its nuclear translocation process. Our results reinforce the importance of functional HSP90 in this process as both inhibition of its ATPase activity and its hyperacetylation, led to impaired PR shuttling. In addition, we found that FKBP52 activity is not essential for PR nuclear translocation, suggesting that FKBP52 plays a role in PR activation after the nuclear accumulation process. Finally, RU486-BODIPY preferentially accumulated in tissues that express high levels of PR in vivo. Thus, RU486-BODIPY’s design and properties make it a potential candidate for in vivo imaging of PR by PET through incorporation of 18F into the BODIPY fragment. Noninvasive whole-body imaging of steroid receptors could be of considerable value in classifying and staging many cancers of the endocrine and reproductive systems.

**ASSOCIATED CONTENT**

† Supporting Information

Synthetic procedures, chemical and spectral characterization, cell culture and imaging methods, alkaline phosphatase assay details, additional figures as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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