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Elements in support of the 'non-identity' of the PGRMC1 protein with the σ_2 receptor

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ABSTRACT

σ_2 Receptor subtype is overexpressed in a variety of human tumors, with σ_2 agonists showing antiproliferative effects towards tumor cells through multiple pathways that depend both on the tumor cell type and on the molecule type. Therefore, σ_2 receptor is an intriguing target for tumor diagnosis and treatment despite the fact that that it has not yet been cloned. One of the last attempts to characterize σ_2 receptors led to identify it as the progesterone receptor membrane component 1 (PGRMC1). Although still controversial, such identity appears to have been accepted. We the aim of contributing to solve this controversy, in this work we stably silenced or overexpressed PGRMC1 protein in human MCF7 adenocarcinoma cells. Western blotting analyses were performed to quantify the presence of PGRMC1 protein on each of the three MCF7 cell lines variants, while scatchard analyses with radioligand were performed in order to determine the expression of the σ_2 receptors. In order to correlate the antiproliferative effect of σ_2 receptor agonist with PGRMC1 density, some σ_2 ligands were administered to each of the three MCF7 cells variants. The results suggested that PGRMC1 and σ_2 receptors are two different molecular entities.

1. Introduction

σ Receptors are classified into two subtypes, σ_1 and σ_2, that display distinct physiological and pharmacological profile in central and peripheral nervous system, with expression in many normal and tumors tissues. These receptors are associated with many cellular processes, including motor function, endocrine function, proliferation, immunoregulation and ion channel modulation (Megalizzi et al., 2012). σ_1 Receptor has been cloned (Hanner et al., 1996), and it has been shown to modulate the release of a number of neurotransmitters. Its role in neuroprotection and neuroplasticity in pathologies such as anxiety, depression, schizophrenia, drug addiction, Parkinson's and Alzheimer's diseases have been demonstrated (Cobos et al., 2008). σ_1 Receptor is involved in the intracellular signaling through modulation of Ca^{2+} levels via inositol 1,4,5-trisphosphate receptors, and its role in modulation of K^+ channels and lipid compartmentalization has been suggested, but it is yet to be understood (Balasuriya et al., 2014; Hayashi and Su, 2003; Kourrich et al., 2013). On the other hand, σ_2 receptor has not yet been cloned. Nevertheless, great interest in σ_2 research is due to the evidence that this subtype is overexpressed in a variety of peripheral and brain human tumors. In particular, Mach et al. (1997) demonstrated that the density of σ_2 receptors in proliferating cells was 10-fold greater than the density observed in quiescent cells (Al-Nabulsi et al., 1999). This observation led to propose the σ_2 receptor as endogenous biomarker for the proliferative status of tumors (Wheeler et al., 2000; ClinicalTrials.gov). Importantly, σ_2 receptors have been shown to exert antiproliferative effects towards tumor cells when activated with σ_2 agonists. Promising results have been also obtained in vivo by treatment of animal tumor xenografts with σ_2 agents (Abate et al., 2012a). Taken together these pieces of evidence make σ_2 proteins intriguing targets for tumor diagnosis and treatment. The antiproliferative mechanisms activated by σ_2 proteins are still under study. Apparently there are multiple pathways through which σ_2 agonists induce cell death which depend both on the tumor cell and on the molecule type (Zeng et al., 2012). Several attempts to characterize the σ_2 subtype have been made. Colabufo et al. (2006) suggested that σ_2 receptor could be related to

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; G418, geneticin; MCF7, human breast adenocarcinoma cells; MCF7_SH, human breast adenocarcinoma cells stably silenced for PGRMC1 protein; MCF7_PGRMC1, human breast adenocarcinoma cells stably overexpressing PGRMC1 protein; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; PEI, polyethylenimine; PGRMC1, progesterone receptor membrane component 1; ROS, reactive oxygen species

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histone proteins (Abate et al., 2010), whereas recent studies proposed σ_2 binding site located in the progesterone receptor membrane component 1 (PGRMC1) (Xu et al., 2011). Although in a few recent papers the identity of the σ_2 receptor with PGRMC1 appears as accepted (Ahmed et al., 2012; Mir et al., 2012, 2013) there is still controversy about that (Ruoho et al., 2013; van Waarde et al., 2014). In order to help in the resolution of this controversy, we selected human MCF7 breast adenocarcinoma cells, to stably silence or overexpress PGRMC1. MCF7 cells overexpress σ_2 receptors with a very low density of the σ_1 subtype, so that these cells are reliably used as mean to study σ_2-mediated action. Scatchard analyses with radioligand were performed on each of the three MCF7 cell variants in order to determine the expression of the σ_2 receptors, while western blotting analyses were performed to quantify PGRMC1 protein. Some σ_2 agonists were administered to each of the three MCF7 cells variants in order to correlate their antiproliferative effect with PGRMC1 density.

2. Materials and methods

2.1. Materials

[^{3}H]-DTG (29 Ci/mmol) and (þ)-[^{3}H]-pentazocine (32 Ci/mmol) were purchased from PerkinElmer Life Sciences (Zavantem, Belgium). DTG was purchased from Tocris Cookson Ltd., U.K. (þ)-Pentazocine was obtained from Sigma-Aldrich-RBI s.r.l. (Milan, Italy). Male Dunkin guinea-pigs and Wistar Hannover rats (250–300 g) were from Harlan, Italy. Cell culture reagents were purchased from EuroClone (Milan, Italy). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide), puromycin, G418 (geneticin), AG205, protease inhibitor cocktail, were obtained from Sigma-Aldrich (Milan, Italy). σ_2 Receptor agonists siramesine, F395, F397, F408 were obtained as reported in Niso et al., 2013, whereas PB28 and PB221 as in Berardi et al., 2009. FuGENE HD Transfection Reagent was purchased from Promega (Milan, Italy). Opti-MEM was obtained from Invitrogen. PGRMC1 MISSION shRNA (SHCLND-NM_006667), Anti-PGRMC1 Prestige antibody produced in rabbit, were purchased from Sigma-Aldrich (Milan, Italy). Anti-β-actin, secondary peroxidase antibodies and all reagents for western blotting were purchased from Life Technologies Italia (Monza, Italy).

2.2. Competition binding assays

All the procedures for the binding assays were previously described. σ_1 and σ_2 receptor binding were carried out according to Matsumoto et al. (1995). The specific radioligands and tissue sources were respectively: (a) σ_1 receptor, (þ)-[^{3}H]-pentazocine, guinea-pig brain membranes without cerebellum; (b) σ_2 receptor, [^{3}H]–DTG in the presence of 1 μM (þ)-pentazocine to mask σ_1 receptors, rat liver membranes. The following compounds were used to define the specific binding reported in parentheses: (a) (þ)-pentazocine (73–87%), (b) DTG (85–96%). Concentrations required to inhibit 50% of radioligand specific binding (IC_{50}) were determined by using six to nine different concentrations of the drug studied in two or three experiments with samples in duplicate. Scatchard parameters (K_d and B_{max}) and apparent inhibition constants (K_i) values were determined by nonlinear curve fitting, using the Prism, version 3.0, GraphPad software (1998).

2.3. Saturation binding assay

The saturation experiments were carried out as described by Vilner et al. (1995) with minor modifications in human MCF7, MCF7_SH and MCF7_PGRMC1 adenocarcinoma breast cell membranes. σ_2 Receptors were radiolabelled using [^{3}H]-DTG concentrations of 0.5–40 nM. Samples containing 200 μg membrane protein, radioligand, 10 μM DTG (to determine non-specific binding), and 1 μM (þ)-pentazocine (to mask σ_1 receptors) were equilibrated in a final volume of 500 μl (50 mM TRIS, pH 8.0) for 120 min at 25 1C. Incubations were stopped by addition of 1 ml icecold buffer (50 mM TRIS, pH 7.4), and then the suspension filtered through GF/C presoaked in 0.5% polyethylenimine (PEI) for at least 30 min prior to use. The filters were washed twice with 1 ml ice-cold buffer. Scatchard parameters (K_d and B_{max}) were determined by nonlinear curve fitting, using the Prism, version 3.0, GraphPad software (1998).

2.4. Cell culture

The human MCF7 breast adenocarcinoma was obtained from Interlab Cell Line Collection (ICLC, Genoa). MCF7_SH and MCF7_PGRMC1 were created in our laboratory. MCF7, MCF7_SH and MCF7_PGRMC1 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 1C with a 5% CO_2 atmosphere.

2.5. Construction of expression vector harboring PGRMC1 receptor complete coding sequence (CDS)

Total RNA was extracted from 1×10^6 MCF7 cells using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) and reverse transcripted with GeneAmp RNA PCR core kit (Applied Biosystem). The full-length coding region of the human PGRMC1 receptor (GenBank accession number NM_006667.4) was amplified from MCF7 cDNA using the iProof High Fidelity DNA Polymerase (Bio-Rad), 10 μl of GC Buffer, 2,5 μl of Dimethyl sulfoxide (DMSO), 10 pmol of each primer (Table 1) and 1 μl of dNTPs (10 mM for each nucleotide) in a final volume of 50 μl. PCR was run under the following conditions: preincubation at 98 1C for 30 s, run for 35 cycles at 98 1C for 10 s, 68 1C for 30 s, and 72 1C for 30 s, extension at 72 1C for 10 min. The PCR amplification product was purified using the High Pure PCR Product Purification Kit (Roche) and digested with HindIII and BamHI (Roche). The digested DNA fragment was ligated with the purified HindIII- and BamHI-digested pcDNA3.1(þ) vector (Invitrogen) in the sense orientation. Top10 chemically competent Escherichia coli cells (Invitrogen) were transformed with the construct described above and the vector amplified. The plasmid DNA was isolated by High Pure Plasmid Isolation Kit (Roche). The fidelity of the final human PGRMC1 receptor insert in pcDNA3.1(þ) plasmid was verified by DNA sequencing using BigDye Terminator Kit (Applied Biosystems) (Infantino et al. 2013) and the primers shown in Table 1.

2.6. MCF7 Transfection with sh_RNA targeting PGRMC1

To develop stably MCF7 without PGRMC1 receptor (MCF7_SH cell lines), MCF7 cells were plated at a density of 3×10^6 cells in 10 ml of growth medium in 100-mm Petri dishes, and incubated at 37 1C overnight. Cells were transfected with 17 μg of the pLKO.1

Table 1
Primers used for amplification and sequencing of PGRMC1 receptor CDS.

Primer name	Primer sequence 5′–3′
FORhindPGRMC1	CGAAAGCTTATGGCTGCCGAGGATGTGGTGG
REVbamPGRMC1	CAGGGATCCTTAATCATTTTTCCGGGCACTC
T7	TAATACGACTCACTATAGGG
Pcdna3rev	TAGAAGGCACAGTCGAGG

Table 2
σ Receptor affinities and antiproliferative activity of σ_2 reference compounds.

Compound	Affinity		Activity
	K_i ± S.E.M. (nM)[a]		EC_{50} ± S.E.M. (μM)[a]
	σ_1	σ_2	MCF7[c]
Siramesine	10.5 ± 2.6	12.6 ± 0.1	12.3 ± 0.6
PB28	0.38 ± 0.10	0.68 ± 0.20	28.4 ± 6.1
PB221	143 ± 18	18.8 ± 5.9	16.0 ± 2.0
F397	139 ± 20	5.34 ± 1.22	17.8 ± 0.4
F395	1.07 ± 0.28	90%[b]	8.86 ± 1.81
F408	219 ± 72	30.04 ± 0.01	28.2 ± 5.0
AG205	34 ± 7	369 ± 410,000	81.2 ± 6.2
DTG		31.5 ± 73.3	
(þ)-pentazocine	3.38 ± 0.31		

[a] Values represent the mean of n ± 2 separate experiments, in duplicate.
[b] Percent of displacement at concentration of 10^{-11} M is reported, when a complete displacement curve was not obtained.
[c] Antiproliferative effect in MCF7 cell line.

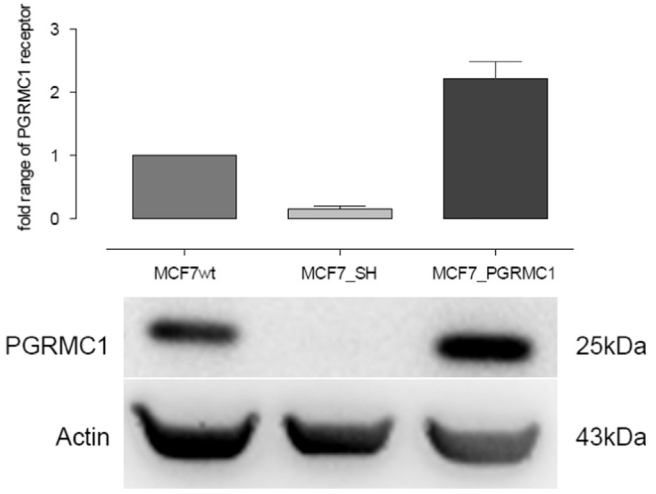

Fig. 1. Expression of PGRMC1 receptor protein in three different cells lines (1-fold unit is given to expression in MCF7 cells). In the figure are reported both histogram and bands of PGRMC1 receptor protein. β-actin protein is used for signal normalization.

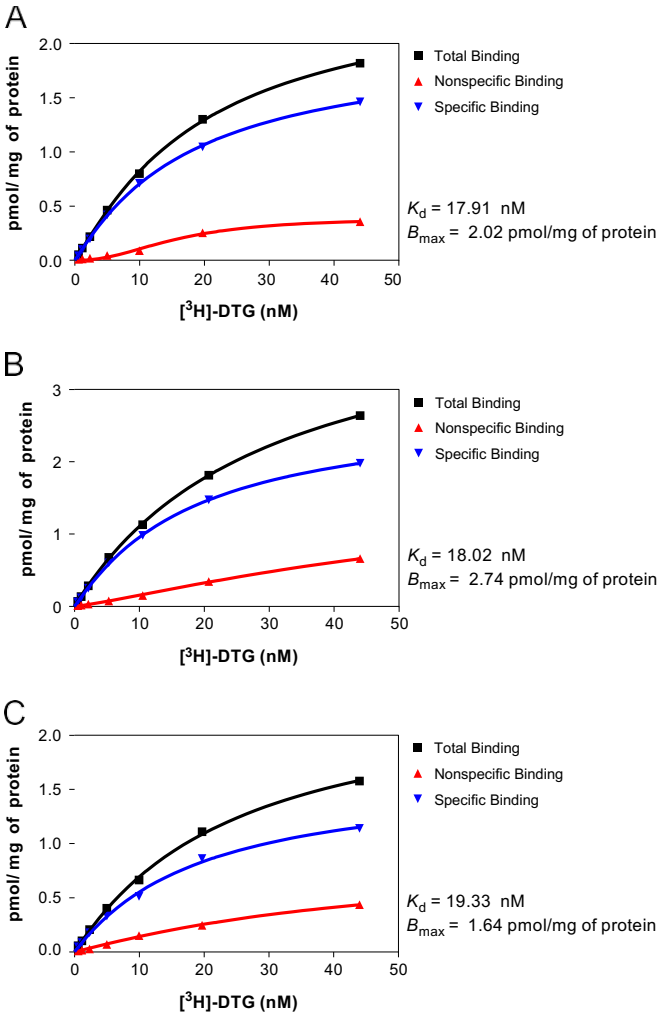

Fig. 2. Saturation analysis of σ_2 receptors in membrane preparations from MCF7 (A), MCF7_SH (B) and MCF7_PGRMC1 (C) cells. In the figure are reported curves of saturation analysis, K_d and B_{max} values for the three cell lines used.

vector containing sh_RNA targeting PGRMC1, as per standard protocol using FuGENE HD Transfection Reagent in Opti-MEM medium without serum. Vector-silencing cells were selected using puromycin. After transfection, cells were placed in normal DMEM growth medium. After 1 day, cells were detached with trypsin/EDTA and replated into DMEM growth medium containing puromycin (2 μg/ml) and cultured for 25 days. Surviving cell clones were picked out and propagated separately in 60-mm Petri dishes in the same medium, with 2 μg/ml puromycin. To suppress reversion of the phenotype, all subsequent cell culture was carried out in DMEM growth medium as described above, supplemented with 2 μg/ml puromycin.

2.7. MCF7 Transfection with PGRMC1 receptor

To develop stably MCF7 overexpressing PGRMC1 receptor (MCF7_PGRMC1 cell lines), MCF7 cells were plated at a density of 3 ~ 10^6 cells in 10 ml of growth medium in 100-mm Petri dishes, and incubated at 37 1C overnight. Cells were transfected with 17 μg of the pcDNA3.1(þ) vector containing the target PGRMC1 DNA sequence, as per standard protocol using FuGENE HD Transfection Reagent in Opti-MEM medium without serum. Vector-expressing cells were selected using geneticin (G418). After transfection, cells were placed in normal DMEM growth medium. After 1 day, cells were detached with trypsin/EDTA and replated into DMEM growth medium containing geneticin (800 μg/ml) and cultured for 25 days. Surviving cell clones were picked out and propagated separately in 60-mm Petri dishes in the same medium, with 800 μg/ml geneticin. To suppress reversion of the phenotype, all subsequent cell culture was carried out in DMEM growth medium as described above, supplemented with 800 μg/ml geneticin (Abate et al., 2012b).

2.8. Cell viability and co-administration assay

Determination of cell growth was performed using the MTT assay at 48 h (Azzariti et al., 2006). On day one, 25,000 cells/well were seeded into 96-well plates in a volume of 100 μl. On day two, the various drugs concentration (0.1 μM–10 μM) were added. In the co-administration assay one day, 25,000 cells/well were seeded into 96-well plates in a volume of 100 μl. On day 2, the drugs (0.1 μM–100 μM) were added alone or in combination with 30 μM of AG205. In all the experiments, the various drug–solvents (EtOH, DMSO) were added in each control to evaluate a possible solvent cytotoxicity. After the established incubation time with drugs (48 h), MTT (0.5 mg/ml) was added to each well, and after 3–4 h incubation at 37 1C, the supernatant was removed. The formazan crystals were solubilized using 100 μl of DMSO/EtOH

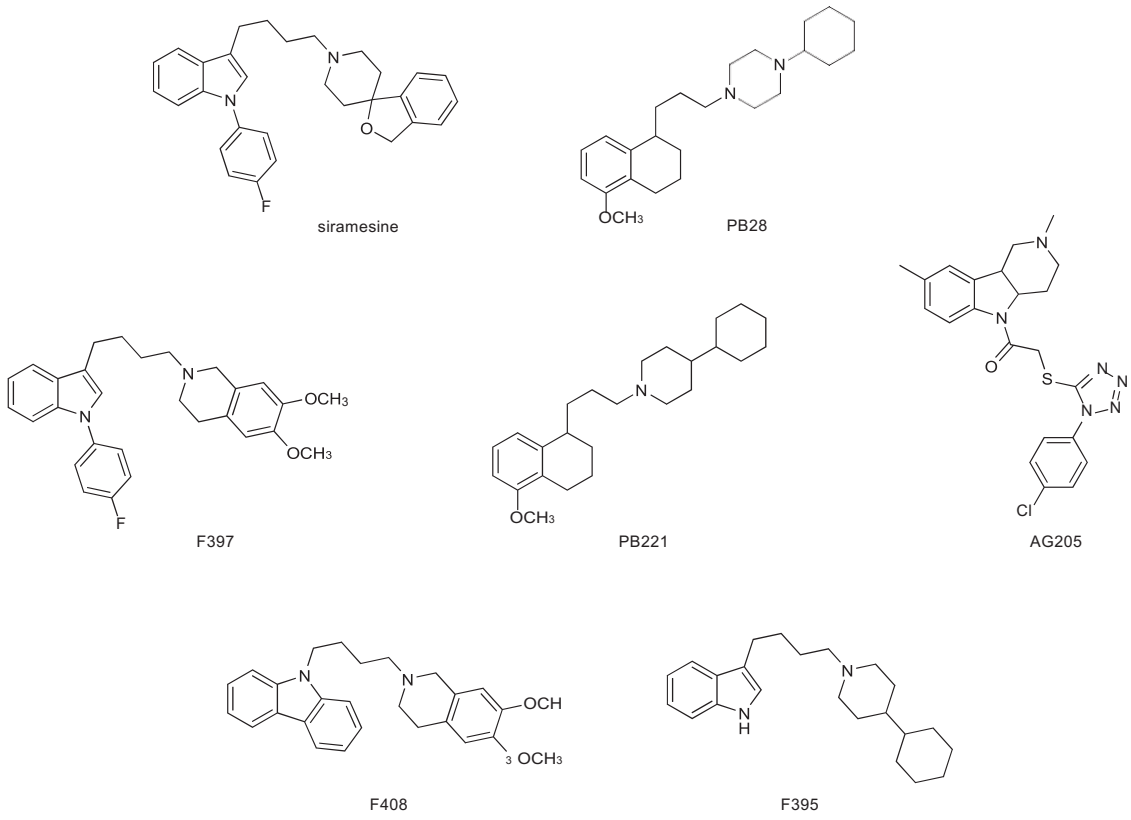

Fig. 3. Chemical structures of σ_2 receptor agonists and PGRMC1 inhibitor AG205.

(1:1) and the absorbance values at 570 and 630 nm were determined on the microplate reader Victor 3 from PerkinElmer Life Sciences.

2.9. Western blotting

All cells were washed twice with 10 ml phosphate-buffered saline (PBS), scraped in 1 ml PBS and centrifuged for 1 min at 11,000 g. Proteins were extracted from cells by homogenization in cold RIPA buffer (Life Technologies) containing 1X protease inhibitor cocktail and centrifuged at 14,000 g for 15 min at 4 1C. The supernatant was recovered and the protein concentration was measured using the microLowry kit. 30 μg of protein extract was separated on 10% polyacrylamide gel (Life Technologies) and then transferred onto a polyvinylidene difluoride membrane (PVDF) by iBlot® Gel Transfer Device (Life Technologies). Membrane was blocked for 30 min at room temperature with blocking buffer (1% BSA, 0.05% Tween 20 in Tris-buffered saline, TBS). The membrane was then incubated for 1 h at room temperature with either anti-PGRMC1 (1:1000 rabbit polyclonal) or anti-β-actin (1:1000 mouse monoclonal) antibodies, diluted in blocking buffer. After incubation time, membrane was washed with washing buffer (0.05% Tween 20 in Tris-buffered saline, TBS) for three times and incubated with a secondary peroxidase antibody (1:3000 anti-rabbit for PGRMC1 and 1:2000 anti-mouse for β-actin) for 1 h at room temperature. After washing, the membrane was treated with the enhanced chemiluminescence (ECL, Life Technologies) according to the manufacturer's instructions and the blot was visualized by UVITEC Cambridge (Life Technologies). The expression level was evaluated by densitometric analysis using UVITEC Cambridge software (Life Technologies) and β-actin expression level was used to normalize the sample values.

3. Results

3.1. Putative PGRMC1 ligand AG205 displays poor binding at the σ receptors

Affinity at the σ receptors of the putative PGRMC1 inhibitor AG205 was evaluated through the standard binding procedures for σ receptor binding determination. The K_i values obtained were very high for both receptors (K_i ¼3478 nM for σ_1 receptor, and K_i410,000 nM for σ_2 receptor, Table 2), indicating a very poor affinity at the σ_1 receptor and a lack of affinity at the σ_2 receptor.

3.2. Silencing and overexpression of PGRMC1 in MCF7 cell lines do not affect σ_2 receptor expression and σ_2 binding

In MCF7 cell line expression of PGRMC1 receptor was stably inhibited or increased through transfection either with pLKO.1-PGRMC1 or with pCDNA3-PGRMC1 vector. Thus, the cell line lacking PGRMC1 (MCF7_SH) and the cell line overexpressing PGRMC1 (MCF7_PGRMC1) were respectively obtained as shown by western blotting techniques (Fig. 1). Compared to MCF7 (1-fold unit is given to PGRMC1), the content of PGRMC1 receptor was reduced to less than 0.2-fold in MCF7_SH whereas it was increased of more than 2-fold in MCF7_PGRMC1 cells. In order to evaluate whether the variation of the content of PGRMC1 affected σ_2 receptors, saturation experiments with the σ_2 receptor reference ligand [^{3}H]-DTG were carried out on the three cell lines (e.g. MCF7, MCF7_SH and MCF7_PGRMC1, Fig. 2A–C). Surprisingly, the K_d and the B_{max} values among the three cell lines were strongly comparable, indicating that the content of the σ_2 receptors as well as the binding to DTG were not affected by the drastic reduction or increase of the PGRMC1.

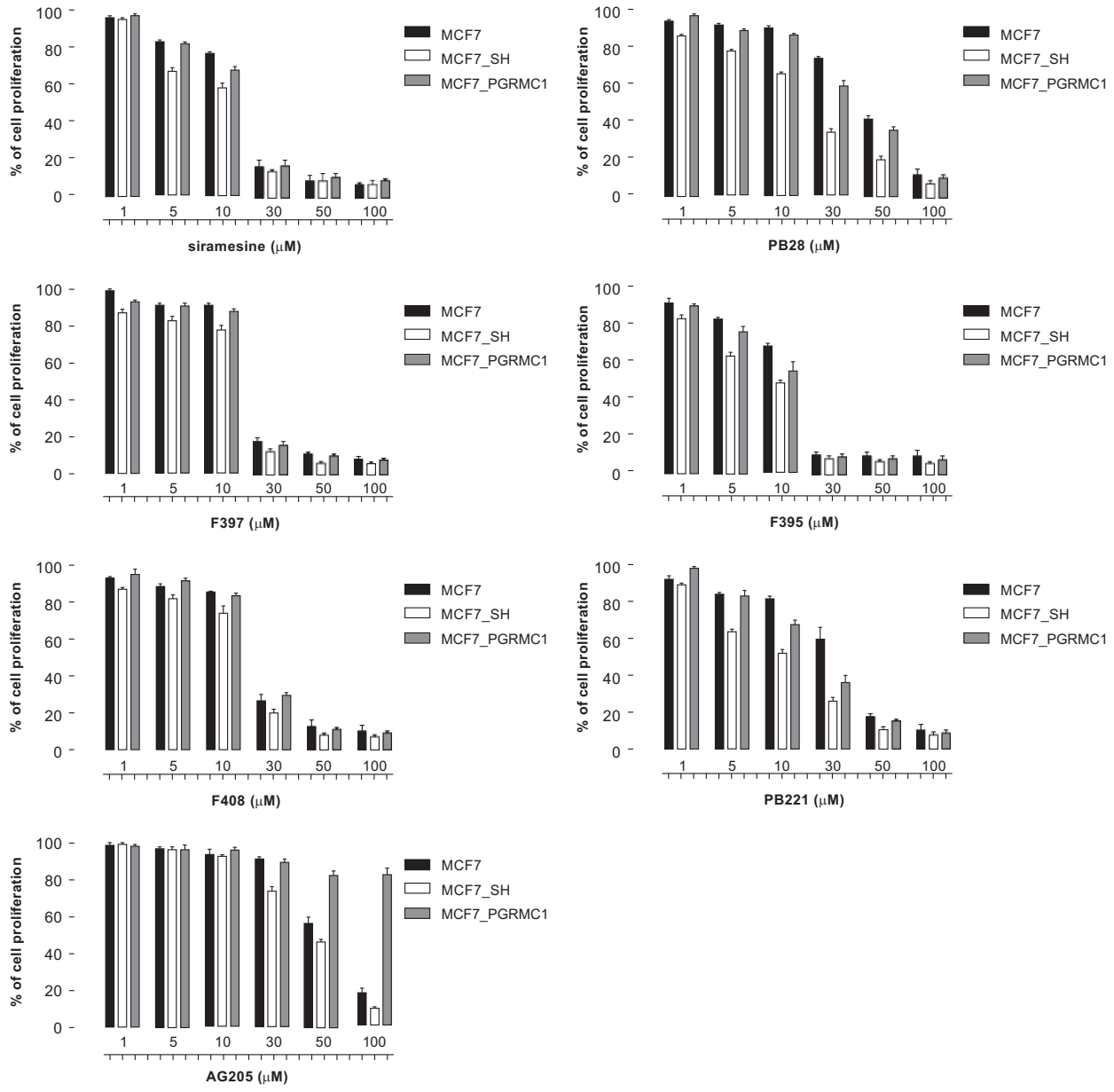

Fig. 4. σ_2 Agonists effect on cell viability. Antiproliferative assay was performed at 48 h. Different concentrations of some σ_2 ligands were administrated in MCF7 (black bars), MCF7_SH (white bars) and MCF7_PGRMC1 (gray bars).

3.3. The overexpression of PGRMC1 does not affect cell viability upon σ_2 receptor agonists administration, whereas the reduction makes cells more sensitive to the σ_2 mediated antiproliferative effect

Based on their pharmacological profile (Table 2), we selected six σ_2 receptor agonists (Fig. 3) which were previously reported to exert σ_2 mediated antiproliferative action. Among these compounds, siramesine and PB28 are two reference σ_2 agonists whose antiproliferative σ_2 mediated effect has been shown in diverse tumor cell lines as well as in vivo tumor models (Zeng et al., 2012; Hornick et al., 2012, 2010; Niso et al., 2013; Abate et al., 2011, 2012b; Berardi et al., 2009). Six concentrations of the compounds were used (from 1 μM to 100 μM) to investigate their antiproliferative action in MCF7, MCF7_SH and MCF7_PGRMC1 cell lines (Fig. 4). Generally, compounds administered at 1 μM were ineffective or barely effective in the three cell lines, whereas the highest concentration (100 μM) caused 90–100% of cells death. Depending on the EC_{50} of the compounds, concentrations at 50 μM (F408 and PB221) and 30 μM (siramesine, F397, F395) resulted toxic with less than 20% viability in all the three cell lines. In all the other cases, no difference or slight and insignificant differences in the antiproliferative effect of siramesine, F397, F408, PB28 and F395 were detected between MCF7 and MCF7_PGRMC1, suggesting that the overexpression of PGRMC1 in these cells does not interfere with the σ_2 mediated processes, at least with these σ_2 ligands. Only PB221 presented a more important difference in the antiproliferative effect in MCF7 and MCF7_PGRMC1 cells when used at 30 μM. By contrast, all the six compounds, used at concentrations below 100 μM, determined an antiproliferative effect in MCF7_SH more potent than in the other two cell lines, supporting the proliferative properties of PGRMC1, whose absence increased the sensitivity of the tumor cells to the σ_2 agonists toxic action. The same experiments were conducted with the putative PGRMC1 inhibitor AG205 which was used from 1 μM to 100 μM in the three cell lines. AG205 was able to exert an antiproliferative effect only at the highest concentrations (50 μM and 100 μM) in MCF7. By contrast, administration of AG205 in MCF7_SH started to be toxic at lower concentration (30 μM determined 25% of cell death), and this effect is curious since the effect of AG205 is more pronounced where its target (i.e. PGRMC1) is missing. Worthy of note

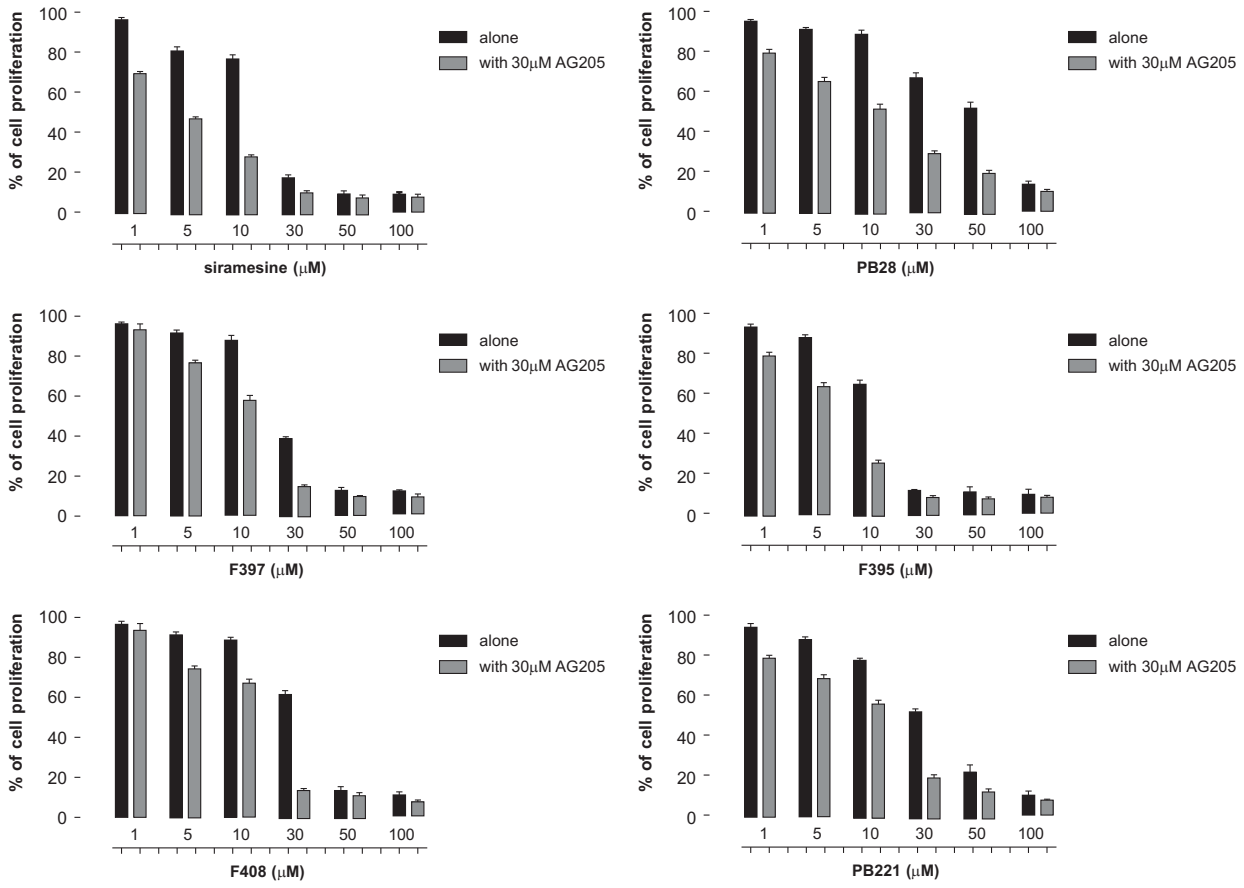

Fig. 5. σ_2 Agonists effect on MCF7 cell viability. Antiproliferative effect of some σ_2 agonists at 48 h. Compounds were administered alone (black bars) or in combination (gray bars) with 30 μM PGRMC1 inhibitor AG205.

is the absence of antiproliferative effect of AG205 in MCF7_PGRMC1, where the abundance of the cell-protective PGRMC1 is likely able to rescue cells from AG205-dependent death.

3.4. Blocking of PGRMC1 with AG205 sensitizes MCF7 cells to σ_2-agonists effect.

MCF7 cells were treated with each of the six σ_2 agonists (1 μM–100 μM) in the absence or presence of an ineffective concentration of AG205 (30 μM) (Fig. 5). The presence of the PGRMC1 inhibitor AG205 notably increased the cytotoxic effect of the σ_2 agonists: siramesine, PB28, F395 and PB221 determined a 20–30% cell death, even at the lowest concentration used (1 μM), whereas F397 and F408 caused the same level of cell death when used at 5 μM. Notably, none of these compounds, administered alone, showed antiproliferative activity at concentrations lower than 10 μM. Generally, the co-presence of 30 μM AG205 together with the σ_2 agonist determined the death of 20–40% cells more than the administration of the σ_2 agonist alone, suggesting that rather than being the same molecular entity, PGRMC1 and σ_2 may act synergistically affecting cell proliferation.

4. Discussion

Since σ_2 receptors were identified as the PGRMC1 protein complex in 2011 (Xu et al., 2011), a number of papers, in which this identity appears to be taken for granted, came out (Mir et al., 2012, 2013; Izzo et al., 2014a, 2014b). However, controversy is still on about the identity of these receptors. PGRMC1 is a 25 kDa protein that binds P450 cytochrome, whereas a molecular mass of 18 kDa has been attributed to σ_2 protein by photoaffinity labeling (Ruoho et al., 2013), with no binding activity at P450. In order to provide insights into the identity and functions of these proteins, which are intriguing targets for tumor therapy and diagnosis, we transfected MCF7 cells with the appropriate shRNA or cDNA to reduce (MCF7_SH) or increase (MCF7-_PGRMC1) the expression of PGRMC1 receptor, as confirmed by the western blotting techniques. In the same cell lines, the density of the σ_2 receptors was investigated by saturation with the reference σ_2 radioligand [^{3}H]-DTG. If σ_2 and PGRMC1 receptors were the same molecular entity, overexpression of PGRMC1 should result in an increased B_{max} in MCF7_PGRMC1 compared to MCF7. Nevertheless, this was not the case, with both MCF7_PGRMC1 and MCF7_SH showing saturation curves, B_{max} and K_d values very similar to the same values in MCF7. Indeed, over- or down- expression of the PGRMC1 did not result in a change of the expression of the σ_2 receptors as well as in the binding affinity to DTG, showing that PGRMC1 and σ_2 receptors (detected by DTG) are two different proteins. The photoaffinity photoprobe containing a σ_2-directing moiety that led to the identification of PGRMC1 (Xu et al., 2011) as the σ_2 receptor, likely binds both PGRMC1 and σ_2 receptor, suggesting that other σ_2 ligands may bind both proteins. Therefore, we selected six already known σ_2 receptor agonists belonging to different structural classes, and we checked whether the variation of the expression of the PGRMC1 in the three MCF7 cell lines affected their antiproliferative actions. We chose two well-established reference compounds (siramesine and PB28) together with other four σ_2 ligands previously shown to exert antiproliferative activity (F397, F395, F408, PB221). The overexpression of the PGRMC1 did not affect the antiproliferative action of these σ_2

ligands, with MCF7 and MCF7_PGRMC1 cells displaying pretty much the same response. By contrast, MCF7_SH cells appeared more sensitive to σ_2 receptor ligands, as expected in cells where the promoter of cell survival PGRMC1 is less expressed. These results led to different hypotheses: 1) σ_2 receptor ligands do not bind (or do not inhibit) PGRMC1, as no difference in their effect between MCF7 and MCF7_PGRMC1 cells was recorded; 2) no additional effect can be exerted by PGRMC1 in MCF7_PGRMC1 cells, in the absence of PGRMC1 binding partners/effectors over-expression. The latter hypothesis was weakened by results obtained with PGRMC1 inhibitor AG205, which was studied in the three MCF7 cell lines (Fig. 4). The PGRMC1 inhibitor did not determine any antiproliferative effect (administered up to 100 μM) in MCF7_PGRMC1, likely because the abundance of PGRMC1 massively promoted cell survival and damage resistance. This result suggested that the cell survival action can be increased by the sole overexpression of PGRMC1 even if the possible PGRMC1 binding partners are not overexpressed along with PGRMC1. However, such overexpression was not able to rescue MCF7_PGRMC1 cells from σ_2 agonist mediated cell death, suggesting that AG205 and σ_2 ligands likely act through different pathways. Curiously, AG205 was more effective in MCF7_SH where its target (i.e. PGRMC1) was only barely expressed, suggesting a possible off-target effect. Therein, 30 μM AG205 determined a 20% cell death, whereas 50 μM were needed in MCF7 to exert some antiproliferative action. Although it has been previously shown that AG205 effect depends on cultures conditions (% of serum and type of cells, Mir et al., 2012), we recorded a weak antiproliferative effect in MCF7 that could be exploited to reinforce σ_2 agonists' action, as already indicated by results from σ_2 agonists in MCF7_SH cells. Therefore, we selected the highest ineffective concentration of AG205 (i.e. 30 μM) in parent MCF7 cells to study the effect of σ_2 receptor ligands when PGRMC1 is pharmacologically inhibited (Fig. 5). The presence of AG205 determined a considerably more potent effect of all σ_2 receptor ligands in comparison to compounds administered alone. This synergic action was already shown at the lowest concentration (1 μM) with siramesine, PB28, F395 and PB221 showing an antiproliferative effect even more pronounced than the effect shown in MCF7_SH. This synergic effect may be due to either the inhibition of the cell protective protein PGRMC1 by AG205 or to AG205 off-target action.

5. Conclusions

Taking all the results together, we suggest that PGRMC1 and σ_2 receptors are two different molecular entities. σ_2-Mediated cell death is not influenced by increased levels of PGRMC1 in MCF7 cells, which appear to be more sensitive to σ_2 ligands in the absence or by the inhibition of the 'pro-survival' protein PGRMC1. Co-administration of PGRMC1 inhibitor AG205 with σ_2 agonists results in notably increased antitumor effect of σ_2 agonists and suggests convenient synergic effects. In conclusions, these results that support the need of further studies about these two proteins, enlighten their importance in cancer-related research. Their exploitation may shed light on novel tumor therapeutic approaches.

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