Novel and Selective Fluorescent σ2 -Receptor Ligand with a 3,4-Dihydroisoquinolin-1-one Scaffold: A Tool to Study σ2 Receptors in Living Cells

Novel and selective σ_2 receptor fluorescent ligand with 3,4-dihydro-isoquinolin-1-one scaffold as tool for σ_2 receptor study in living cells

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Although sigma-2 (σ_2) receptors are still enigmatic proteins, they are promising targets for tumor treatment and diagnosis. With the aim of contributing to clarify their role in oncology, we developed a σ_2-selective fluorescent tracer (compound **5**) as a specific tool to study σ_2 receptors. Using **5** by flow cytometry, we successfully performed competition binding studies on three different cell lines where we also detected σ_2 receptors' content, avoiding the inconvenient use of radioligands. Comparison with a previously developed mixed σ_1/σ_2 fluorescent tracer (**1**) allowed the detection of σ_1 receptors within these cells, as well. Results obtained by flow cytometry with the two tracers **1** and **5** were confirmed by standard methods (western-blot for σ_1, and Scatchard analysis for σ_2 receptors). Thus, we produced powerful tools for σ receptor research whose reliability and adaptability to a number of fluorescence techniques will help to elucidate σ receptors roles in oncology.

Introduction

Since they were first proposed in 1976, sigma (σ) receptors have been thoroughly studied. In the early 1990s two subtypes were pharmacologically recognized, namely σ_1 and σ_2, and since then, only the σ_1 has been cloned. This subtype works as an Endoplasmic Reticulum (ER) chaperone resulting in a number of functions such as Ca^{2+} signaling mediated by IP3 receptor, cholesterol compartmentalization, neurotransmitters release and microglia modulation.[1] Involvement in amyotrophic lateral sclerosis, Alzheimer's and Parkinson's diseases has been reported for this protein.[2-4] Different hypotheses have been made about the identity of the σ_2 subtype. After σ_2 receptors were proposed to be histone related proteins,[5] they were then identified as the progesterone receptor membrane component 1 (PGRMC1).[6] Although the latter hypothesis seems rather accepted, some controversy about the nature of this subtype is still on.[7,8] Despite the fact that the identity and the physiological roles of σ_2 receptor have to be still elucidated, scientific interest is on the increase because these receptors are overexpressed in a number of tumors. Moreover, when activated by specific ligands, σ_2 receptors cause cell death through diverse pathways that appear to depend on the cell types and on the molecule types.[9] Caspases involvement, as well as authophagy and reactive oxygen species (ROS) have been reported for cell death mediated by σ_2 ligands.[10-13] In order to fully understand the pharmaceutical potentials of these proteins to be exploited for both tumor diagnosis and therapy, more specific and powerful pharmacological tools are needed. With this aim, we developed different series of σ_2 receptor ligands for the visualization of σ_2 proteins *in vivo* - by Positron Emission Tomography[14-16] - and *in vitro* - by fluorescence microscopy.[17-19] For the latter aim, we developed diverse series of fluorescent

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ligands looking for the best compromise between pharmacological and fluorescent properties. Among all the compounds synthesized, 2-(6-{1-[3-(4-cyclohexylpiperazin-1-yl)propyl]-1,2,3,4-tetrahydronaphthalen-5-yloxy}hexyl)-5-dimethylamino-1*H*-isoindole-1,3(2*H*)-dione (**1**, Figure 1) emerged as a σ receptor high affinity ligand with optimal fluorescent properties for living cell visualization.[19] σ_2-Dependent up-take of the compound was shown by fluorimetric experiments in human breast adenocarcinoma MCF7 cells, where σ_2 are overexpressed. By confocal microscopy we followed **1** distribution, and by flow cytometry we verified the compound suitability to perform σ_2 binding experiments demonstrating that **1** is a useful and safe alternative to radioligands. However, if only σ_2 receptors have to be detected, **1** would not be the ligand of choice in cells that express both σ subtypes, as **1** binds equally well to σ_1 and σ_2 receptors, visualizing a dual nonselective up-take. With the aim of developing a σ_2 selective fluorescent ligand, we based our design on the 3,4-dihydro-isoquinolin-1-(2*H*)-one scaffold which demonstrated to selectively bind σ_2 receptors when linked to 6,7-dimethoxytetrahydroisoquinoline as basic moiety.[14,20] Therefore, we functionalized such scaffold with the fluorescent tag present in **1**. Following this approach, we obtained a σ_2 selective fluorescent ligand (i.e. **5**) that was successfully used in flow cytometry to detect σ_2 receptor density and to perform σ_2 binding experiments. Herein we show that σ_2 selective compound **5** is as promising as **1** and appears as a powerful alternative tool to radioligands when σ_2 receptors want to be studied.

Results and Discussion

Design and Synthesis. 1-Cyclohexyl-4-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)-*n*-propyl]piperazine (**PB28**, Figure 1) is one of the highest affinity σ_2 agonists known,[9] and several series of compounds were developed starting from it as the lead compound. In addition, different series of fluorescent ligands were developed from its structure, and Structure Affinity Relationship (SAfiR) studies indicated the position on **PB28** where to insert the fluorescent tag through the most appropriate linker in order to obtain optimal σ_2 binding.[18] Excellent results in terms of σ_2 affinity and fluorescent properties were obtained with ligands such as **1**.[19] Nevertheless, as demonstrated by several SAfiR studies, the presence of 1-cyclohexylpiperazine as the basic moiety is responsible for the high affinity at both σ receptor subtypes in the **PB28**-related structures (e.g. **1**), so that selectivity is hard to be reached.[10, 20-22] Therefore, we focused our attention on 2-(3-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl)propyl)-5-methoxy-3,4-dihydroisoquinolin-1(2*H*)-one (**2**, Figure 1)[14] belonging to the series of the 3,4-dihydro-isoquinolin-1-(2*H*)-one derivatives. Its σ_2 *vs* σ_1 selectivity was excellent (Table 1) and its structure was suitable for the functionalization with a fluorescent tag.

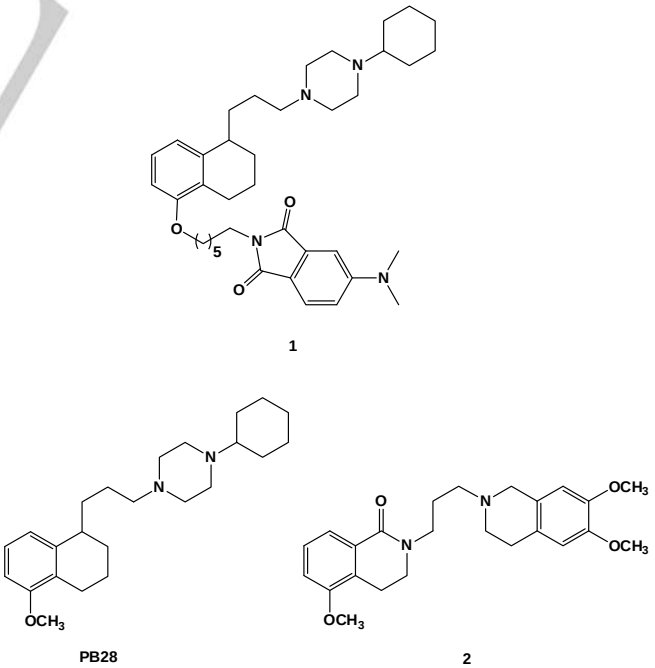
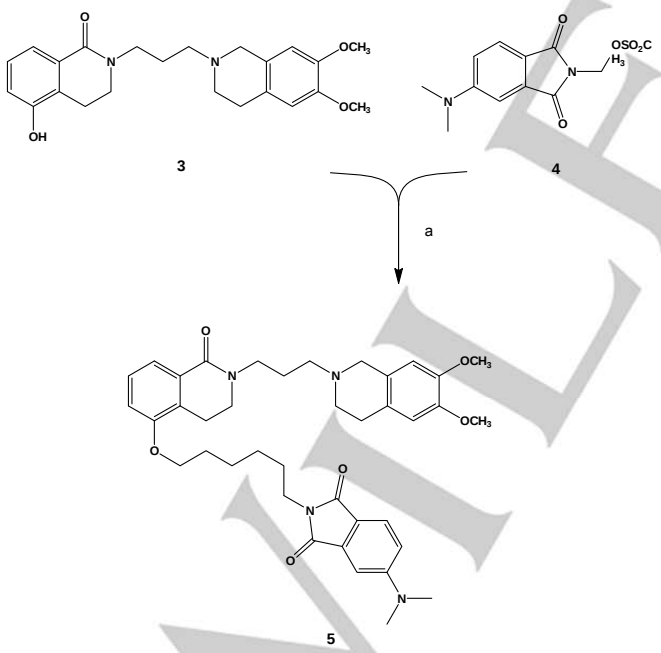


Figure 1. σ_2 Reference ligands.

The methoxy group in 5-position on the 3-4-dihydroisoquinolinone ring could conveniently be replaced by a hexamethylenoxy group, taking advantage of the results obtained with the **PB28**-related fluorescent ligands.[18,19] As fluorescent tag, 4-*N*,*N*-dimethylphthalimide was selected because of its recognized environment-sensitive properties able to reflect the changes in polarity that often occurs in organized media. This tag, which was previously inserted into peptides, brought excellent results also when linked to small molecules in assay with living cells.[19, 23, 24] Therefore, according to two previously followed synthetic pathways, we obtained 2-(3-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl)propyl)-5-hydroxy-3,4-dihydroisoquinolin-1(2*H*)-one (**3**)[14] and 6-(5-(dimethylamino)-1,3-dioxoisoindolin-2-yl)hexyl methanesulfonate (**4**).[19] These two key intermediates were reacted in DMF in the presence of K_2CO_3 to afford the final fluorescent compound **5** (Scheme 1).



Scheme 1. Synthesis of fluorescent compound **5**.

Reagents and conditions: a) K_2CO_3, DMF 150°C, overnight.

σ Receptors binding. Results from binding assays are expressed as inhibition constants (K_i values) in Table 1. The novel compound **5** kept the same selectivity displayed by the lead compound **2**, and a σ_2 receptor affinity similar to the value displayed by **1**. There was an important decrease in the σ_1 binding (K_i > 5000 nM) and just a slight decrease in the σ_2 affinity (K_i = 10.2 nM) compared to compound **2**, so that the σ_2 *vs* σ_1 selectivity was > 500-fold. This result confirmed **2** as an excellent pharmacophore for obtaining highly σ_2 *vs* σ_1 selective ligands, despite the functionalization with bulky substituents in the 5-position.

Fluorescence Spectroscopy

The fluorescent properties are listed in Table 1. The excitation and emission spectra were recorded for **5** both as hydrochloride salt and as free base in EtOH at a concentration of 10^{-4} mol/L. For comparison purposes, fluorescent properties of compound **1**, which were extensively studied previously, were measured in EtOH at a concentration of 10^{-4} mol/L. The two forms of compound **5** (free base and hydrochloride salt) displayed the same maximum excitation wavelength (λex = 390 nm) and the same maximum emission wavelength (λem = 525 nm). An important difference between λex and λem (i.e., Stokes shift) was recorded. As for **1**, measurement of quantum yield (QY) demonstrated the environment sensitive properties of novel compound **5**, that displayed low fluorescence intensity in polar solvent (QY = 0.02 in EtOH), but high fluorescence in nonpolar solvents, or when bound to hydrophobic sites (QY = 0.5 in $CHCl_3$). As expected, PBS solutions of compound **5** provided QY values close to 0, as also recorded for **1**. Despite the similar QY values, the hydrochloride form of **5** displayed an intensity of the fluorescent signal at λem around 2-fold lower than the compound as the free base (Figure 1, Supporting Information).

Table 1. Receptor Affinities and Fluorescence Properties of Final and Reference Compounds.

	K_i nM ± SEM (nM)[a]					
compound	**σ_1**	**σ_2**	**λex nm**[b]	**λem nm**[b]	**QY EtOH**	**QY $CHCl_3$**
1[c]	5.37 ± 1.96	6.9 ± 2.84	400	520	0.015	0.6[d]
2[d]	2435 ± 995	4.24 ± 0.84				
5	> 5000	10.2 ± 2.4	390	525	0.02	0.5
DTG		32.1 ± 3.1				
(+)-pentazocine	2.87 ± 0.57					

[a] Values are the means of n ≥ 3 separate experiments, in duplicate. [b] Fluorescence properties of compound **5** were evaluated on compound both as free base or hydrochloride salt in EtOH solutions; compound **1** was evaluated as hydrochloride salt. [c] From ref 19. [d] From ref 14.

Detection of σ Receptors by Flow Cytometry

Up-take of compounds **1** and **5** was studied in three human tumor cell lines, which were selected according to their content in σ receptors. Human breast MCF7 tumor cells had been previously characterized for their overexpression of σ_2 receptors by both Scatchard analysis and fluorescence microscopy.[19,25] By contrast, σ_1 receptors' density is very low,[25] so that this cell line is widely used when σ_2-receptor mediated actions want to be studied. Human glioblastoma U87 cells were reported to express σ_1 receptors, but no indications on the σ_2 receptors content has been reported, at the best of our knowledge.[26] Human leukemic monocyte THP1 cells were reported to express σ_1 receptors with a 3-fold higher density than σ_2 receptors, but with a high K_d that corresponded to a low-affinity σ_1 binding site.[27] Since the up-take of the fluorescent compounds increased in a dose-dependent manner reaching a plateau around 100 nmol/L, all the experiments were performed incubating the three cell lines with either **1** or **5** at 100 nmol/L. Incubation of the three cell lines with **5**·HCl resulted in a significantly lower fluorescence accumulation in comparison to the incubation with the corresponding free base, in accordance with the lower intensity of the fluorescence signal detected by fluorescence spectroscopy. Therefore, all of the experiments were performed with **5** as free base. In order to set up a σ_2 receptor binding assay, we studied the best incubation time for the three cell lines. We incubated the cells for 45 min, or 75 min or 180 min at 37 °C with increasing concentrations (from 1 nmol/L to 10 μmol/L) of either 1,3-di-(2-tolyl)guanidine (DTG) or PB28 as σ_2 reference compounds, followed by 100 nmol/L of either **1** or **5**. In all cases, we detected a progressive decrease of fluorescence when the concentrations of reference compounds increased (Figure 2, Supporting Information), indicating that both fluorescent tracers specifically bind σ_2 receptors. For the three cell lines, the best results in terms of fluorescence intensity related to dose-dependent displacement were obtained with incubation time of 75 min so that binding curves were generated for PB28 and DTG, using both **1** and **5** as fluorescent tracers (fluo-ligands) in spite of [^{3}H]-DTG radioligand (Figure 3, Supporting Information). In MCF7, we detected IC_{50} values of PB28 and DTG with fluo-ligand **5**, and for comparison purposes we re-evaluated the same values with fluo-ligand **1**. IC_{50} value obtained with **5** (IC_{50} values = 14.1 nM for DTG and 5.24 nM for PB28, Table 2) reliably matched the value obtained with **1** (IC_{50} values = 13.7 nM for DTG and 4.90 nM for PB28, Table 2). This data were in accordance with what we previously found by radioligand competition binding assay where [^{3}H]-DTG was displaced by PB28 in MCF7 cell membranes.[19] In THP1 and in U87 cells, IC_{50} values obtained through fluo-ligand **5** (IC_{50} values = 19.7 nM for DTG and 8.36 nM for PB28, in THP1; IC_{50} values = 27.5 nM for DTG and 10.6 nM for PB28, in U87, Table 2) again matched values obtained through fluo-ligand **1** (IC_{50} values = 22.9 nM for DTG and 9.78 nM for PB28, in THP1; IC_{50} values = 25.9 nM for DTG and 11.2 nM for PB28, in U87; Table

2). In both these cell lines, the σ_2 affinity values displayed by the two reference compounds were slightly lower than the affinity values obtained in MCF7.

Table 2. PB28 and DTG binding by fluo-ligand assay**.**

Cell Lines	compd	IC_{50} (μM)[a]	
		1	**5**
MCF7	DTG	13.7±1.3	14.1±1.6
	PB28	4.90±0.9	5.24±1.1
THP1	DTG	22.9±2.1	19.7±1.8
	PB28	9.78±1.3	8.36±1.1
U87	DTG	25.9±1.9	27.5±2.3
	PB28	11.2±0.9	10.6±1.2

[a] Values are the means of n ≥ 3 separate experiments, in duplicate.

In MCF7, we detected the highest intensity of the fluorescent signal with very low differences between the two fluorescent tracers. This result reflected the expression of σ receptors in MCF7 cell line: both the σ_2 selective tracer **5** and the σ unselective tracer **1** label only σ_2 subtype, given the very low density of σ_1 receptors. No difference between the two fluorescent tracers was detected in the other two cell lines studied (e.g. U87 and THP1), where we expected to detect higher fluorescence intensity upon labeling of both σ_1 and σ_2 receptors with **1**, compared to **5** that preferentially labels σ_2 subtype. As in MCF7, this result indicated a very low density of the σ_1 subtype in U87 and THP1 cells. In addition, in both these cell lines fluorescence intensity was 5-times lower than in MCF7 cells, suggesting that σ_2 receptors are less expressed in U87 and THP1 cells than in MCF7 cells (Figure 3).

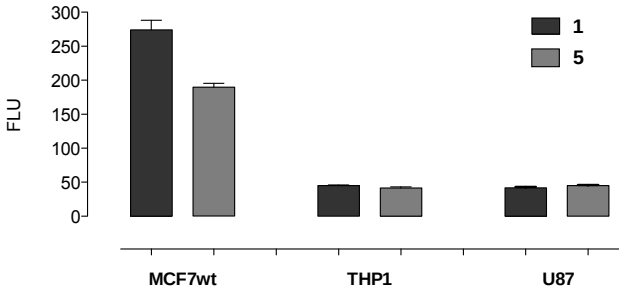


Figure 3. Fluorescent intensity by incubation of the cells with 100 nM/L of fluo-ligand **1** or **5**.

Detection of σ_1 Receptors by Western Blotting. In order to test these hypotheses we detected the presence of the σ_1 receptors by immunoblotting techniques, in the three cell lines. As anticipated by flow cytometry experiments, the density of σ_1 receptors in U87 and THP1 cells was as low as in MCF7, with U87 displaying a slightly lower content, and THP1 displaying a slightly higher content compared to MCF7 (Figure 4; 1-fold is attributed to MCF7). To support these data, we detected the presence of the σ_1 subtype in the MCF7 σ_1-overexpressing cell line (MCF7σ_1), that we previously obtained by transfection of the MCF7 cells with the σ_1-cDNA.[25] The stably transfected MCF7σ_1 cells displayed a 6-fold higher content of the σ_1 subtype than in the three cell lines (Figure 4).

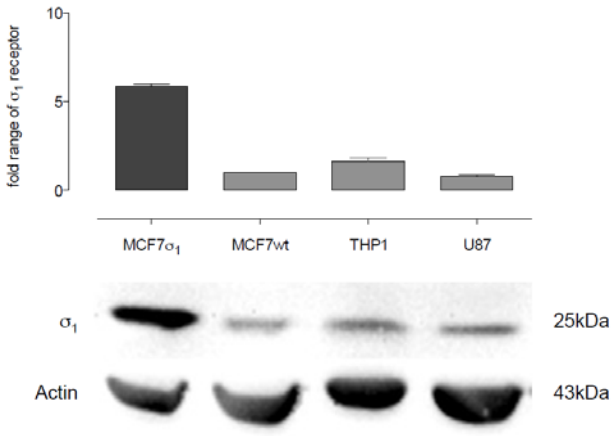
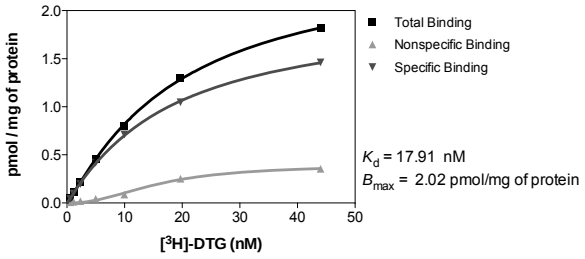


Figure 4. Expression of σ_1 receptors (1-fold unit is given to expression in MCF7 cells).

Detection of the σ_2 receptor by Scatchard Analysis. Since the σ_2 protein is still unknown, no immunoblotting technique can be applied for the σ_2 receptor detection. Therefore, we performed Scatchard analyses on the three cell lines to determine the protein content by saturation with the radioligand [^{3}H]-DTG (Figure 5) according to standard methods. MCF7 cells showed a B_{max} = 2.02 pmol/mg of σ_2 proteins with a K_d = 17.91 nM, in accordance to the values previously found. By contrast, B_{max} values from U87 and THP1 could not be reliably detected, since acceptable saturation curves could not be obtained, with the non-specific binding almost reaching the specific binding. For the same reasons, K_d could not be determined for [^{3}H]-DTG in these cells. These results were in accordance with fluorescence intensity obtained by flow cytometry that showed a σ_2 receptors content in THP1 and U87 cells 5-fold lower than in MCF7 cells.

A



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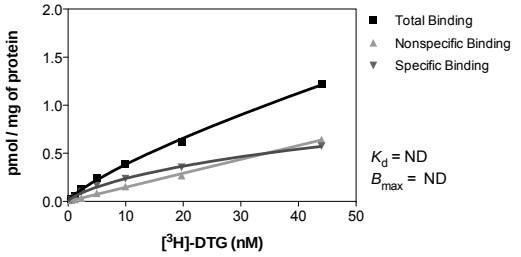

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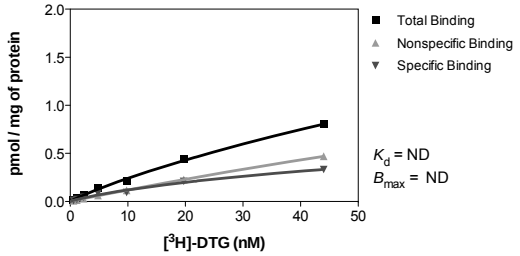

Figure 5. Saturation analysis of σ_2 receptors in membrane preparations from MCF7 (A), THP1 (B), U87 (C) cells.

Conclusions

In conclusion, we designed and obtained a highly selective and high-affinity σ_2 fluorescent ligand (**5**) and demonstrated that it is a versatile tool to detect σ_2 receptors in living cells and to perform σ_2 receptor competition binding assays avoiding the inconvenient use of the radioligand ([^{3}H]-DTG). We also demonstrated that in the absence of the σ_2-antibody, this tracer may be conveniently employed by flow cytometry to evaluate the presence and the amount of σ_2 receptors within tumor cells. By comparison of the results obtained from **5** with the results obtained from the mixed σ_1/σ_2 fluorescent tracer **1**, we also obtained indications about the σ_1 receptors' content which were then confirmed by western-blotting. Indeed, these tracers were shown to be reliable tools for σ receptors study, and their wide applicability to a number of techniques (e.g. fluorimetry, flow cytometry, fluorescence microscopy) will likely contribute to the clarification of σ receptors roles in oncology and to their possible exploitation for tumor treatment and diagnosis.

Experimental Section

Chemistry. Column chromatography was performed with 60 Å pore size silica gel as the stationary phase (1:30 w/w, 63–200 µm particle size from ICN). Purity of tested compound was

established by combustion analysis, confirming a purity ≥ 95%. Elemental analyses (C, H, N) was performed on an Eurovector Euro EA 3000 analyzer; the analytical results were within ± 0.4% of the theoretical values. ^{1}H-NMR (300 MHz) spectrum was recorded on a Mercury Varian using $CDCl_3$ as solvent. The following data were reported: chemical shift (δ) in ppm, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), integration and coupling constant(s) in Hertz. Recording of mass spectra was done on an Agilent 1100 series LC-MSD trap system VL mass spectrometer; only significant m/z peaks, with their percentage of relative intensity in parentheses, are reported. Chemicals were from Sigma-Aldrich and Alfa Aesar and were used without any further purification.

2-{6-[2-(3-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl)propyl)-3,4-dihydroisoquinolin-1(*2H*)-one-5-yloxy]hexyl}-5-(dimethylamino)isoindoline-1,3-dione (5): The already described intermediates 2-(3-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl)propyl)-5-hydroxy-3,4-dihydroisoquinolin-1(2*H*)-one (**3**)[14] (0.15 g, 0.38 mmol) and 6-(5-(dimethylamino)-1,3-dioxoisoindolin-2-yl)hexyl methanesulfonate (**4**)[18] (0.15 g, 0.42 mmol) were mixed in DMF (5 mL) in the presence of K_2CO_3 (0.06 g, 0.42 mmol). After the reaction mixture was stirred at 150°C overnight, the solvent was removed under reduced pressure, and H_2O was added to the crude residue which was extracted with CH_2Cl_2 (3 × 10 mL). The organic layers were dried over Na_2SO_4 and concentrated under reduced pressure to afford a crude residue which was purified by column chromatography with CH_2Cl_2/MeOH (98:2) as eluent to afford the title compound as yellow oil (0.18 g, 73% yield). ^{1}H NMR δ 1.37-1.99 (m, 10H, $OCH_2(CH_2)_4$, $NCH_2CH_2CH_2$), 2.55-2.96 (m, 8H, 2 $ArCH_2$, $N(CH_2)_2$), 3.10 (s, 6H, $N(CH_3)_2$), 3.52-3.66 (m, 8H, $ArCH_2N$, $(CO)_2NCH_2$, $CON(CH_2)_2$), 3.82 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3) 3.90-3.97 (m, 2H, OCH_2), 6.51 (s, 1H, aromatic), 6.57 (s, 1H, aromatic), 6.75-6.78 (dd, 1H, CH aromatic, *J* = 9 Hz, *J*' = 2.4 Hz), 6.91-6.94 (d, 1H, *J* = 9 Hz, CH aromatic), 7.04-7.05 (d, 1H, *J* = 2.4 Hz, CH aromatic), 7.22-7.23 (m, 1H, aromatic), 7.60-7.68 (m, 2H, aromatic) ; LC-MS (ESI^+) m/z: 691 $[M+Na]^+$. Anal. ($C_{39}H_{48}N_4O_6 \cdot 2HCl \cdot ¼H_2O$) C, H, N.

Fluorescence Spectroscopy

Fluorescence spectra of fluorescent compounds were obtained with a PerkinElmer LS55 spectrofluorometer, on solutions 10^{-4} M in EtOH.

Biology

Materials. [^{3}H]-DTG (50 Ci/mmol), and (+)-[^{3}H]-pentazocine (30 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). DTG was purchased from Tocris Cookson Ltd., U.K. (+)-Pentazocine was obtained from Sigma-Aldrich (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). Anti-SIGMAR1 prestige antibody, was purchased from Sigma-Aldrich (Milano, Italy). Anti-β-actin, secondary peroxidase antibodies and all reagent for western blotting were purchased from Life Technologies Italia (Monza, Italy). Stock solutions of tested compounds were prepared at 10 mM concentration in EtOH and diluted to the final concentrations in the appropriate buffer.

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.[28] The specific radioligands and tissue sources were respectively: (a) σ_1 receptor, (+)-[^{3}H]-pentazocine, guinea-pig brain membranes without cerebellum; (b) σ_2 receptor, [^{3}H]-DTG in the presence of 1 μM (+)-pentazocine to mask σ_1 receptors, rat liver membranes.

Permission for animals' tissues use was provided by Ministero della Sanità-Italy. The following compounds were used to define the specific binding reported in parentheses: (a) (+)-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 GraphPad software (version 3.0).[29]

Saturation binding assay. The saturation experiments were carried out as described by Vilner et al.[27] with minor modifications in human MCF7, THP1 and U87 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 (+)-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 °C. 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).[29]

Cell cultures. Human MCF7 breast adenocarcinoma cell line was purchased from ICLC (Genoa, Italy). Human leukemic monocyte THP1 and Human glioblastoma U87 cells were from ATCC (Manassas, VA). MCF7 and U87 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 % CO2 atmosphere. THP1 cells was grown in RPMI 1640 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 % CO2 atmosphere.

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 (Sigma Aldrich, St.Louis, MO, USA) and centrifuged at 14,000 g for 15 min at 4°C. 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 1h at room temperature with either anti-SIGMAR1 (1:300 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 SIGMAR1 and 1:2000 anti-mouse for β-actin) for 1h 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.

Flow Cytometry. MCF7, THP1 and U87 cells were incubated with increasing concentrations (1 nmol/L, 10 nmol/L, 100 nmol/L, 1 μmol/L, 10 μmol/L – in cell culture medium) of **PB28** or DTG followed by 100 nmol/L of either compound **1** or **5** for 45 min, 75 min and 180 min at 37 °C. In addition, MCF7 cells were incubated with increasing concentrations (1 nmol/L, 10 nmol/L, 100 nmol/L, 1 μmol/L, 10 μmol/L – in cell culture medium) of the more specific σ_2 ligand F390 before 100 nmol/L of **1** or **5** were added. The best up-take of the fluorescent compounds in the three cell lines was obtained with 75 min incubation. Masking of the σ_1 receptors in MCF7 and THP1 was performed with (+)-pentazocine (1 μmol/L) and increasing concentrations (1 nmol/L, 10 nmol/L, 100 nmol/L, 1 μmol/L, 10 μmol/L – in cell culture medium) of DTG followed by 100 nmol/L of compound **1** or **5** for 75 min at 37 °C. 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 min 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 FACSCalibur system (Becton Dickinson Biosciences, San Jose, CA), with a 530 nm band pass filter. For each analysis 50,000 events were collected and analysed with the Cell Quest software (Becton Dickinson Biosciences).

Abbreviations: ER, Endoplasmic Reticulum; MCF7σ_1, MCF7 σ_1-overexpressing cell line; PBS, phosphate-buffered saline; PEI, polyethylenimine; PGRMC1, progesterone receptor membrane component 1; PVDF, polyvinylidene difluoride membrane; QY, quantum yield; ROS, reactive oxygen species; SAfiR, Structure Affinity Relationship; TBS, Tris-buffered saline.

Keywords: σ receptors, σ receptor fluorescent ligands, flow cytometry, cancer, fluorescent probes.

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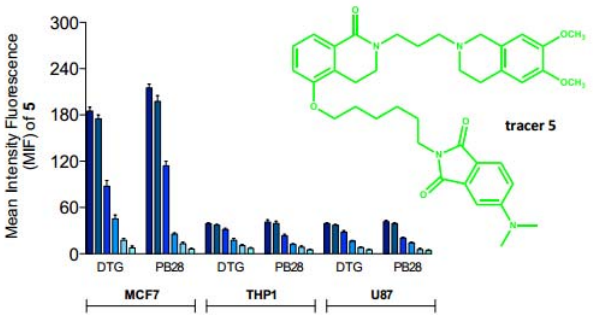

Making σ_2 receptors fluorescent: We developed a σ_2 selective fluorescent tracer and validated it as an alternative and powerful tool for σ_2 receptor research.

*Mauro Niso, Chiara Riganti, Maria Laura Pati, Dario Ghigo, Berardi Francesco, Carmen Abate**

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Novel and selective σ_2 receptor fluorescent ligand with 3,4-dihydro-isoquinolin-1-one scaffold as tool for σ_2 receptor study in living cells

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Supporting Information

Novel and Selective Fluorescent σ_2-Receptor Ligand with a 3,4-Dihydroisoquinolin-1-one Scaffold: A Tool to Study σ_2 Receptors in Living Cells

Mauro Niso,[a] Chiara Riganti,[b] Maria Laura Pati,[a] Dario Ghigo,[b] Francesco Berardi,[a] and Carmen Abate*[a]

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Supporting Information

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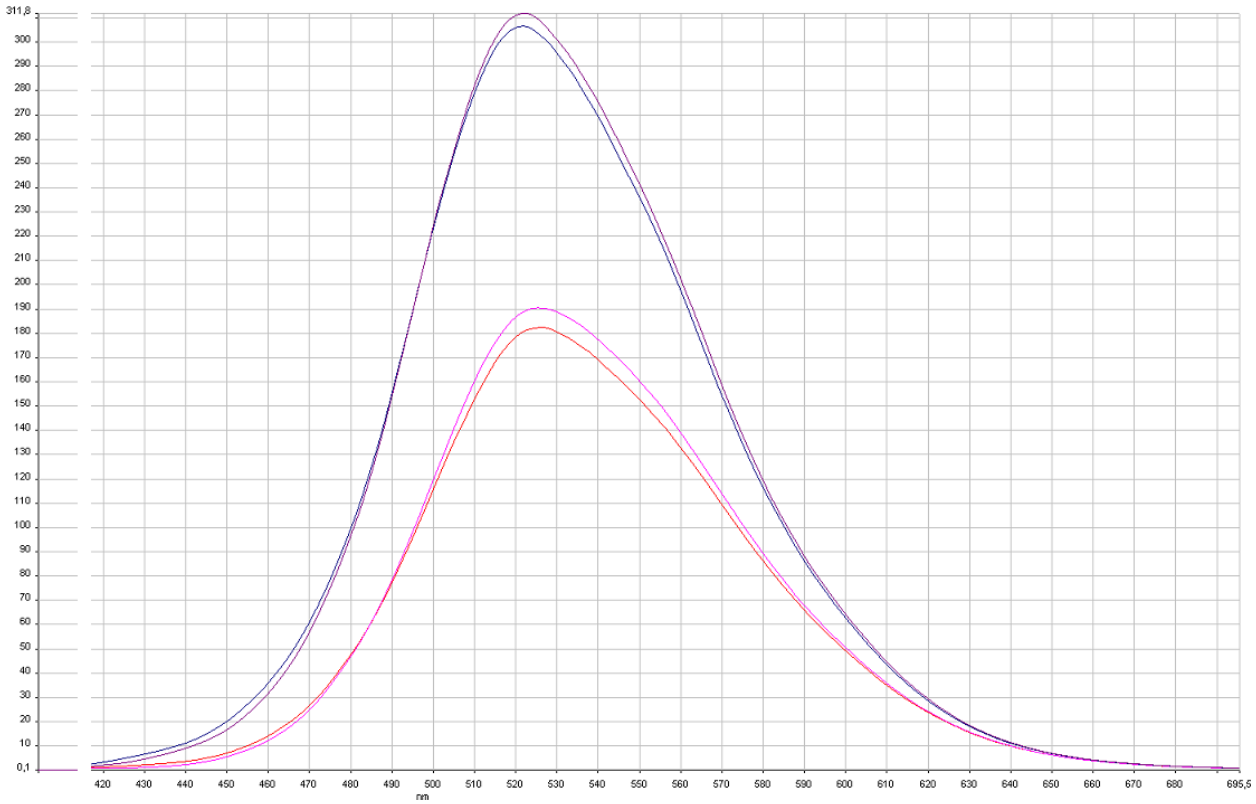
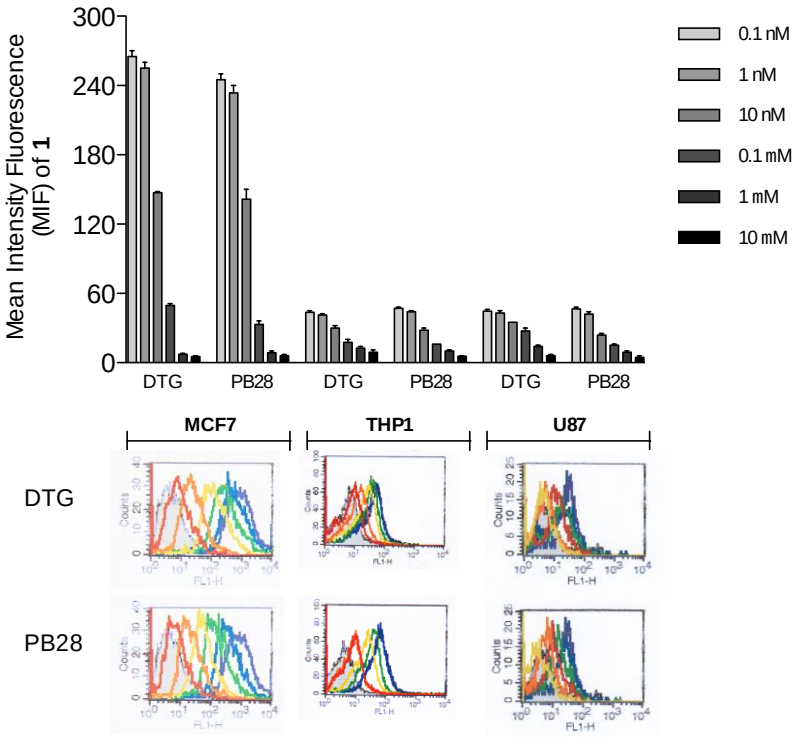


Figure 1. Emission spectra of compound **5** in EtOH **(**10^{-4} M): higher curves (purple and violet) from the free base; lower curves (red and pink) from the hydrochloride salt.

A



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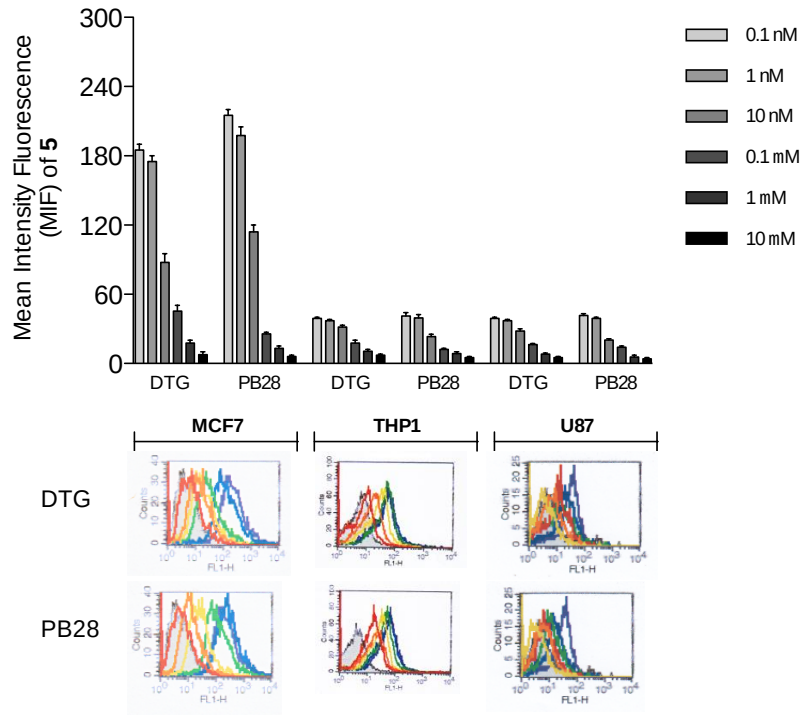

Figure. 2. Flow Cytometry analysis of MCF7, THP1 and U87 cells exposed to **1** and **5.** A) Displacement of **1** (100 nM) with increasing concentration of either DTG or PB28, B) Displacement of **5** (100 nM) with increasing concentration of either DTG or PB28. Upper panels: mean intensity fluorescence of the representative experiments shown in lower panel. Lower panels: cell associated-fluorescence-associated versus cell count of one representative experiment out of

three. For MCF7 and THP1 cells: grey curve: untreated cells; violet curve: 0.1 nM DTG or PB28; blue curve: 1 nM DTG or PB28; green curve: 10 nM DTG or PB28; yellow curve: 0.1 µM DTG or PB28; orange curve: 1 µM DTG or PB28; red curve: 10 µM DTG or PB28. For U87 cells: grey curve: untreated cells; violet curve: 0.1 nM DTG or PB28; blue curve: 1 nM DTG or PB28; green curve: 10 nM DTG or PB28; red curve: 0.1 µM DTG or PB28; orange curve: 1 µM DTG or PB28; yellow curve: 10 µM DTG or PB28.

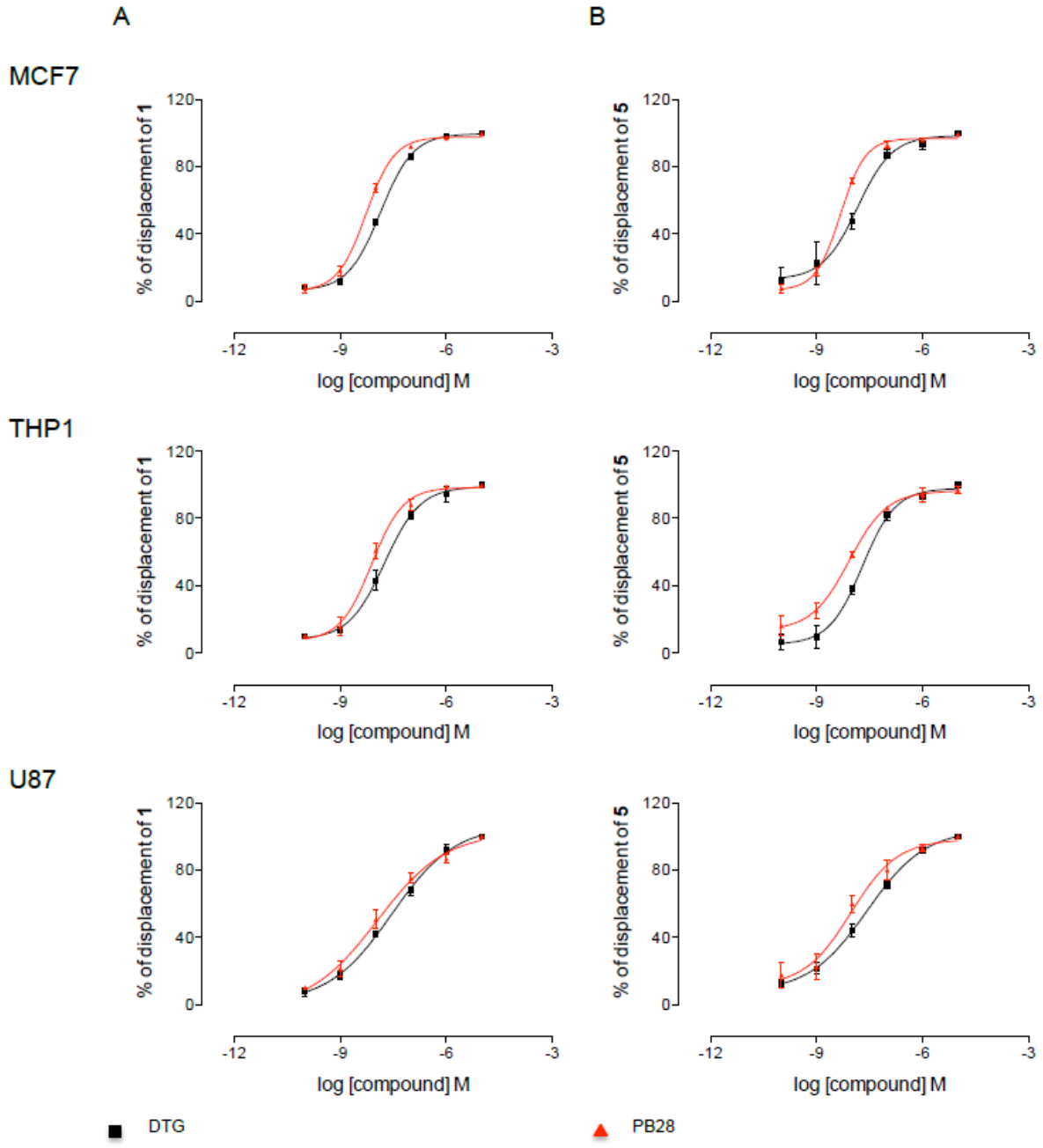

Figure 3. Binding curves generated by Flow Cytometry experiments shown in Figure 2.