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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A R T I C L E   I N F O

A B S T R A C T

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 reported to be identical to PGRMC1. Because a misidentification of these proteins will lead to biased future research hampering the possible diagnostic and therapeutic exploitation of the two targets, there is the need to solve the debate on their identity. With this aim, we have herein investigated uptake and distribution of structurally different fluorescent sigma-2 receptor ligands by flow cytometry and confocal microscopy in MCF7 cells, where together with intrinsic sigma-2 receptors, PGRMC1 was constitutively present or alternatively silenced or overexpressed. HCT116 cells, with constitutive or silenced PGRMC1, were also studied. These experiments showed that the fluorescent sigma-2 ligands bind to their receptor irrespective of PGRMC1 expression. Furthermore, isothermal titration calorimetry was conducted to examine if DTG and PB28, two structurally distinct nanomolar affinity sigma-2 ligands, bind to purified PGRMC1 proteins that have recently been revealed to form both apo-monomeric and heme-mediated dimeric forms. While no binding to apo-PGRMC1 monomer was detected, a micromolar affinity to heme-mediated dimerized PGRMC1 was demonstrated in DTG but not in PB28. The current data provide evidence that sigma-2 receptor and PGRMC1 are not identical, paving 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 further needs to be pursued.

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 '90 s 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 downstream pathways still need clarification. Overall, the interest in the sigma-2 subtype waxed and waned during the years. However, the evidence that this receptor is overexpressed in a variety of tumors and the consequent important associated therapeutic and diagnostic perspectives, 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. In addition, several sigma-2 radioligands have

Compound	K_i (nM)		Fluorescence Properties	
	sigma-1	sigma-2	hex	hem
PB28 [a]	0.38	0.68		
DTG		24.6		
F412[b]	5.37	6.9	390	500
PB385[c]	78.7	10.8	467	520
NO1[d]	>5000	10.2	390	525

[a] Reference [23].
[b] Reference [19].
[c] Reference [20].
[d] Reference [21].

also been developed [4–6] with the most promising already tested in a Phase I clinical trial for the imaging of breast tumors [7]. Nevertheless, the identity of the sigma-2 protein remains ambiguous. While in 2006 it was suggested that sigma-2 receptor could be related to histones [8,9], later studies reported that this protein is identical to progesterone receptor membrane component 1 (PGRMC1) [10]. However, this identification is still matter of debate, since data supporting the concept that the sigma-2 receptor differs from PGRMC1 have been reported [11,12]. In this context, we have shown 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 [3H]-DTG was not altered, while DTG and haloperidol affinity for PGRMC1 (obtained by competition with [3H]-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]. Recently, the crystal structure of PGRMC1 has been explored revealing that PGRMC1 exhibits heme-mediated dimerization that exerts its function to regulate EGF receptor-mediated cell proliferation in cancer cells [14]. This protein seems to play a crucial role as gas sensor. In particular, it responses to CO: CO supplementation interferes with PGRMC1 dimerization suppressing the downstream effects [15]. However, it remains unknown if the sigma-2 ligands bind to PGRMC1. As PGRMC1 and sigma-2 proteins represent promising targets for the therapy and diagnosis of tumors, as well as AD disease-modifying targets [16–18], their misidentification would seriously hamper their future clinical assessment. In order to clarify the controversial relationship between sigma-2 and PGRMC1, we have herein employed structurally different fluorescent ligands with nanomolar affinity for sigma-2 receptor. The uptake of these ligands was studied by flow cytometry and confocal microscopy in breast cancer MCF7 cells, where together with intrinsic sigma-2 receptors, PGRMC1 was constitutively present or alternatively silenced or overexpressed. Also, colon cancer HCT116 cells, with constitutive or silenced PGRMC1, were employed in flow cytometry and confocal microscopy to give further support to our investigation. Furthermore, we determined the binding of two high-affinity sigma-2 ligands, DTG and PB28, to purified PGRMC1 proteins by calorimetric assays.

2. Materials and methods

2.1. Materials

Fluorescent compounds F412 [19], PB385 [20], NO1 [21] and PB28 [22,23] were synthesized according to previously reported procedures. Cell culture reagents were purchased from Euro-
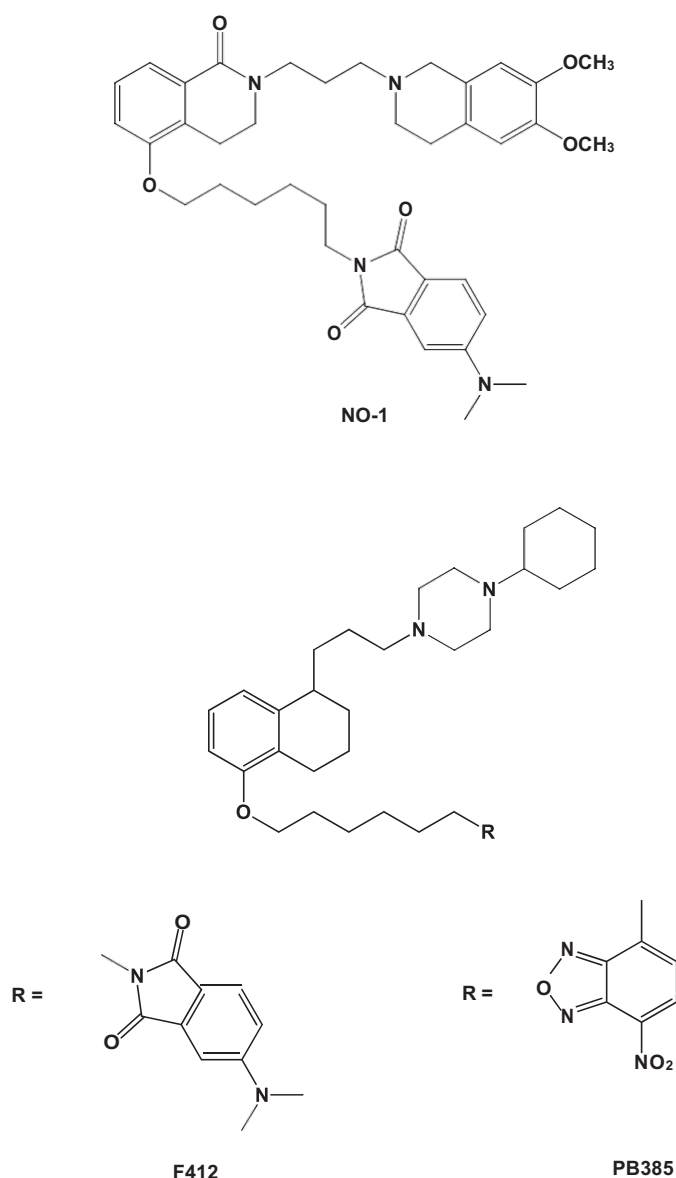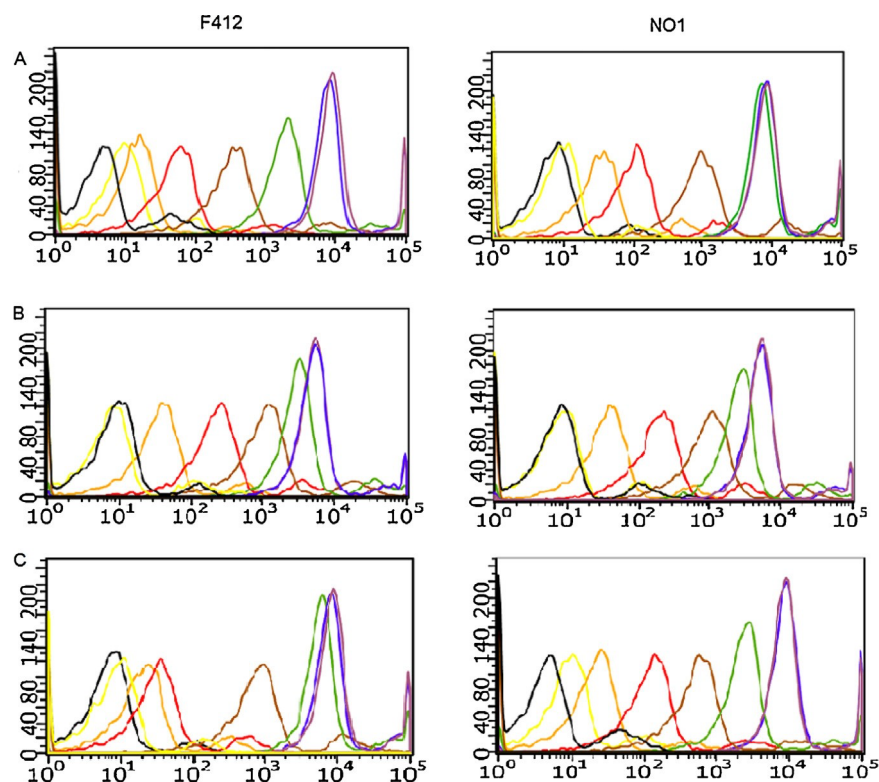

Fig. 1. Sigma-2 fluorescent compounds.
Chemical structures of the sigma-2 fluorescent compounds.

Clone (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. For

Fig. 2. Flow Cytometry analyses of MCF7 wt, MCF7 SH and MCF7 PGRMC1 cells labeled with sigma-2 fluorescent compounds, in the presence of PB28. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Flow Cytometry analysis showing cell-associated fluorescence versus cell count of one representative experiment out of three in MCF7 wt (A), MCF7 SH (B) and MCF7 PGRMC1 (C). Left Panel displacement of F412 (100 nM) with increasing concentrations of PB28 or DTG; Right panel: displacement of NO1 (100 nM) with increasing concentrations of PB28 or DTG (1 nM, 25 nM, 100 nM, 1 μM, 10 μM, 30 μM); black curve: untreated cells; violet curve: 0 nM DTG or PB28; blue curve: 1 nM DTG or PB28; green curve: 10 nM DTG or PB28; brown curve: 100 nM DTG or PB28; red curve: 1 μM DTG or PB28; orange curve: 10 μM DTG or PB28; yellow curve: 30 μM DTG or PB28.

flow-cytometry analyses in colon cancer HCT116 cells, control or PGRMC1-knockdown cells were prepared as reported previously [14]. HCT116 wt or HCT116 PGRMC1 knockdown cells were treated with or without 10 μM NO1 for 75 min at 37 °C, and the fluorescent intensity was detected by using a Gallios Flow Cytometer (Beckman Coulter, Brea, CA).

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 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 [21]. 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 13,000g 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 analyzed with InCyte[TM] software (Millipore).

2.4. Confocal microscopy analysis

1 x 10^5 cells were grown on sterile 8-well Thermo Scientific[TM] 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. The NO1 staining and immunostaining by using anti-PGRMC1 antibody (Sigma-Aldrich) in HCT116 cells were performed as mentioned above. The PGRMC1 staining was performed by using an Alexa568-conjugated secondary antibody. The cells were visualized by using LSM710 Zeiss confocal microscope (Oberkochen, Germany).

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 soni-cated and centrifuged at 20,000g for 30 min. The supernatant was incubated with glutathione Sepharose 4 B (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 chromatogra-phy according to previous studies [14].
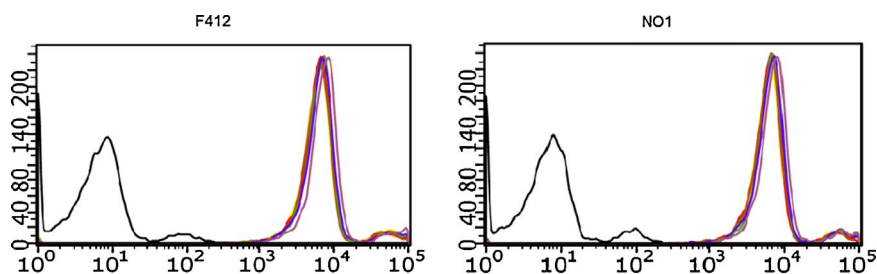
Fig. 3. Flow Cytometry analyses of MCF7 wt, MCF7_SH and MCF7_PGRMC1 cells labeled with sigma-2 fluorescent compounds. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Flow Cytometry analysis showing cell-associated fluorescence 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.
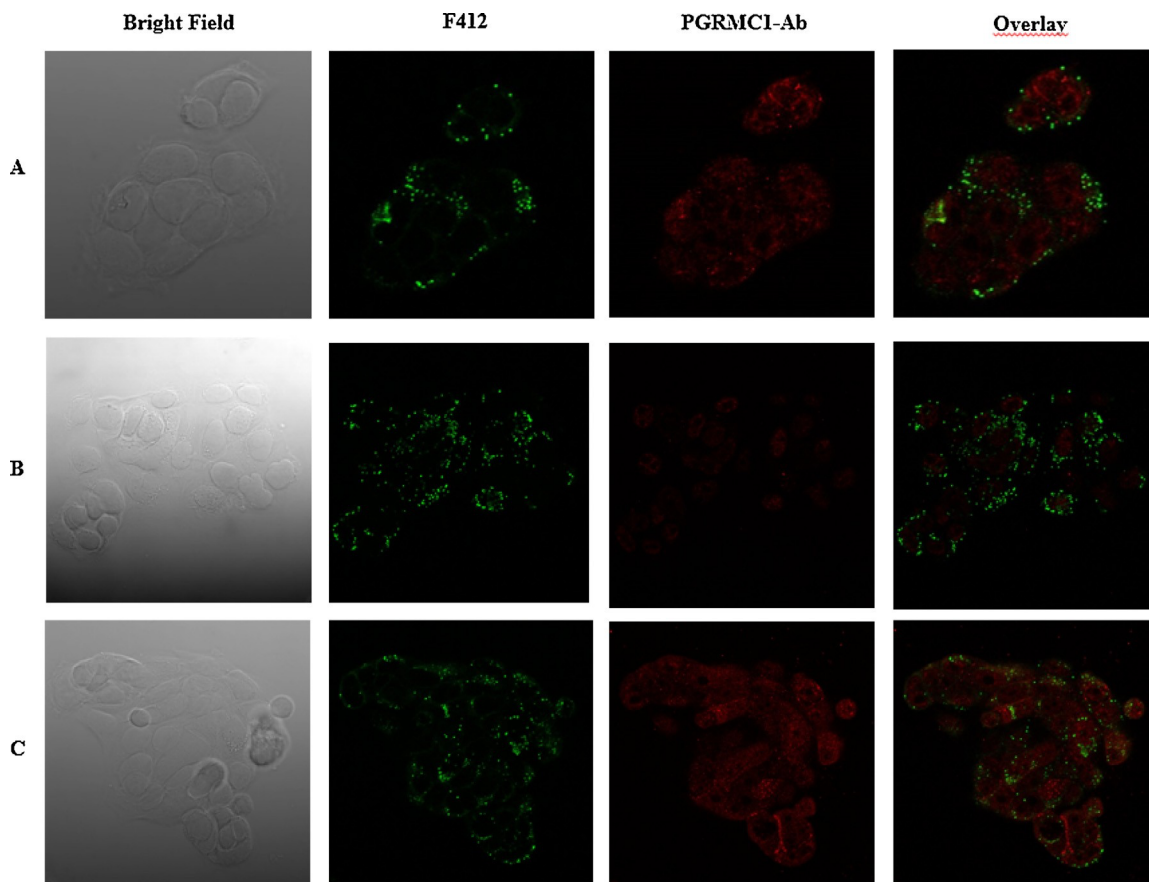
Fig. 4. Confocal microscopy analyses of MCF7 wt, MCF7 _SH and MCF7 _PGRMC1 cells labeled with PGRMC1-antibody and sigma-2 fluorescent compound F412. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Confocal microscopy analysis showing the three different cell lines MCF7 wt (A), MCF7 SH (B), MCF7_PGRMC1(C). Bright field (first column); staining with fluorescent sigma-2 receptor ligand F412 (10 μM, green, second column); staining with anti-PGRMC1 antibody (red, third column); overlay (fourth column). The images are representative of one experiment out of three.

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 [24]. 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

As human breast cancer MCF7 cells have been thoroughly characterized for the sigma-2 receptor expression becoming a model for sigma-2-related investigations, flow-cytometry studies were performed on three MCF7 cell lines: MCF7 wild-type (MCF7wt) and the corresponding cells devoid of (MCF7_SH) or overexpressing PGRMC1 (MCF7_PGRMC1) [11]. These cells were treated with increasing concentrations of the sigma-2 receptor ligands PB28
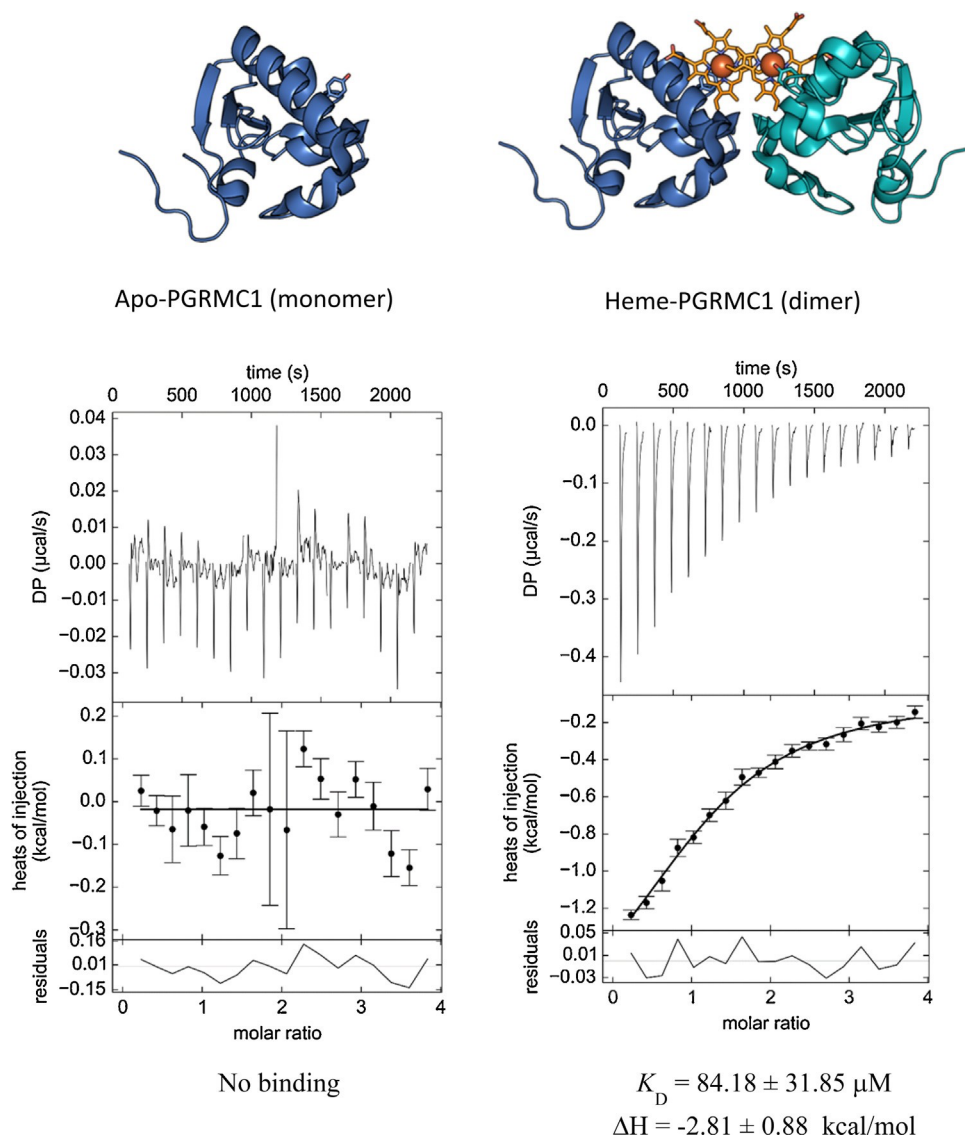
| Bright Field | NO-1 | PGRMC1-Ab | Overlay |
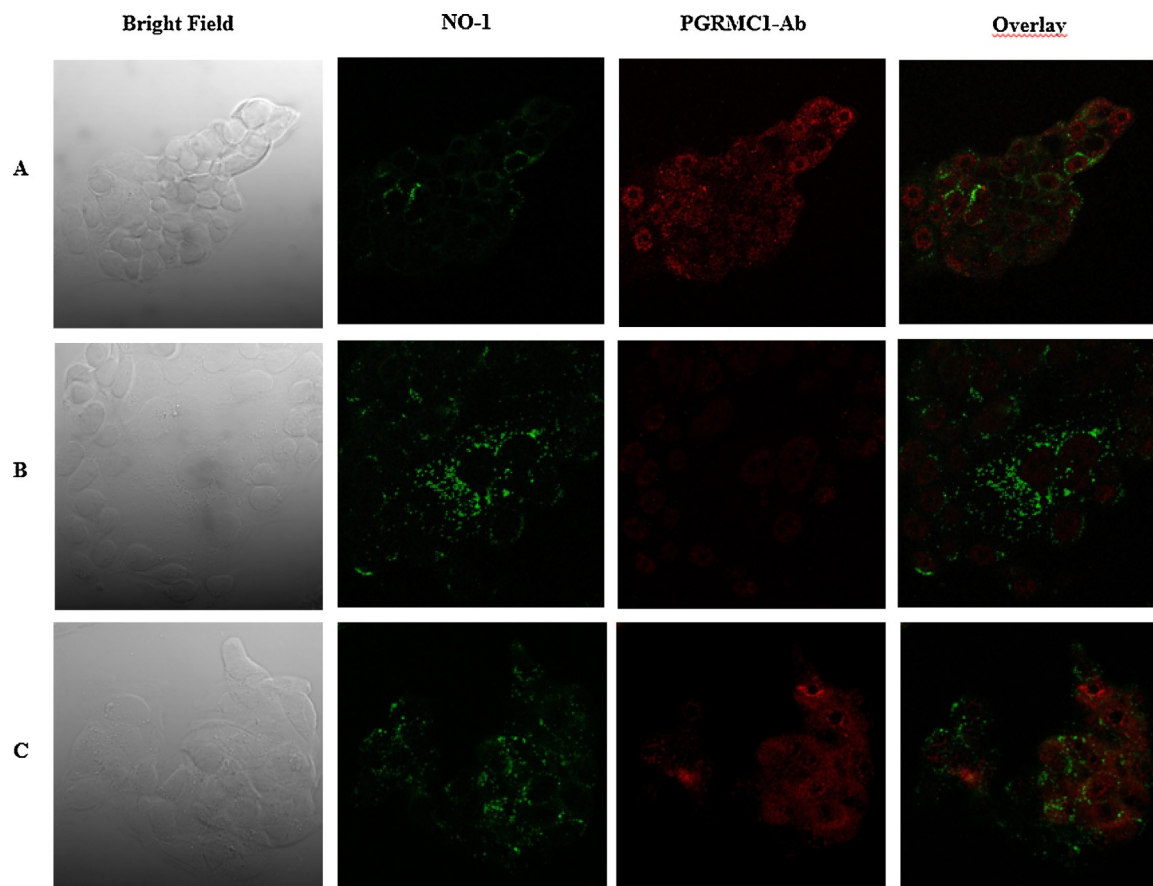
Fig. 5. Confocal microscopy analyses of MCF7 wt, MCF7 SH and MCF7 PGRMC1 cells labeled with PGRMC1-antibody and sigma-2 fluorescent compound NO1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) Confocal microscopy analysis showing the three different cell lines MCF7 wt (A), MCF7 SH (B), MCF7 PGRMC1(C). Bright field (first column); staining with fluorescent sigma-2 receptor ligand NO1 (10 μM, green, second column); staining with anti-PGRMC1 antibody (red, third column); overlay (fourth column). The images are representative of one experiment out of three.

[19,20] or DTG (K_i values in Table 1), or with the PGRMC1 inhibitor AG205. Afterwards, MCF7 cells were alternatively labeled with three previously developed sigma-2 receptor-specific fluorescent ligands having two different sigma-2 receptor-targeting moieties and fluorescent tags (Fig. 1). F412 [19] and PB385 [20] are analogues of the well known sigma-2 receptor ligand PB28 with high sigma-2 receptor affinity but moderate selectivity for sigma-1 (Table 1). On the other hand, NO1 [21] carrying the same fluorescent tag as F412, displayed high sigma-2 affinity and selectivity towards the sigma-1 receptors, in accordance with the dihydroisoquinolinone 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 [20,21]. As shown in Fig. 2, the two fluorescent compounds F412 (right panel) and NO1 (left panel) were dose-dependently displaced by PB28 or by DTG (from 1 nM to 30 μM) to a similar extents in all the three cell lines (Fig. 2, A–C: representative showing displacement with PB28). The less powerful fluorescent ligand PB385 (lower quantum yield) was also similarly dose-dependently displaced by PB28 or DTG (Supplementary Fig. 1). Binding curves from the three cell lines obtained by displacement of the fluorescent ligands with PB28 showed a substantial overlap at all the concentrations used (Supplementary Fig. 2). The curves generated by DTG also showed a significant overlap except for the lowest concentrations (10^{-9} M and 10^{-8} M, Supplementary Fig. 2). 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 tested cell lines (Fig. 3; representatively showing the experiment in PGRMC1 overexpressing cells labeled with F412 and NO1). Furthermore, we also determined the effect of the NO1 accumulation in human-derived colon cancer cell line HCT116 with intrinsic PGRMC1 (HCT116wt) or treated with shRNA for PGRMC1 (HCT116 SH) [14]. Between these cells, no difference in the NO1-derived fluorescent signal was observed (Supplementary Fig. 3). Taken together, these data indicate that fluorescent high affinity sigma-2 receptor ligands (with different structures in the hydrophobic, basic and fluorescent moieties), which comparably label sigma-2 receptors, do not label PGRMC1, supporting the notion that PGRMC1 and sigma-2 are two different proteins.

3.2. Confocal microscopy

As a next step, three cell lines MCF7wt, MCF7 SH or MCF7 PGRMC1 were then incubated with either F412 or NO1 for 45 min. Then cells were fixed with paraformaldehyde, permeabilized with TRITON-X 100 and PGRMC1 was visualized using a specific primary and TRITC-conjugated secondary antibody system. The three cell lines were then analyzed by confocal microscopy: sigma-2 receptors (F412 in Fig. 4, and NO1 in Fig. 5; green) and PGRMC1 (PGRMC1-Ab in Figs. 4 and 5; red) were clearly and specifically stained by their corresponding markers. No co-localization of the sigma-2 green emitting molecules with the PGRMC1 red signal was revealed (Overlay in Figs. 4 and 5). We also analyzed the cellular localization of NO1 and PGRMC1 in HCT116 cells. The NO1

Apo-PGRMC1 (monomer)　　　　　　Heme-PGRMC1 (dimer)

No binding

$K_D = 84.18 \pm 31.85\ \mu M$

$\Delta H = -2.81 \pm 0.88\ $ kcal/mol

Fig. 6. 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.

staining (green) was clearly observed irrespective of PGRMC1 presence (no difference between HCT116 wt or HCT116_SH). While a part of NO1 staining (green) was co-localized with PGRMC1 staining (red), overall the distribution was different in HCT116 wt cells (Supplementary Fig. 4).

3.2. Binding assay

In order to investigate the binding affinity of sigma-2 ligands with PGRMC1, the *in vitro* drug-binding assay 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]. In line with the intracellular co-localization studies, results from the calorimetric titrations curves (Fig. 6) showed no affinity of the nanomolar high affinity sigma-2 agonist DTG with the monomeric PGRMC1 protein. However, a modest interaction with the heme-dimerized form of PGRMC1 ($K_D = 84.18_{\pm} 31.85$ μM) was detected. In addition, we also analyzed the binding of the 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 (Fig. 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 affinity, supporting the notion that sigma-2 receptor and PGRMC1 are different proteins.

4. Discussion

Researchers need to 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 the target of these proteins. In this work, we used three structurally different fluorescent sigma-2 ligands (F412, NO1 and PB385) to stain their binding proteins in three MCF7 cell lines (MCF7 wt, 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 showed a dose-dependent displacement of the fluorescent ligands by two reference sigma-2 compounds (PB28 and DTG), but not by the reference PGRMC1 inhibitor (AG205). This is in accordance with the previously reported results of AG205 lacking affinity for sigma-2 receptors ($K_i > 10,000$ nM) [11]. The displacement occurred to comparable extents 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 interfere with PGRMC1 (Figs. 2 and 3; Supplementary Fig. 1). Binding curves generated by flow cytometry experiments significantly overlapped for the three MCF7 cell lines (Supplementary Fig. 2) with just slight differences that may be due to the diverse K_i values of DTG and PB28 (Table 1). Confocal images of the same cell lines, in which PGRMC1 was labeled with a fluorescent secondary antibody (red emitting), showed no co-localization with the proteins labeled by the small fluorescent molecules (F412 and NO1), while the staining of the sigma-2 proteins was in agreement with already found patterns in MCF7 cells [19], although the experiment was conducted for longer timescale in order to achieve the PGRMC1 co-staining. Red emission was in line with previously reported data from Western Blot analysis: in MCF7_PGRMC1 cells red emission was higher reflecting the overexpression of PGRMC1, while a faint red signal was detected from MCF_SH because of a residual expression of PGRMC1. Green emission was in agreement with previously reported Scatchard analyses detecting a slightly 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]. Flow cytometry and confocal microscopy experiments were also conducted on a different cell line: colon cancer HCT116 wild-type and the corresponding PGRMC1-knocked down. Also in these cells, the sigma-2 receptor labeling was independent of PGRMC1 presence, and an intense green emission due to the sigma-2 fluorescent ligand NO1 was detected from the PGRMC1-knocked down cells. While the affinity of the putative PGRMC1 inhibitor AG205 for sigma-2 receptors was already excluded by radioligand binding assays [11], herein we established the affinity of DTG and PB28 for the human purified PGRMC1. The crystallization of the PGRMC1 showing the heme-dependent dimerization through the stacking interaction of two protruding heme molecules was recently published. Therefore, 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 had no affinity at all for the apo-monomer, while DTG but not PB28 exhibited 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 [17] 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 further 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 the 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.

Conflict of interest

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

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2016.12.023.

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