Sigma-2 receptor and progesterone receptor membrane component 1 (PGRMC1) are two different proteins: Proofs by fluorescent labeling and binding of sigma-2 receptor ligands to PGRMC1

Sigma-2 Receptor and Progesterone Receptor Membrane Component 1 (PGRMC1) are two different Proteins: Proofs by Fluorescent Labeling and Binding of Sigma-2 Receptor Ligands to PGRMC1

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Abstract

A controversial relationship between sigma-2 and progesterone receptor membrane component 1 (PGRMC1) proteins, both representing promising targets for the therapy and diagnosis of tumors, exists since 2011, when the sigma-2 receptor was identified as the PGRMC1. Because a misidentification of these proteins will lead to biased future research hampering the possible diagnostic and therapeutic exploitation of these targets, there is an urgent need to finally solve the debate on their identity. With this aim, we employed structurally different sigma-2 receptor fluorescent ligands by flow cytometry and confocal microscopy in MCF7 cells, where together with sigma-2 receptors, PGRMC1 was constitutively present or alternatively silenced or overexpressed. In addition, because the PGRMC1 crystal structure was recently explored and its functional heme-dependent dimerization was demonstrated, we measured the affinity of two structurally distinct nanomolar affinity sigma-2 ligands, DTG and PB28, to purified PGRMC1 proteins that were both in the apo-monomeric and heme-dependent dimeric forms. While no binding to apo-PGRMC1 monomer was detected, a modest affinity (micromolar) to heme-mediated dimerized PGRMC1 was demonstrated by DTG. Altogether the data obtained by this work provide the evidence that sigma-2 receptor and PGRMC1 are not the same binding sites, and pave the pathway for future unbiased research in which these two attractive targets are treated as different proteins while the identification of the true sigma-2 protein needs to be pursued.

Keywords: Sigma-2 receptor, PGRMC1, heme-PGRMC1 dimer, apo-PGRMC1 monomer, flow-cytometry, confocal microscopy.

Abbreviations: Progesterone receptor membrane component 1 (PGRMC1); Amyotrophic Lateral Sclerosis (ALS); Epidermal growth factor (EGF); isothermal titration calorimeter (iTC).

1. Introduction

After four decades from their discovery [1], the enigma about sigma receptors remains to be solved. Based on the different pharmacological profile, in the early '90s they were classified in two subtypes, namely sigma-1 and sigma-2. Since then, only the sigma-1 has been cloned and appears to be involved in a plethora of CNS diseases such as depression, Alzheimer's disease (AD), schizophrenia, as well as in Amyotrophic Lateral Sclerosis (ALS), neuropathic pain and tumors [2,3]. However, no endogenous ligand has been identified yet, and the pathways activated still need clarification. Interest in the sigma-2 subtype waxed and waned during the years. The evidence that this receptor is overexpressed in a variety of tumors and the consequent important therapeutic and diagnostic perspectives associated with it, lately rejuvenated scientific research interest. A number of sigma-2 ligands with antiproliferative activity have been developed during the years and some were evaluated in preclinical animal tumor models with promising results. Several sigma-2 radioligands have also been developed [4-6] with one of them under study in Phase I clinical trial for the imaging of breast tumors [7]. Nevertheless, the identity of the sigma-2 protein is still ambiguous. While in 2006 it was suggested that sigma-2 receptor could be related to histone proteins [8,9], later studies identified it as the progesterone receptor membrane component 1 (PGRMC1) [10]. Such identification is a contemporary matter of debate, since we and others have reported data supporting the concept that the sigma-2 receptor differs from PGRMC1 [11-12]. We previously showed that the expression of sigma-2 receptor is independent of PGRMC1, and that the sigma-2 mediated activity is independent of the density of PGRMC1 in adenocarcinoma breast tumor cells (MCF7). Similarly, Ruoho et al. found that, in motor neuron-like NSC34 cells devoid of or overexpressing PGRMC1, binding of the putative sigma-2 receptor ligand [^{3}H]-DTG was not altered, while DTG and haloperidol affinity for PGRMC1 (obtained by competition with [^{3}H]-progesterone) was more than three orders of magnitude lower than that determined for the sigma-2. Nevertheless, at the same time, Mach et al. published that the fluorescent sigma-2 receptor ligand SW120 correlates with PGRMC1 expression in rat brain cells concluding that 'PGRMC1 is the

sigma-2 receptor binding site' [13]. On the other hand, the crystal structure of PGRMC1 has recently been explored; the study revealed that PGRMC1 exhibits heme-mediated dimerization that exerts its function to regulate EGF receptor-mediated cell proliferation in cancer cells [14]. However, how the sigma-2 ligands can bind to PGRMC1 has never been examined yet. It goes without saying that a misidentification of these proteins would seriously hamper their future therapeutic or diagnostic exploitation. Both PGRMC1 and sigma-2 proteins represent promising targets for the therapy and diagnosis of tumors, as well as they hold promises as AD disease-modifying targets, as recently suggested [15,16]. With the aim of contributing to the clarification of the controversial relationship between sigma-2 and PGRMC1, we have herein employed structurally different fluorescent ligands with nanomolar affinity for sigma-2 receptor by flow cytometry and confocal microscopy in MCF7 cells in which, together with sigma-2 receptors, PGRMC1 was constitutively present, silenced or overexpressed. Furthermore, we determined the affinity of two high-affinity sigma-2 ligands, DTG and PB28, with purified PGRMC1 proteins by calorimetric assays.

2. Materials and Methods

2.1. Materials. Fluorescent compounds F412 [17], NO1 [18] and PB28 [19,20] were synthesized according to previously reported procedures. Cell culture reagents were purchased from EuroClone (Milan, Italy). Puromycin, G418 (geneticin), AG205, were obtained from Sigma-Aldrich (Milan, Italy). Anti-PGRMC1 antibody produced in rabbit was purchased by Sigma-Aldrich (catalog number: HPA002877, lot number: C95870). Rabbit anti-Mouse IgG Secondary Antibody, TRITC conjugate was purchased from ThermoFisher/Invitrogen (catalog number: T6778; lot number: 047K4760). DTG (1,3-di-*o*-Tolylguanidine) was purchased from Tocris Cookson Ltd., U.K.

2.2. Cell lines. The human MCF7 breast cancer cells were obtained from Interlab Cell Line Collection (ICLC, Genoa). MCF7_SH and MCF7_PGRMC1 were created in our laboratory [11]. MCF7 (passage 18), MCF7_SH (passage 15), and MCF7_PGRMC1 (passage 18) cells were grown

in DMEM high glucose supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, in a humidified incubator at 37 °C with a 5% CO_2 atmosphere. DMEM growth medium contain puromycin (2 μg/ml) or geneticin (800 μg/ml) for MCF7_SH and MCF7_PGRMC1, respectively.

2.3. Flow cytometry analysis. Cells were incubated with increasing concentrations (1 nM, 25 nM, 100 nM, 1 μM, 10 μM, 30 μM) of PB28, DTG or AG205 for 75 min, followed by 100 nM of ~~either~~ F412, NO1 or PB385 for 75 min at 37 °C. This experimental condition was the best one allowing the up-take of the fluorescent compounds in MCF7 cells [18]. At the end of the incubation periods, cells were washed twice with PBS, detached with 200 μl of Cell Dissociation Solution (Sigma Chemical Co.) for 10 m in at 37°C, centrifuged at 13,000 × *g* for 5 min and re-suspended in 500 μL of PBS. The fluorescence was recorded using a Guava® easyCyte flow cytometry (Millipore, Billerica, MA), with a 530 nm band pass filter. For each analysis 10,000 events were collected and analysed with InCyte™ software (Millipore).

2.4. Confocal microscopy analysis. 10^5 cells were grown on sterile 8-well Thermo Scientific™ Diagnostic Slides, treated for 45 min with F412 or NO1, rinsed and fixed with 4% w/v paraformaldehyde for 15 min, then rinsed and permeabilized with 0.5% TRITON-X 100 for 20 min. To visualize PGRMC1, the samples were washed with PBS and stained with an anti-PGRMC1 antibody (Sigma-Aldrich) for 1 h. After washing, samples were incubated with a TRITC-conjugated secondary antibody (Invitrogen) for 1 h and re-washed. The coverslips were mounted with Vectashield Mounting Medium and examined with a Zeiss LSM 700 Olympus (Carl Zeiss AG, Oberkochen, Germany). For each experimental spot, a minimum of three microscopic fields were examined.

2.5. Preparation of recombinant PGRMC1 protein. Recombinant PGRMC1 protein (cytosolic domain: a.a.44-195) was prepared as described previously [14]. Briefly, the GST tagged PGRMC1 was expressed in BL21 (DE3) by induction with 1 mM isopropyl-β-thiogalactopyranoside (IPTG).

The cells were sonicated and centrifuged at 20,000 × *g* for 30 min. The supernatant was incubated with glutathione Sepharose 4B (GE Healthcare), and the GST tag was cleaved by addition of Factor Xa (GE Healthcare). The apo-PGRMC1 was prepared by eliminating the bacterial holo-PGRMC1 with size-exclusion chromatography (Superdex 200; GE Healthcare). Heme-bound PGRMC1 were prepared by treatment with 100 μM hemin, and purified by size-exclusion chromatography according to previous studies [14].

2.6. Isothermal titration calorimetry (ITC). ITC experiments were performed at 298 K with ITC-Buffer (50 mM Phosphate-NaOH (pH 7.0), 2% DMSO) using a MicroCal iTC200 (Malvern). DTG and PB28 were dissolved at 2 mM with ITC-Buffer, and titrated into 100 μ M of apo- or heme-bound PGRMC1. The titration was performed by injecting 2 μL of the syringe solution at intervals of 120 s. The binding isotherms were analyzed with SEDPHAT [21]. The K_D values of DTG with heme-PGRMC1 were calculated as mean of the data from five independent experiments.

3. Results

3.1. Flow cytometry. Flow-cytometry studies were performed on three human MCF7 breast cancer cell lines. MCF7 cells have been thoroughly characterized for the sigma-2 receptor expression becoming a model for sigma-2-related studies. In this study, we pre-incubated MCF7 wild-type (MCF7wt) and the corresponding cells devoid of (MCF7_SH) or overexpressing PGRMC1 (MCF7_PGRMC1) [11] with increasing concentrations of the sigma-2 receptor ligands PB28 [19,20] or DTG (K_i values in Table 1), or with the PGRMC1 inhibitor AG205. Then, we alternatively labeled the three cell lines with two previously developed sigma-2 receptor specific fluorescent ligands having ~~two~~ different sigma-2 receptor targeting moieties (Figure 1). F412 [17] is the analogue of the well known sigma-2 receptor ligand PB28 with high affinity for sigma-2 receptor but moderate selectivity for sigma-1 (Table 1). On the other hand, NO-1 [18] carrying the same fluorescent tag as F412, displayed high sigma-2 affinity and selectivity towards the sigma-1 receptors, in accordance with the dihydroisquinolinone structure and the 6,7-

dimethoxytetrahydroisoquinoline basic moiety (Table 1) [4]. Concentrations of the fluorescent compounds to be used and incubation time were selected according to previously reported experiments [18,22]. As shown in Figure 2, the two fluorescent compounds F412 (right panel) and NO1 (left panel) were dose-dependently displaced by PB28 or DTG (from 1 nM to 30 μM) to a very similar extent in all the three cell lines (Figure 2, A-C: representative showing displacement with PB28; Figure 3, A-C: representative showing displacement with DTG). PB385 also had a similar displacement kinetic (Supplementary Figure 1). On the other hand, the three fluorescent compounds were not displaced by the PGRMC1 inhibitor AG205 even at its highest concentration, with all the curves from 1 nM to 30 μM perfectly superimposed in all the three cell lines (Figure 3, Supplementary Figure 2; representatively showing the experiment in PGRMC1 overexpressing cells). Binding values of each compound are reported in Table 2/Supplementary Table 1: for each sigma-2 ligand, using the same concentration of PB28 or DTG, there were not statistically significant differences among each cell line. Taken together, these data show that high affinity sigma-2 receptor fluorescent ligands (with different structures both in the hydrophobic and basic moiety), which equally well label sigma-2 receptors, do not label PGRMC1. This finding is strongly supporting the hypothesis that PGRMC1 and sigma-2 are two different proteins.

3.2. Confocal Microscopy. The three cell lines MCF7wt, MCF7_SH or MCF7_PGRMC1 were then incubated with either F412 (Figure 4, upper panels) or NO1 (Figure 4, lower panels) for 45 min and analyzed by confocal microscopy. Previous to microscopy, cells were fixed with paraformaldehyde, permeabilized with TRITON-X 100 and PGRMC1 visualized using a specific primary and TRITC-conjugated secondary antibody system. In the three cell lines, sigma-2 receptors and PGRMC1 were clearly and specifically stained by their corresponding markers and no colocalization of the green emitting molecules (F412 or NO1) with the PGRMC1 signal (red) was detected.

3.3. Binding assay. The *in vitro* drug-binding assay with PGRMC1 was performed by isothermal titration calorimeter (MicroCal iTC200) with purified human PGRMC1 cytosolic domain (a.a. 44-195) prepared as apo-monomer or its heme-dimerized form as previously reported [14]. Results from the calorimetric titrations curves (Figure 5) show no affinity of the nanomolar high affinity sigma-2 agonist DTG with the monomeric PGRMC1 protein. On the other hand, a modest interaction with the heme-dimerized form of PGRMC1 (K_D = 84.18 ± 31.85 μM) was detected. In addition, we also attempted to analyze the binding of another high-affinity sigma-2 agonist by ITC i.e. PB28. PB28 did not bind to PGRMC1 monomer but showed a weak binding affinity with heme-dimerized PGRMC1 (Figure 1, Supplementary Information). These results suggest that the sigma-2 ligands such as DTG or PB28 do not bind to PGRMC1 monomer but to heme-mediated dimerized form of PGRMC1 only with micromolar levels, supporting the notion that sigma-2 receptor and PGRMC1 protein are different proteins.

4. Discussion

Researchers need to urgently address the controversial identification of the sigma-2 receptor with the PGRMC1 to take full advantage of the therapeutic and diagnostic potentials that could derive from targeting these proteins. In this work we used two structurally different fluorescent sigma-2 ligands (F412, NO1) to stain their target proteins in three MCF7 cell lines (MCF7wt, MCF7_SH, MCF7_PGRMC1) in which different levels of PGRMC1 expression were achieved. The choice of these ligands was made to lower the probabilities of off-target labeling (which could be the reason for the identification of PGRMC1 as being the same protein as sigma-2) since it is unlikely that differently structured ligands bind the same off-target proteins. Flow-cytometry curves clearly show a dose-dependent displacement of the fluorescent ligands by two reference sigma-2 ligands (PB28 and DTG), but not by the reference PGRMC1 inhibitor (AG205). This is in accordance with the previously reported results of AG205 lacking the affinity for sigma-2 receptors (K_i > 10,000 nM) [11]. The sigma-2 ligands used have slightly different Ki value (Table 1). Moreover, to the best of

our knowledge, it is not known if the ligands' and displacer's binding sites are coincident: the binding of a compound in a specific binding site may produce conformation changes that in turn produce different affinity/binding of the displacer. These features may account for the slightly different displacement kinetics (Figure 2 and 3). The displacement occurred to the same extent among the three cell lines labeled with the differently structured sigma-2 fluorescent ligands, showing how these compounds, that specifically target sigma-2 receptors do not target PGRMC1 (Figure 2 and 3; Table 2?). Confocal images of the same cell lines, in which PGRMC1 is labeled with a fluorescent secondary antibody (red emitting), show no colocalization with the proteins labeled by the small fluorescent molecules (F412 and NO1). While red emission is higher in MCF7_PGRMC1 cells reflecting the overexpression of PGRMC1, interestingly, green emission is higher in MCF7_SH, due to the sigma-2 receptor labeling with small molecules. This result is in agreement with data from Scatchard analyses reporting a higher expression of sigma-2 receptors in PGRMC1 silenced cells (K_D = 2.74 pmol/mg protein) compared to wild-type (K_D = 2.02 pmol/mg protein) or PGRMC1-overexpressing (K_D = 1.64 pmol/mg protein) cells [11]. Despite the activity of sigma-2 ligands being independent of the expression of PGRMC1, which we have previously demonstrated, this sort of compensatory effect in the expression of sigma-2 receptors when PGRMC1 is silenced or overexpressed is an additional factor explaining why the displacement kinetics of sigma-2 ligands are slightly different in cell lines with different PGCRM1 levels. On the one hand, our data suggest that sigma-2 receptors and PGCRM1 are distinct proteins with distinct localization and ligands. On the other hand, our data strongly support the idea that sigma-2 receptors and PGCRM1 expression are interconnected: we are currently investigating the molecular/biochemical basis of this linkage and the possible functional and patho-physiological meaning.

While the affinity of the putative PGRMC1 inhibitor AG205 for sigma-2 receptors was previously excluded by radioligand binding assays [11], herein we established the affinity of DTG and PB28 for the human purified PGRMC1. In light of the recently published crystallization of the PGRMC1

showing the heme-dependent dimerization through the stacking interaction of two protruding heme molecules, we measured the affinity of these compounds for both the apo-monomer and the heme-dimer forms of PGRMC1. As shown by the titration calorimetric curves, DTG and PB28 shows no affinity at all for the apo-monomer, while DTG but not PB28 exhibits modest binding to the PGRMC1 dimer. These observations demonstrate that PGRMC1 does not account for sigma-2 receptor. On the other hand, the current results lead us to investigate functional implications of PGRMC1 on tumor development [14] and dementia [16] or to further examine whether compounds such as DTG might help protein-protein interactions between unidentified sigma-2 receptor and the heme-mediated PGRMC1 dimer.

5. Conclusion

Herein, we combined flow cytometry, confocal microscopy and isothermal titration calorimetry experiments to provide a strong evidence of the non-identity of sigma-2 and PGRMC1 binding sites. For the first time, data from the heme-dimer form of PGRMC1 were measured and excluded the possibility that a binding site for the sigma-2 ligands is formed upon the heme-driven dimerization of the PGRMC1. As a consequence, room is left for an urgent identification of the real sigma-2 receptor, and studies to define the functions of PGRMC1 and sigma-2 receptor within the cells and their possible relationship (such as protein-protein interactions between sigma-2 and heme-PGRMC1 dimer) are encouraged. The potentials of these two proteins in oncology and neurodegenerative fields, as shown by recent literatures, will boost scientific interest, and the present work will help scientific community to properly address future research based on a clear definition that PGRMC1 and sigma-2 receptors are not one and the same protein.

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Conflict of interest. The authors declare that they have no conflicts of interest with the contents of this article.

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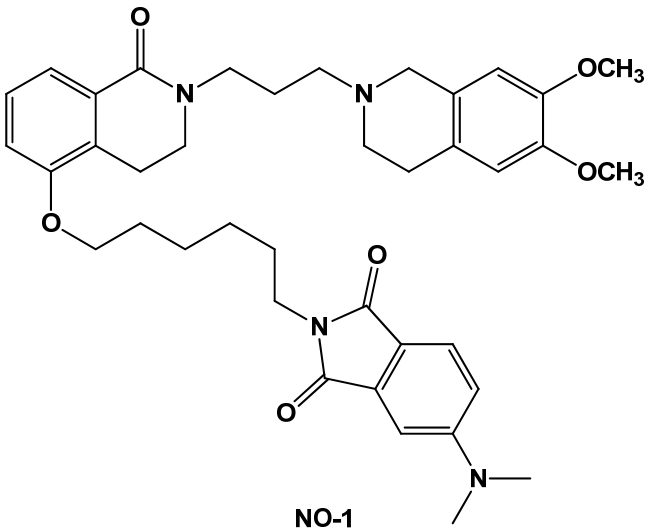
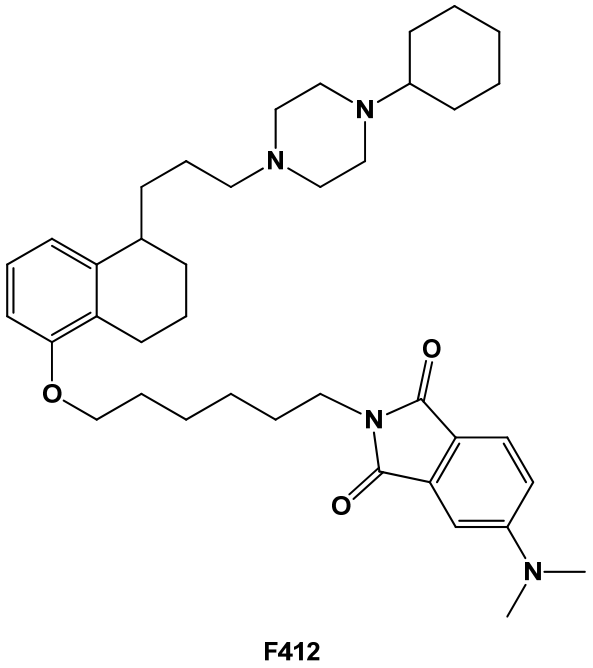
Table 1. Binding affinities and fluorescent properties of sigma ligands

Compound	K_i (nM)		Fluorescence Properties	
	sigma-1	**sigma-2**	**λex**	**λem**
PB28 [a]	0.38	0.68		
DTG		24.6		
F412[b]	5.37	6.9	390	500
NO1[c]	>5000	10.2	390	525

[a] Reference [20,22]; [b] Reference [17]; [c] Reference [18].

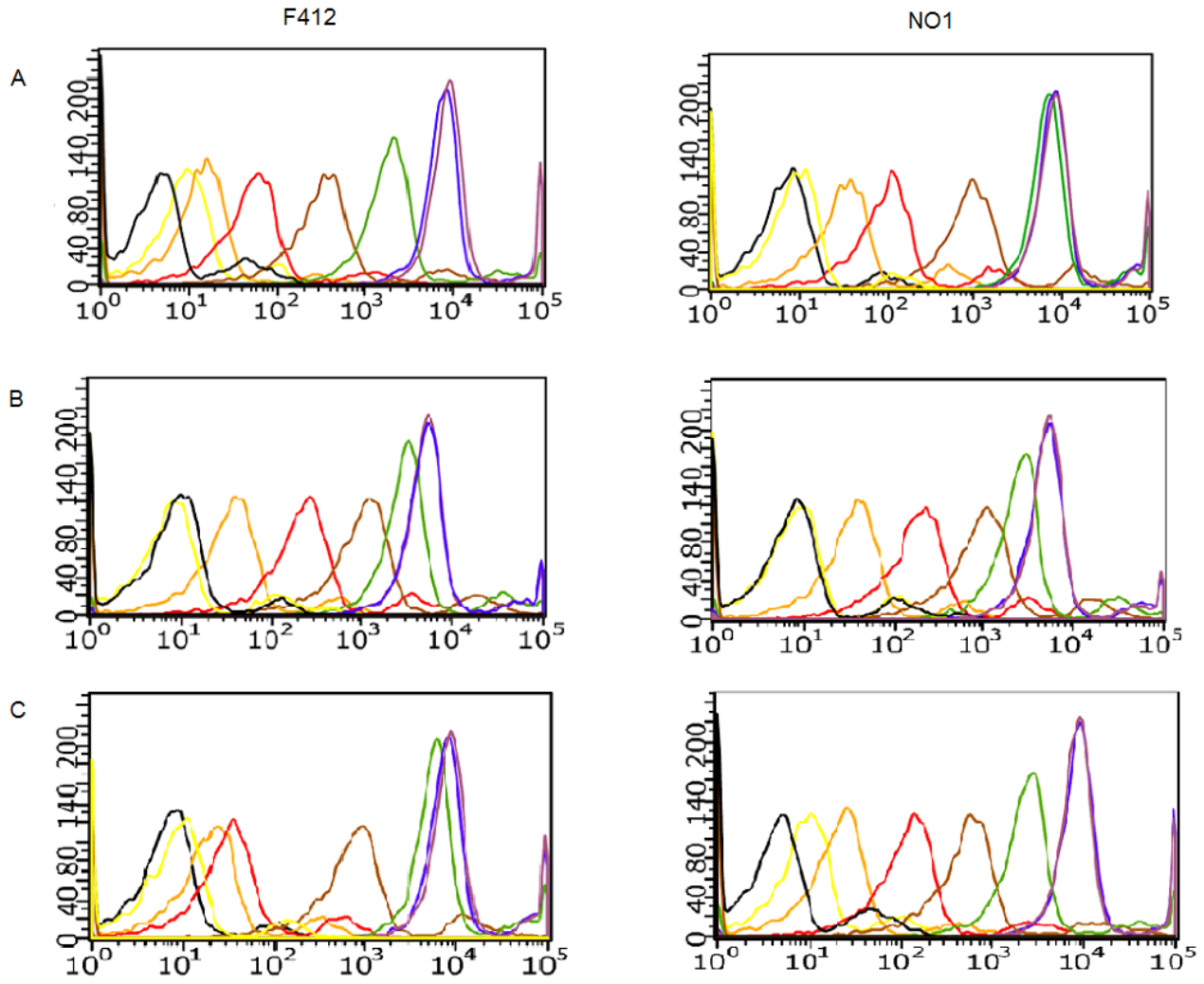
Figure 1. Sigma-2 fluorescent compounds.





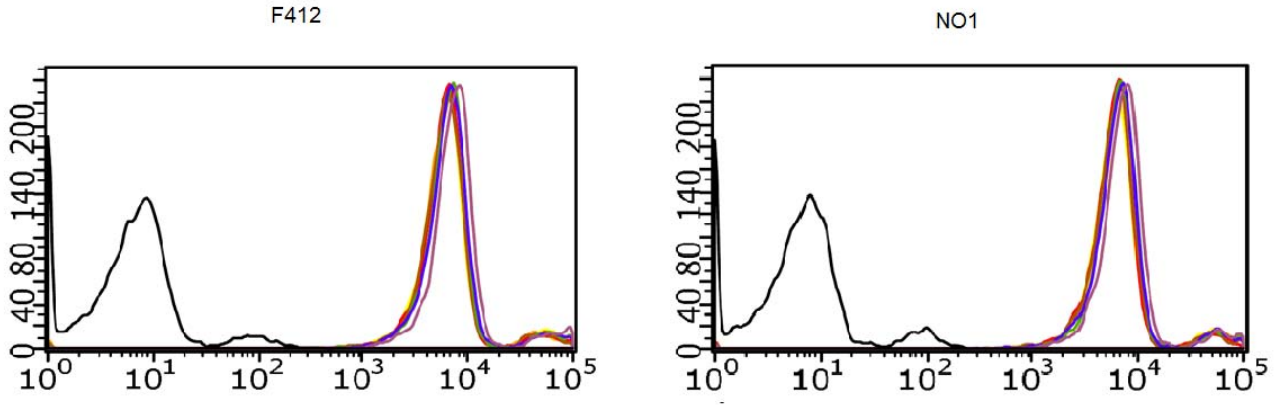
Chemical structures of the sigma-2 fluorescent compounds.

Figure 2. Flow Cytometry analyses of MCF7wt, MCF7_SH and MCF7_PGRMC1 cells labeled with sigma-2 fluorescent compounds, in the presence of PB28.



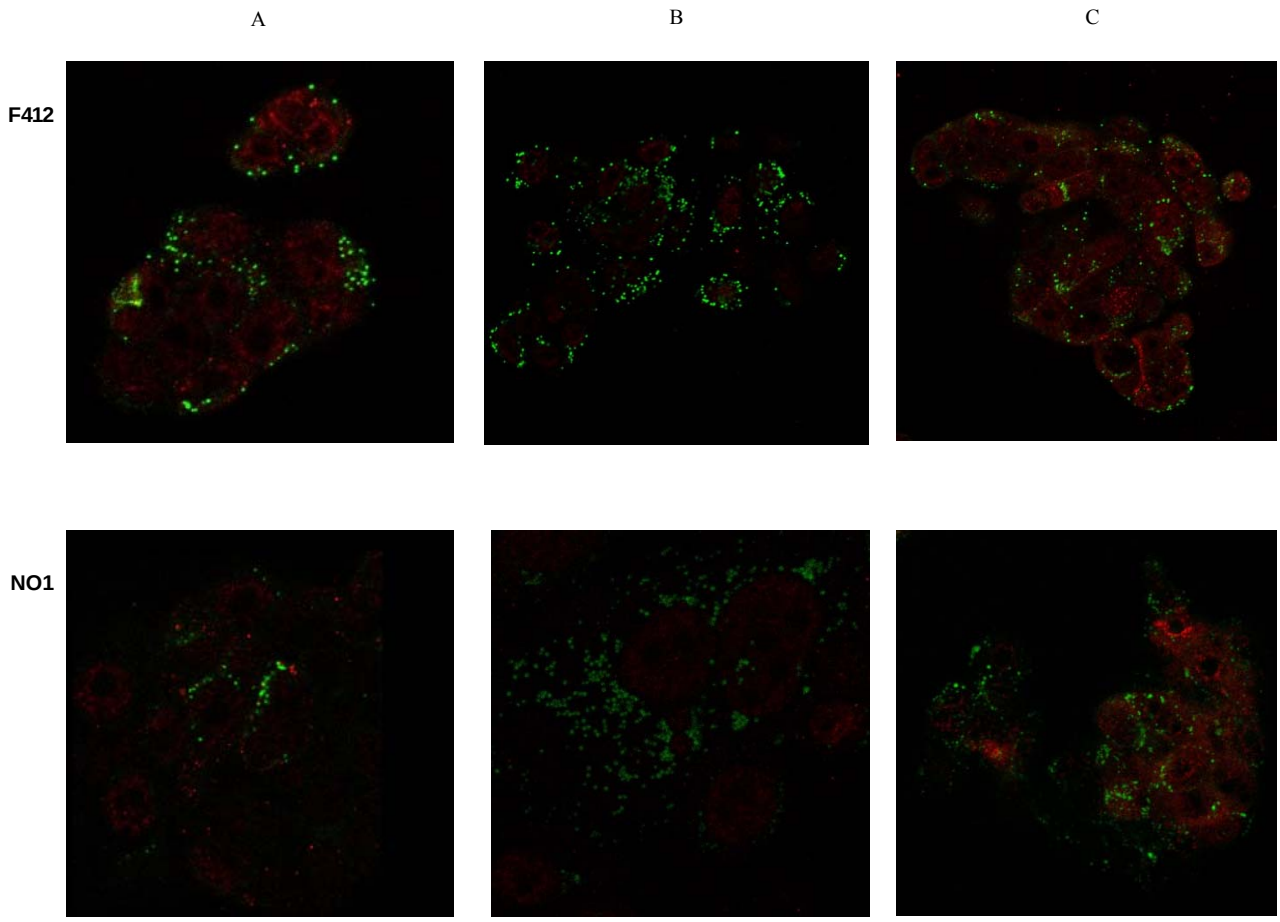
Flow Cytometry analysis showing cell-associated fluorescence versus cell count of one representative experiment out of three in MCF7wt (A), MCF7_SH (B) and MCF7_PGRMC1 (C). Left Panel displacement of F412 (100 nM) with increasing concentrations of PB28; Right panel: displacement of NO1 (100 nM) with increasing concentrations of PB28 (1 nM, 25 nM, 100 nM, 1 μM, 10 μM, 30 μM); black curve: untreated cells; violet curve: 0 nM PB28; blue curve: 1 nM PB28; green curve: 10 nM PB28; brown curve: 100 nM PB28; red curve: 1 μM PB28; orange curve: 10 μM PB28; yellow curve: 30 μM PB28.

Figure 3. Flow Cytometry analyses of MCF7_PGRMC1 cells labeled with sigma-2 fluorescent compounds.



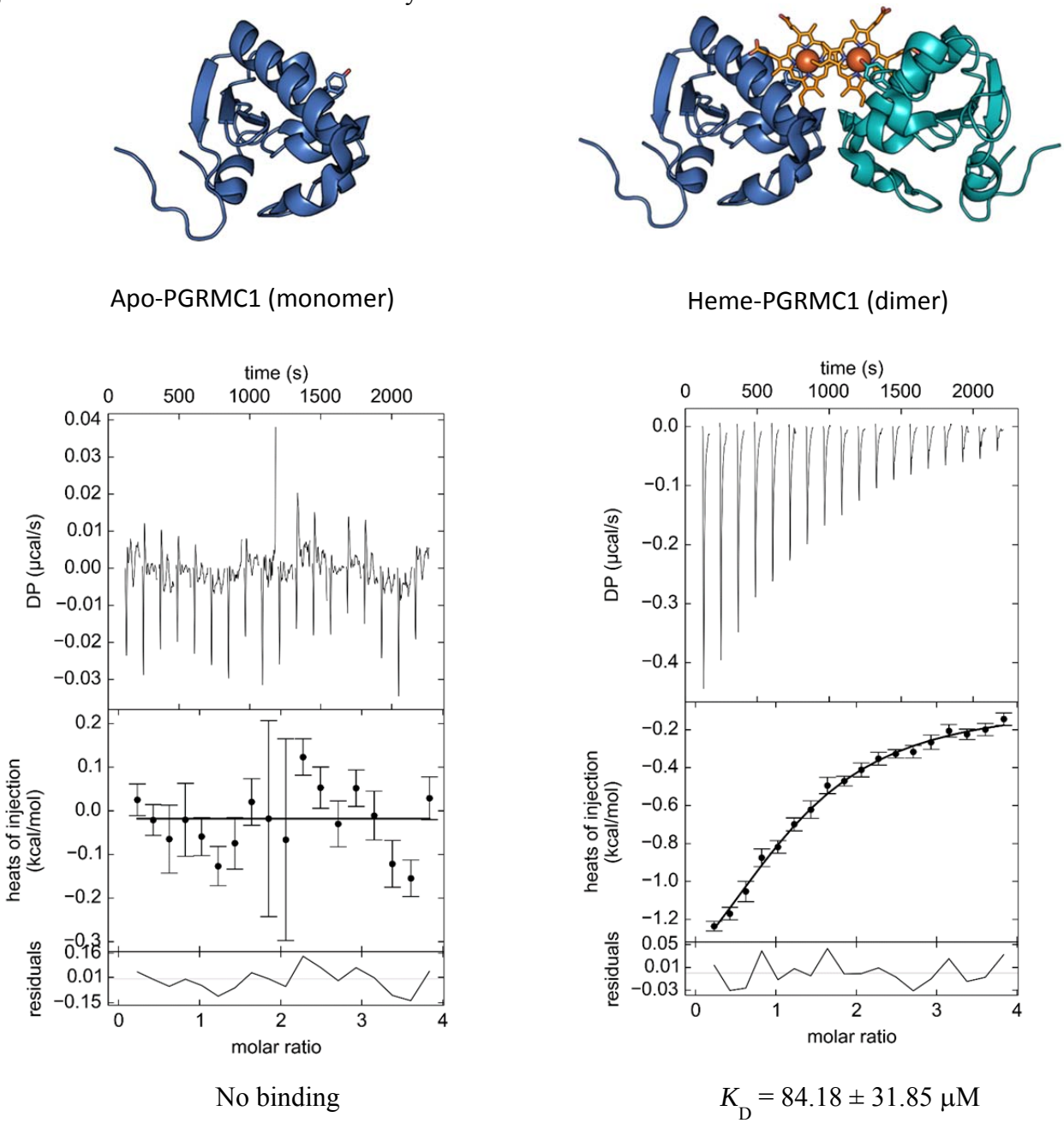
Flow Cytometry analysis showing cell-associated fluorescence versus cell count of one representative experiment out of three in MCF7_PGRMC1. Left Panel displacement of F412 (100 nM) with increasing concentrations of AG205; Right panel: displacement of NO1 (100 nM) with increasing concentrations of AG205 (1 nM, 25 nM, 100 nM, 1 μM, 10 μM, 30 μM); black curve: untreated cells; violet curve: 0 nM AG205; blue curve: 1 nM AG205; green curve: 10 nM AG205; brown curve: 100 nM AG205; red curve: 1 μM AG205; orange curve: 10 μM AG205; yellow curve: 30 μM AG205.

Figure 4. Confocal microscopy analyses of MCF7wt, MCF7_SH and MCF7_PGRMC1 cells labeled with PGRMC1-antobody and sigma-2 fluorescent compounds.



Confocal microscopy analysis showing the three different cell lines MCF7wt (A), MCF7_SH (B), MCF7_PGRMC1(C) stained with the fluorescent sigma-2 receptor ligand F412 or NO1 (10 μM, green), and anti-PGRMC1 antibody (red). The images are representative of one experiment out of three.

Figure 5. Isothermal titration calorimetry



Isothermal titration calorimetry of DTG into PGRMC1. DTG at a concentration of 2 mM was titrated into 100 µ M of apo- or heme-bound PGRMC1 (a.a.44-195). Degrees of polymerization (DP) of the experimental thermogram (upper panel). Titration isotherm (with error bars). The isotherm was fit to a one-to-one binding model (solid line) using the program SEDPHAT with a 1:1 binding model (middle panel). The single dissociation constant and enthalpy change are indicated in

the inset (lower panel). The K_D and ΔH values of DTG with heme-PGRMC1 were calculated as mean from the data of five independent experiments.

Supplementary Information

Sigma-2 Receptor and Progesterone Receptor Membrane Component 1 (PGRMC1) are two Different Proteins: Proofs by Fluorescent Labeling and Binding of Sigma-2 Receptor Ligands to PGRMC1

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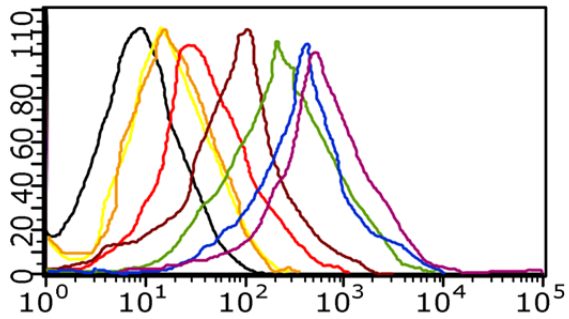
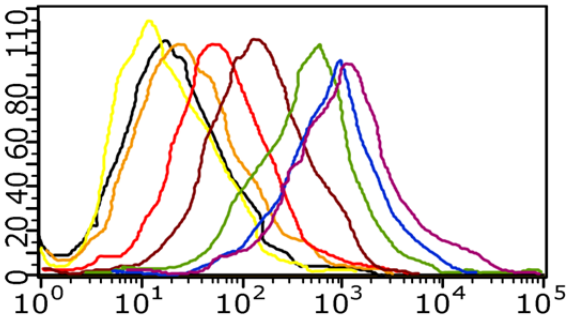
Figure 1. Flow Cytometry analyses of MCF7wt, MCF7_SH and MCF7_PGRMC1 cells labeled with sigma-2 fluorescent compound PB385.

PB385

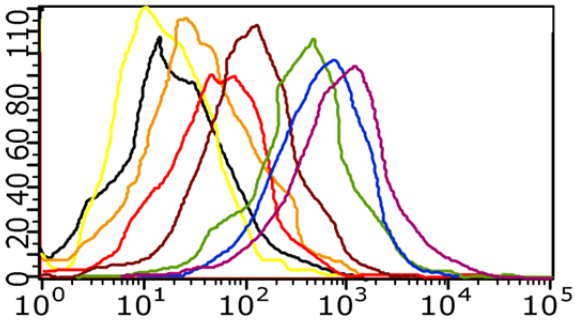
A



B

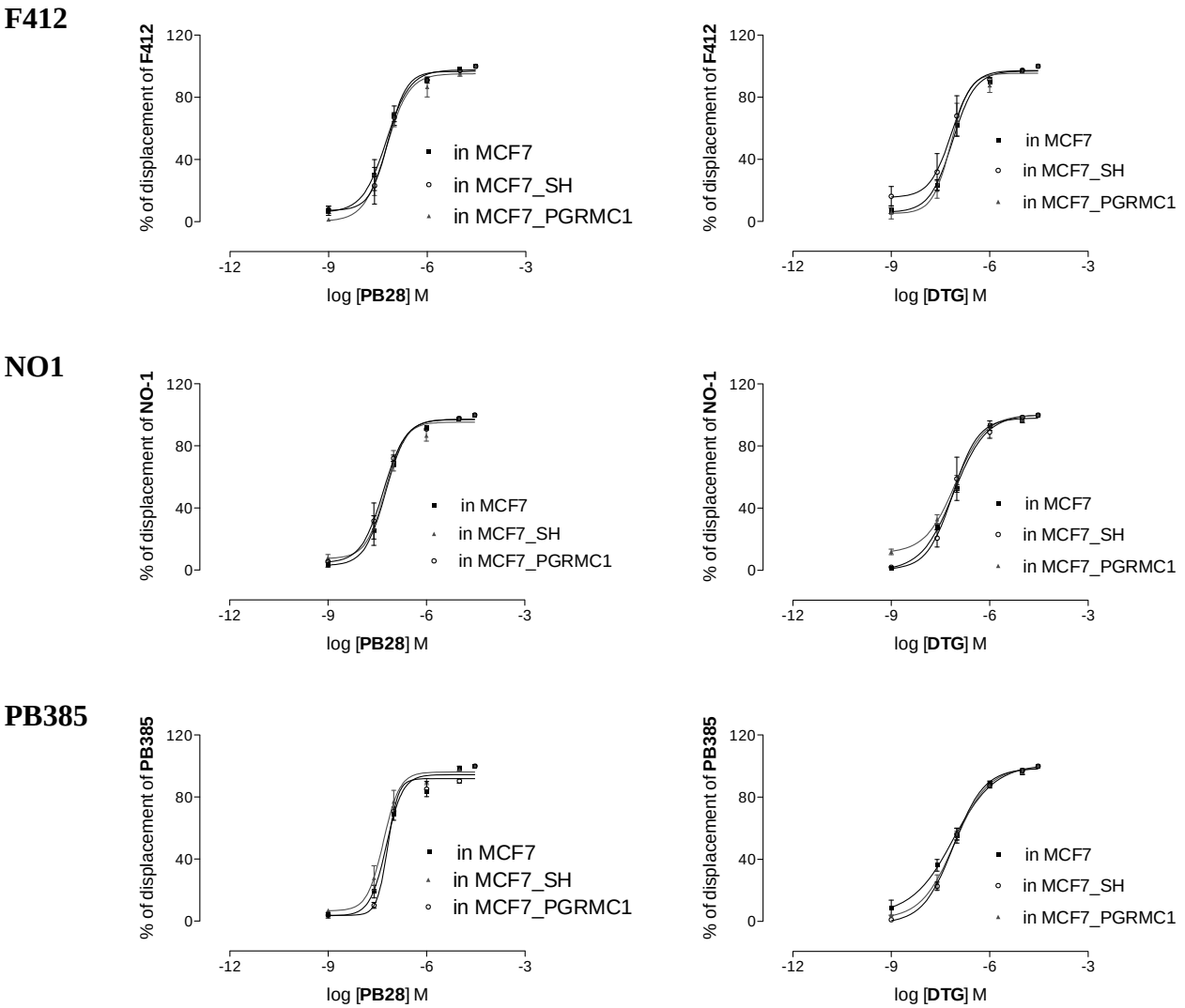


C



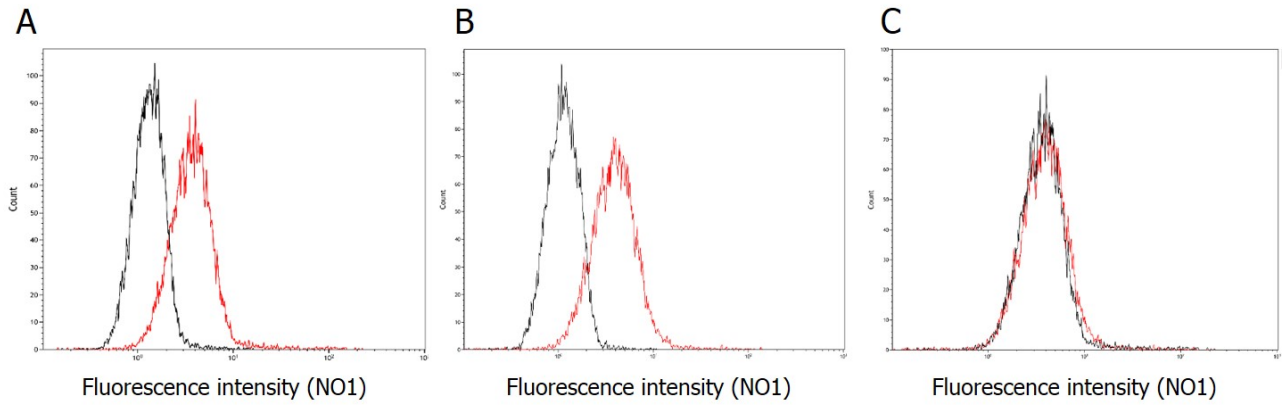
Flow Cytometry analysis showing cell associated-fluorescence-associated versus cell count of one representative experiment out of three in MCF7wt (A), MCF7_SH (B) and MCF7_PGRMC1 (C). Displacement of PB385 (100 nM) with increasing concentrations of PB28 (1 nM, 25 nM, 100 nM, 1 µM, 10 µM, 30 µM); black curve: untreated cells; violet curve: 0 nM PB28; blue curve: 1 nM PB28; green curve: 10 nM PB28; brown curve: 100 nM PB28; red curve: 1 µM PB28; orange curve: 10 µM PB28; yellow curve: 30 µM PB28.

Figure 2. Binding curves of PB28 and DTG by flow cytometry in MCF7 cell lines.



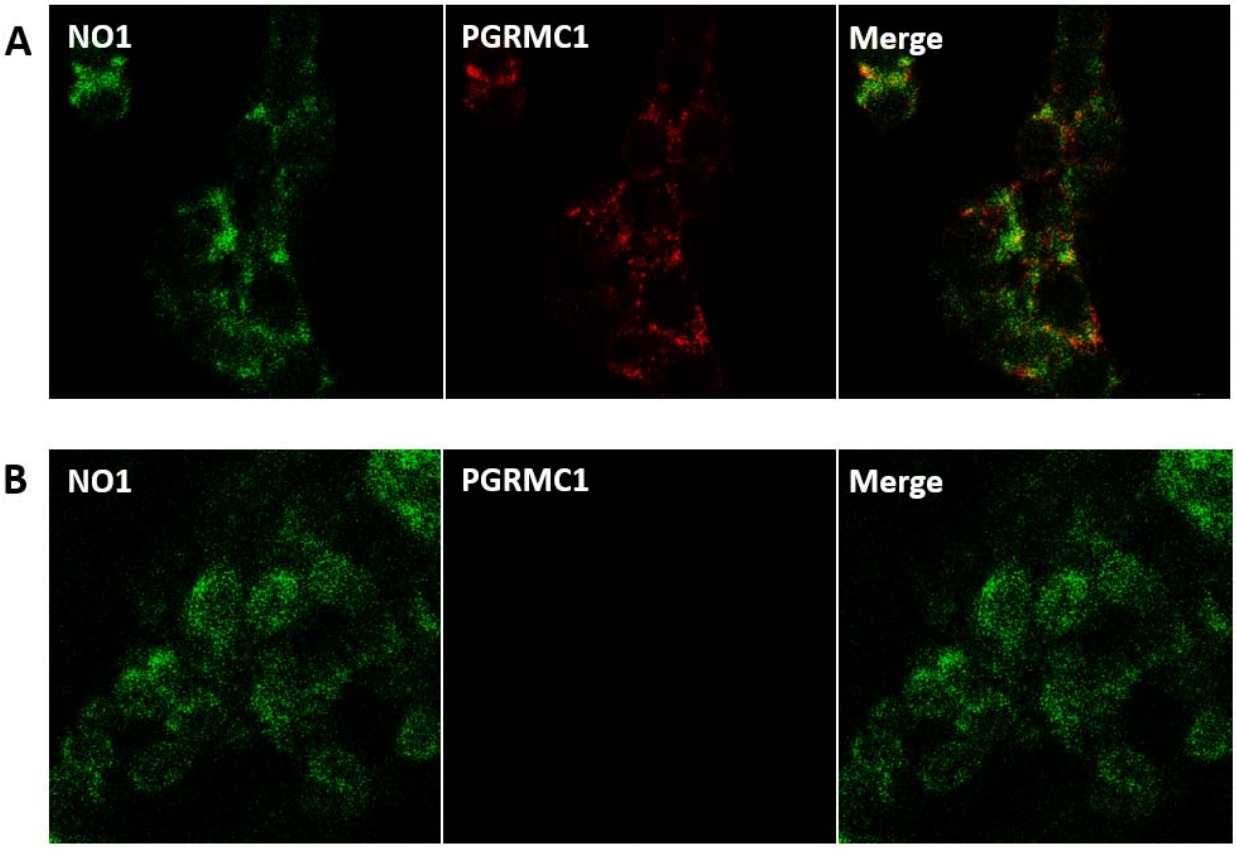
Displacement curves of three fluorescent sigma ligands (**F412**, **NO1**, **PB385**) with **PB28** and **DTG** in MCF7, MCF7_SH and MCF7_PGRMC1 cell lines. The curves were generated from three different experiments in duplicate. Statistical analysis with ANOVA one-way test was performed and no significant differences were observed.

Figure 3. Flow Cytometry analyses of HCT116wt and HCT116_SH cells labeled with sigma-2 fluorescent compound NO1.



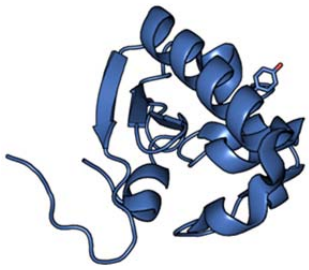
Flow Cytometry analyses of HCT116wt and HCT116_SH cells labeled with sigma-2 fluorescent compound NO-1. Cells were incubated with 10 μM NO1 and the fluorescence intensity was analyzed by flow cytometer. (A) (B) Flow cytometry analysis showing cell-associated fluorescence versus cell count of one representative experiment out of three in HCT116wt (A) and HCT116_SH (B). black curve: untreated cells; red curve: 10 μM NO1. (C) Flow Cytometry analysis showing cell-associated fluorescence versus cell count of one representative experiment out of three in 10 μM NO1 staining cells. black curve: HCT116wt; red curve: HCT116_SH.

Figure 4. Confocal microscopy analyses of HCT116wt and HCT116_SH cells labeled with PGRMC1-antobody and sigma-2 fluorescent compounds.

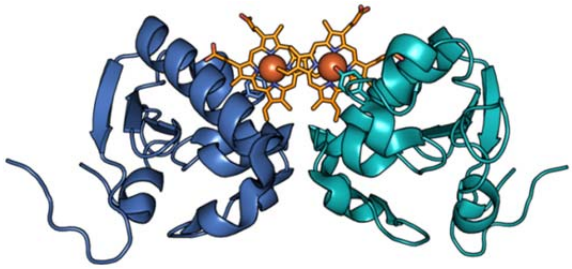


Confocal microscopy analysis showing the two different cell lines HCT116wt (A) and HCT116_SH (B) stained with the fluorescent sigma-2 receptor ligand 10 μM NO1 (green), and anti-PGRMC1 antibody (red). The cells were examined using an LSM710 Zeiss confocal microscope. Scale bar = 10 μm.

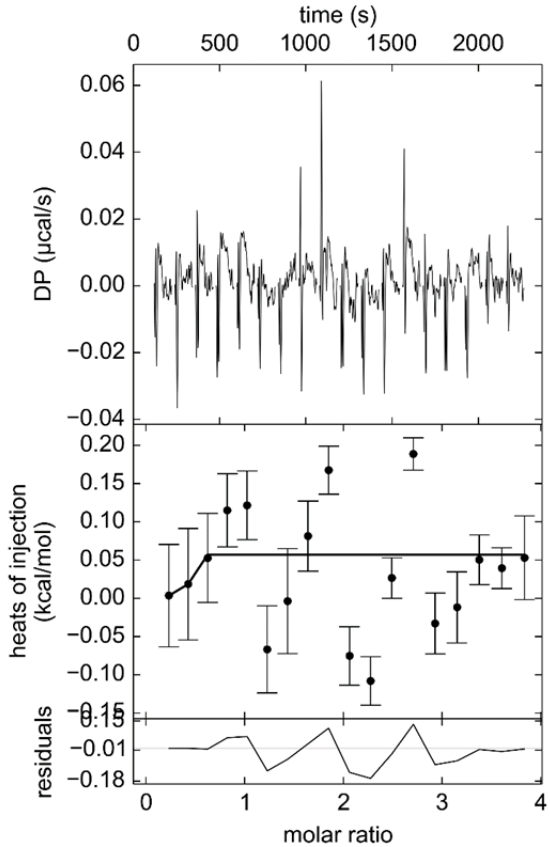
Figure 5. Isothermal titration calorimetry of PB28 into PGRMC1



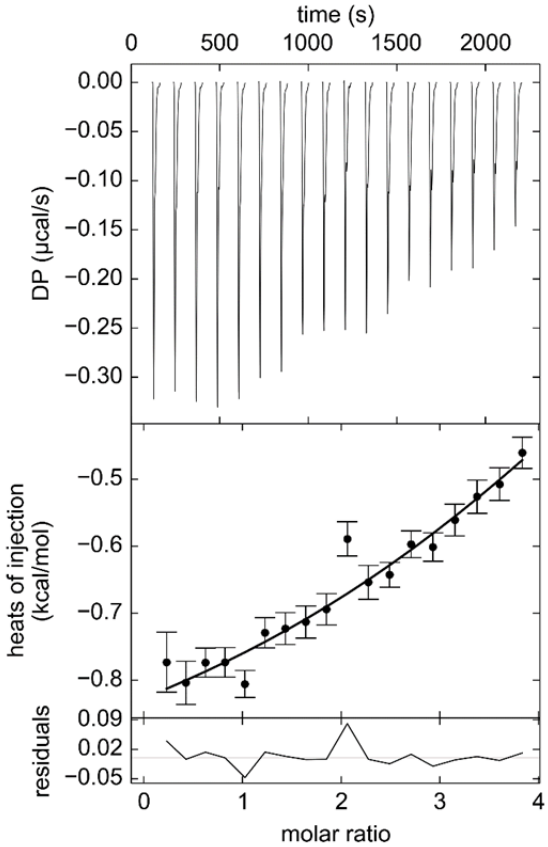
Apo-PGRMC1 (monomer)



Heme-PGRMC1 (dimer)



No binding



Weak binding

PB28 at a concentration of 2 mM was titrated into 100 μ M of apo- or heme-bound PGRMC1 (a.a.44-195). Degrees of polymerization (DP) of the experimental thermogram (upper panel). Titration isotherm (with error bars). The isotherm was fit to a one-to-one binding model (solid line) using the program SEDPHAT with a 1:1 binding model (middle panel). The single dissociation constant and enthalpy change are indicated in the inset (lower panel).