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A Bodipy as Luminescent Probe for Detection of the G Protein Estrogen Receptor (GPER)

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We report the rational design, based on docking simulations, and synthesis of the first fluorescent and selective probe of GPER for bioimaging purposes and functional dissecting studies. It has been conceived as a Bodipy derivative and obtained by an accessible and direct synthesis. Its optical properties have been measured in different solvents, showing insensitivity to their polarity. Its binding to GPER was achieved by competition assays with [3H]E2 and [5,6-3H] nicotinic acid in ER-negative and GPER-positive SkBr3 breast cancer cells. SkBr3 cells, transfected with a GPER expression vector containing a FLAG tag, were used to confirm that the fluorophore binds to GPER in a specific manner.

The identification of the G protein estrogen receptor (GPER, formerly known as GPR30) deeply contributed to a better understanding of the multifaceted action elicited by estrogens in a variety of physio-pathological processes.[1-5] For instance, ligand-activated GPER has been shown to trigger diverse transduction pathways and biological responses in both normal and malignant cells upon exposure to numerous chemicals, including antagonists of the classical estrogen receptor (ER).[1-3] The role of GPER is still a focus of investigation as a target for cancer therapy.[1,5-6] Moreover, in vivo studies have demonstrated that the expression of GPER correlates with negative clinical features of breast, endometrial and ovarian tumors.[6]

The rational design and synthesis of a selective agonist, 1-[(3a*S*,4*R*,9b*R*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]ethanone, known as **G-1** (Fig. 1),[7] represented a milestone in the development of research focusing on GPER-mediated signalling.[1,8,9] Thereafter, our and other groups developed further probes,[10] all contributing to the increasing interest to this research field. Overall, the development of selective GPER ligands for diagnostic imaging using single photon emission computed tomography (SPECT) or positron emission tomography (PET) has been reported.[11]

Noninvasive imaging technology offers great promise for the in vivo characterization of estrogen-dependent cancers. Near InfraRed Fluorescent (NIRF) imaging has a good sensitivity to visualize tumor using optical probes, comparable to that of other diagnostic imaging technologies, such as PET or SPECT, that appear much more invasive and expensive.[12] NIRF needs the use of fluorescent imaging agents and the simplest method of choice is represented by estrogen receptor ligands labeled with a fluorescent tag. However, such approach is often accompanied by insufficient binding affinity towards the receptor and competition problems with the natural agonist.

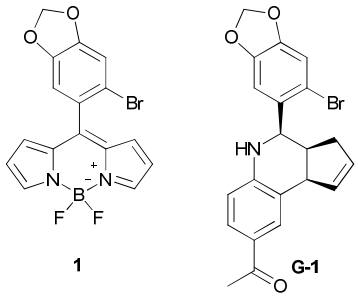

Figure 1. Structures of Bodipy **1** and **G-1**.

Herein, we describe the first model of an inherently fluorescent probe for GPER. Molecule **1** (Fig. 1) has been rationally designed and synthesized with the intention of achieving a class of new diagnostic imaging agents to be used in confocal scanning laser microscopy and possibly in novel therapeutic strategies for estrogen-sensitive tumors. It has been conceived as a borondipyrromethene difluoride (Bodipy) derivative possessing in its skeleton the bromobenzodioxolyl substituent, already present in the **G-1** skeleton and recognized as important structural motif for GPER affinity. Bodipy dyes are strongly UV-visible absorbing small molecules that exhibit relatively sharp and tunable fluorescence with

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high quantum yields and biocompatibility, so that they have been widely used for biological studies.[13]

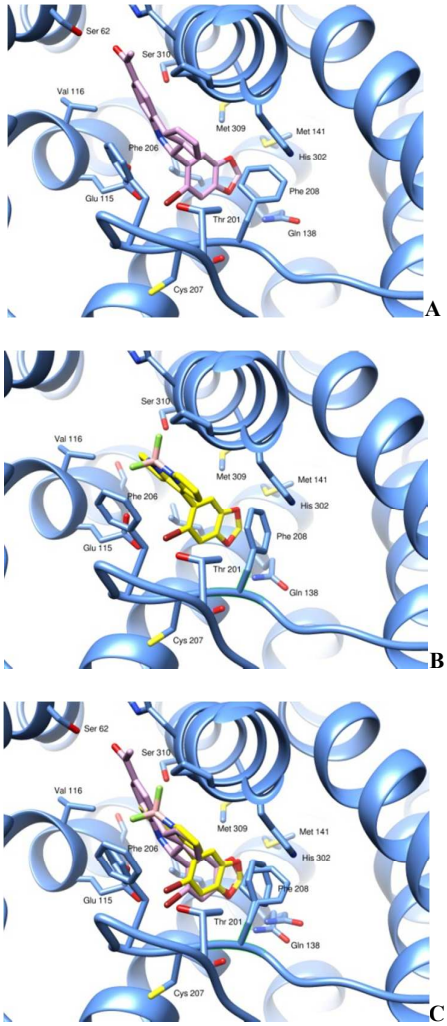
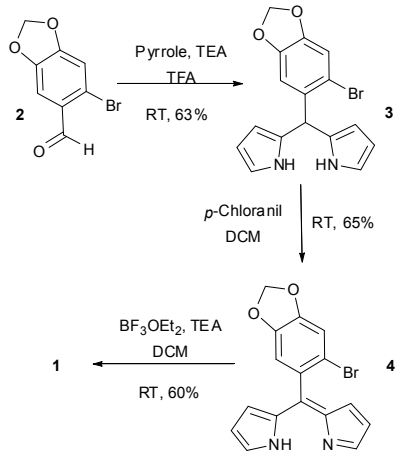


Figure 2. Visual representation of ligand binding modes to GPER (light blue ribbons). Protein residues that are involved in ligand binding are drawn as sticks Panel A) The agonist **G-1** (pink sticks), Panel B) Bodipy **1** (yellow sticks) binding modes. Panel C) Superposition of the two tested moieties.

To validate *in silico* the potential binding modes of Bodipy **1** to GPER, we performed docking simulations using as target the three dimensional structure of GPER modeled in our previous studies.[10a-d,14] Affinity of Bodipy **1** to GPER and its possible binding modes have been evaluated in comparison with the selective agonist **G-1** [see Supporting Information (SI)].
The binding pocket of the protein is a deep cleft encompassed by the GPER transmembrane (TM) helices and is composed by both hydrophobic and hydrophilic residues. Particularly, residues belonging to TM III, V, VI and VII are surrounding the binding site. As previously demonstrated both in silico and in vitro,[7,10a-d] our docking simulations confirmed a good affinity for the agonist **G-1** moiety (Fig. 2, Panel A). Using the same settings and parameters, a further simulation performed for Bodipy 1 showed a good affinity, with a binding mode to GPER like **G-1** (Fig. 2, Panel B). Bodipy **1** forms hydrogen bonds with Gln 138 and with the carboxyl of Pro 303, an halogen bond with Glu 115, and hydrophobic contacts with residues Val 116, Leu 137, Met 141, Phe 206 and Phe 208 contribute to stabilize the complex. Furthermore Phe 208 is in $\pi-\pi$ stacking with the bromobenzodioxole moiety. The same arrangement is adopted by the agonist moiety **G-1**, which, due to its longer side chain, also interacts with Ser 62 through the formation of a hydrogen bond (Fig. 2, Panel C).



Scheme 1. The synthetic procedure for Bodipy **1**.

Bodipy **1** was obtained in a three step synthesis from the commercially available 6-bromo-1,3-benzodioxole-5-carboxaldehyde (**2**) (Scheme 1).[15] Condensation of aldehyde **2** with an excess of freshly distilled pyrrole afforded dipyrromethane **3** in 63% yield. In the second step, dipyrromethane **3** was oxidized to dipyrromethene **4** in 65% yield, using an excess of 2,3,5,6-tetrachloro-1,4-benzoquinone (*p*-chloranil). The reaction of dipyrromethene **4** with BF_3OEt_2 afforded Bodipy **1** in 60% yield. Single crystals of Bodipy **1** suitable for X-ray crystallography were grown by slow evaporation from acetone. The structure shown in Fig. 3 (crystallographic details in SI) exhibits a C_S symmetry, with the bromo-benzodioxole and the borondipyrromethene difluoride moieties constrained into two skewed planes with a dihedral angle of 86°.

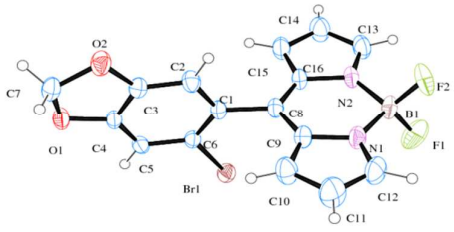


Figure 3. X-ray ORTEP rendition of Bodipy **1**.

The optical properties of Bodipy **1** have been investigated in dichloromethane (DCM), methanol and phosphate buffered solution (PBS). The absorbance and fluorescence properties are summarized in Table 1.

The absorption spectra (Fig. 4 and SI) display typical narrow bands with two absorption maxima in the visible region: an intense 0–0 band at about 500 nm attributed to π–π* transitions (S0→S1) and a shoulder on the high-energy edge of the main band, which is ascribed to the 0–1 vibrational transition.

Table 1. Absorption and emission data of Bodipy **1**.

solvent	Absorption	Emission		
	λ_{max},nm (ε, M^{-1} cm^{-1})[a]	λ_{max},nm	τ, ns	Φ
DCM	507 (71000)	523	2.2	0.10
MeOH	504 (71800)	520	2.5	0.08
PBS	504 (70500)	521	2.5	0.05

[a] Only the lowest-energy band maxima are reported.

Upon excitation at its absorption bands, Bodipy **1** exhibits intense fluorescence peaked at 520 nm. Although the luminescence quantum yield decreases moving from organic solvent to aqueous solution, the emission bands occur at almost the same position in the different solvents.

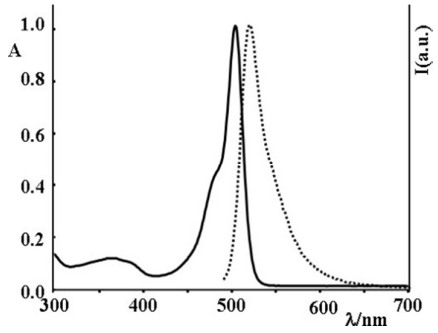


Figure 4. Absorption and emission spectra of Bodipy **1** in MeOH.

The insensitivity to the polarity of solvents is a very important factor in the bioimaging, in fact the dye labelled on a biomolecule should emit stable fluorescence and the emission energy should be independent from various external environments. Moreover, it is interesting to note that no significant changes have been observed (at the used concentrations), from a photophysical point of view, with respect to the analogous aryl substituted Bodipy species in which the bromobenzodioxole residue is not present.[16]

In order to investigate the ability of Bodipy **1** to bind to GPER, we performed competition assays using radiolabeled E2 as tracer in ER-negative and GPER-positive SkBr3 breast cancer cells.[10a-b,14] In line with the results obtained in docking simulations, Bodipy **1** showed the capability to displace [3H]E2 like E2 and **G-1** (Fig. 5, Panel A). In our previous study, nicotinic acid (NA) induced stimulatory effects in breast cancer cells by binding to GPER and activating its mediated signalling.[17]

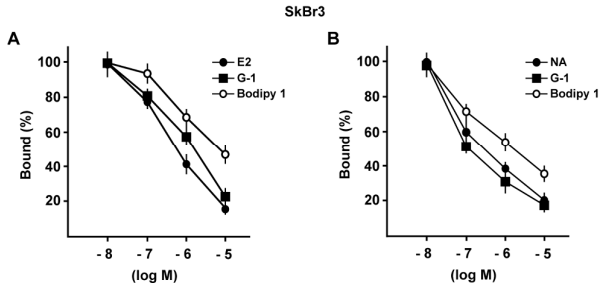


Figure 5. Bodipy **1** is a ligand of GPER. (A) Bodipy **1** competes with [3H]E2 for the binding to GPER in SkBr3 cells. Competition curves of increasing concentration of unlabelled E2, **G-1** and Bodipy **1** expressed as a percentage of maximum specific [3H]E2 binding. (B) Bodipy **1** competes with [5,6-3H]NA for the binding to GPER in SkBr3 cells. Competition curves of increasing concentration of unlabelled NA, **G-1** and Bodipy **1** expressed as a percentage of maximum specific [5,6-3H]NA binding. Each data point represents the mean ± SD of three separate experiments performed in triplicate.

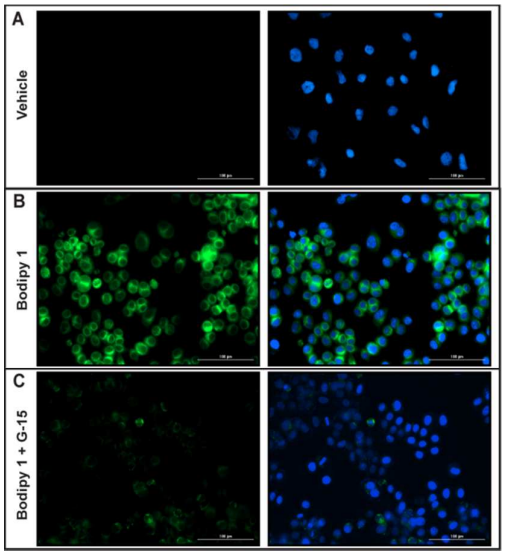


Figure 6. Fluorescence micrographs of live SkBr3 cells treated with Bodipy **1**. (A) SkBr3 cells treated with vehicle, nuclei were stained by DAPI (blue signal). Fluorescence micrograph upon 20 min incubation with 10 μM Bodipy **1** alone (green signal) (B) or in combination with 1 μM GPER antagonist **G-15** (C). Fluorescence was not observed in SkBr3 cells treated with **G-15** alone (1 μM) (data not shown). Each experiment shown is representative of 20 random fields observed each in three independent experiments.

In order to provide additional evidence on the ligand properties of Bodipy **1** to GPER, we performed competition assays using [5,6-3H]NA as tracer in SkBr3 cells that do not express the nicotinic acid receptors namely GPR109A and

GPR109B.[17] Worthy, Bodipy **1** displaced the radiolabeled tracer in a dose-dependent manner NA and **G-1** (Fig. 5, Panel B). Collectively, these results demonstrate that Bodipy **1** may be considered as a novel ligand of GPER.

Next, we aimed to characterize the fluorescence properties of Bodipy **1** by performing fluorescence-based assays in SkBr3 cells. First, cells were treated with increasing concentrations of Bodipy **1** (ranging from 1 nM to 10 μM) and monitored starting from 5 min up to 2 h. Cells treated with vehicle did not exhibit autofluorescence under our experimental conditions, whereas Bodipy **1** treated cells showed an intracellular green fluorescent labeling which was concentration- but not time-dependent (data not shown).

As shown in Fig. 6, the Bodipy **1** fluorescence signal was no longer evident in live SkBr3 cells treated also with the selective GPER antagonist G-15, further corroborating the binding specificity of Bodipy **1** to GPER. Next, we carried out immunofluorescence experiments in SkBr3 cells that were engineered to express a recombinant GPER-FLAG before Bodipy **1** treatment and fixation with paraformaldehyde.

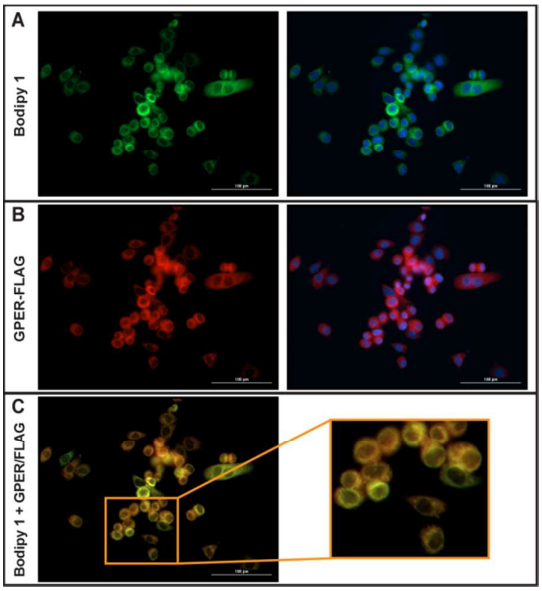


Figure 7. Fluorescent signal of Bodipy **1** co-localizes with GPER. SkBr3 cells were transfected for 36 h with a GPER expression vector containing a FLAG tag and thereafter treated with 10 μM Bodipy **1** (green signal, panel A). Then, cells were immunostained with the anti-FLAG antibody (red signal, panel B) and DAPI (blue signal of nuclei, panels A and B). The co-localization of Bodipy **1** and GPER-FLAG signals generates the orange signal visualized in panel C. Each experiment shown is representative of 20 random fields observed each in three independent experiments.

Notably, we observed a spatial overlap (characterized by the orange signal) between the fluorescence of Bodipy **1** (green signal) and that of the anti-FLAG antibody (red signal) (Fig. 7). These observations strongly confirm that Bodipy **1** binds to GPER in a specific manner. Collectively, our data suggest that Bodipy **1** could be considered as a selective GPER imaging agent useful to visualize the receptor localization and to elucidate key elements involved in GPER trafficking and interaction with other signal molecules and pathways.

In summary, we have conceived and synthesized the first fluorescent, selective ligand of GPER. Its unusual Bodipy skeleton opens great opportunities for structural modifications to improve both its affinity to GPER and its photochemical properties, the possibility of absorption and emission in the NIR included. Our results offer insights in the use of such promising probe not only as tool for diagnostic purposes but also for further investigations on GPER role and prospective therapeutic implications. Work is ongoing in this direction.

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