Chemical Probes |*Hot Paper*|

Chemoproteomic Approach to Explore the Target Profile of GPCR ligands: Application to 5-HT_{1A} and 5-HT_6 Receptors

Ana M. Gamo,[a] Juan A. González-Vera,[a] Ainoa Rueda-Zubiaurre,[a] Dulce Alonso,[a] Henar Vázquez-Villa,[a] Lidia Martín-Couce,[a] Óscar Palomares,[b] Juan A. López,[c] Mar Martín-Fontecha,[a] Bellinda Benhamú,[a] María L. López-Rodríguez,*[a] and Silvia Ortega-Gutiérrez*[a]

Abstract: Determination of the targets of a compound remains an essential aspect in drug discovery. A complete understanding of all binding interactions is critical to recognize in advance both therapeutic effects and undesired consequences. However, the complete polypharmacology of many drugs currently in clinical development is still unknown, especially in the case of G-protein-coupled receptor (GPCR) ligands. In this work we have developed a chemoproteomic platform based on the use of chemical probes to explore the target profile of a compound in biological systems. As proof of concept, this methodology has been applied to selected ligands of the therapeutically relevant serotonin 5-HT_{1A} and 5-HT_6 receptors, and we have identified and validated some of their off-targets. This approach could be extended to other drugs of interest to study the targeted proteome in disease-relevant systems.

Introduction

In drug discovery, most researchers pursue high affinity for a single therapeutic target combined with high selectivity, believing that this will maximize efficacy and minimize side effects. Indeed, activity at nontherapeutic targets can lead to toxicity and cause adverse effects, and a number of drugs have been withdrawn by the pharmaceutical industry for this reason. However, in recent years, some multitarget drugs have become unprecedentedly efficacious and safer. In current research, polypharmacology is increasingly being appreciated since it holds opportunities for drug discovery and drug repurposing. In this regard, the identification of the different proteins targeted by a compound has helped to explain mechanisms of action, therapeutic activities, and undesirable side effects of marketed drugs such as aspirin,[1,2] thalidomide,[3] dasatinib,[4] or tivantinib,[5] just to name a few examples. Hence, the development of chemical tools that facilitate the study of polypharmacology for ligands of therapeutically relevant proteins is of outmost interest and it is attracting much attention.[6] Among them, chemical probes have emerged as efficacious tools for global protein profiling. Two different approaches can be applied depending on the type of probe used. The first methodology, activity-based protein profiling (ABPP), uses probes characterized by the presence of an electrophilic group that will react in a covalent manner with the nucleophile in the active site of an enzyme. In this manner, the availability of probes directed towards different enzyme families has facilitated the throughput identification of enzyme targets across the proteome.[7] Since this approach is limited to enzymes with known catalytic mechanisms, alternative methods, based on the development of metabolite-based probes have emerged. In this second type of approach, binding to the target is directed by the affinity of the scaffold and not by intrinsic chemical reactivity. This methodology has been recently applied to the identification of the protein targets of different lipid metabolites.[8] In all cases, the chemical probe bears a reporter tag that enables protein visualization and/or enrichment prior to mass spectrometry based identification. Considering the potential of chemoproteomic approaches for studying target profiles, we wondered whether such a strategy could be applied to G-protein-coupled receptors (GPCRs) to facilitate the nonbiased profiling of GPCR ligands. Given the importance of GPCRs as drug targets, specific panels of proteins are commonly screened for during the first steps of drug discovery. However, this approach is normally restricted to proteins that belong to the same family (that is, other GPCRs) or proteins with known sequence similarities. Therefore, the development of methods aimed at unravelling unexpected interactions and at

[a] *Dr. A. M. Gamo,[+] Dr. J. A. González-Vera,[+] A. Rueda-Zubiaurre, Dr. D. Alonso, Dr. H. Vázquez-Villa, Dr. L. Martín-Couce, Prof. Dr. M. Martín-Fontecha, Dr. B. Benhamú, Prof. Dr. M. L. López-Rodríguez, Prof. Dr. S. Ortega-Gutiérrez*
Departamento de Química Orgánica I, Facultad de Ciencias Químicas
Universidad Complutense de Madrid, 28040 Madrid (Spain)
E-mail: mluzlr@ucm.es
siortega@quim.ucm.es

[b] *Dr. Ó. Palomares*
Departamento de Bioquímica y Biología Molecular I
Facultad de Ciencias Químicas
Universidad Complutense de Madrid, 28040 Madrid (Spain)

[c] *Dr. J. A. López*
Proteomics Unit
Centro Nacional de Investigaciones Cardiovasculares, CNIC
28029 Madrid (Spain)

[+] *These authors contributed equally to this work*

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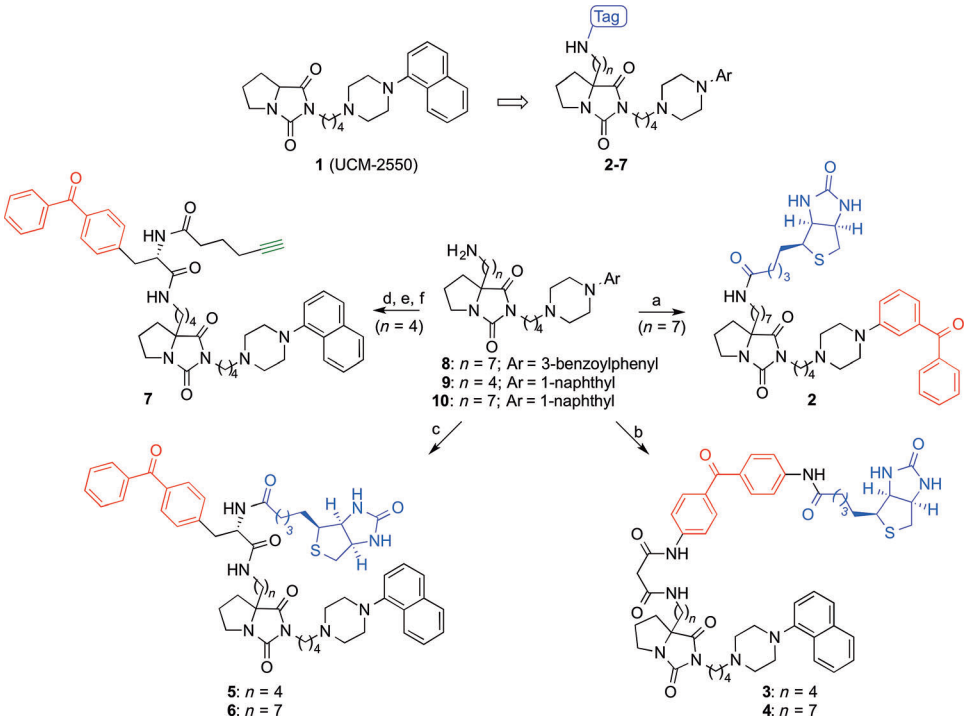
associating these with additional effects (both therapeutic and non-desired) has attracted intense interest. In this regard, bioinformatic efforts have contributed to the generation of computational tools for the prediction and identification of unexpected off-targets.[9] However, an experimental approach that facilitates the high-throughput straightforward and non-biased study of polypharmacology for known ligands of GPCRs is currently lacking. Toward this end, we have developed a chemoproteomic platform based on the use of chemical probes that enables the study of the polypharmacology of GPCR ligands. As proof of concept, this approach has been used to identify additional targets of selected serotonin receptor ligands. Among the different serotonin receptors, we have focused our attention on two of them, the clinically validated 5-HT_{1A} receptor (5-HT_{1A}R)[10] and the currently under validation 5-HT_6 receptor (5-HT_6R).[11] Herein, high-affinity ligands of these receptors have been modified with appropriate tag(s) for covalent binding and subsequent enrichment of the bound proteins followed by their identification using mass spectrometry (MS). These receptor-directed probes work in gel-based assays to enable protein profiling and they bind their cognate receptor as well as several additional targets in proteomic experiments. In particular, HSP60 and prohibitin proteins have been identified as off-targets of the studied 5-HT_{1A} and 5-HT_6 receptor ligands, respectively. These proteins have been validated and their relevance assessed in cellular systems. This approach could be extended to other systems to study the proteome targeted by the compound of interest in disease-relevant samples.



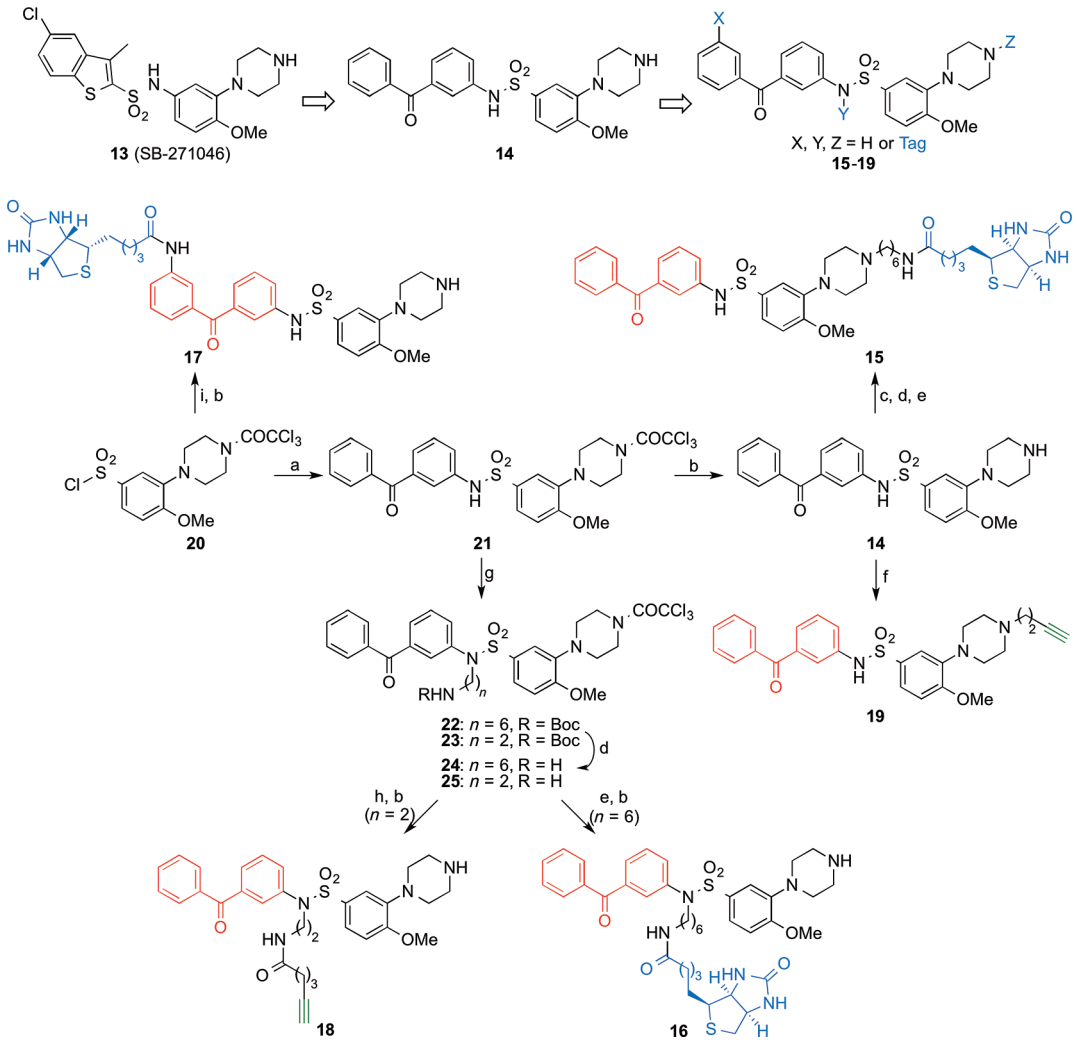
Scheme 1. Design and synthesis of probes for serotonin 5-HT_{1A}R: a) biotin, DCC, HOBt, DMAP, DMF, RT, 39%; b) 3-oxo-3-({4-[4-(biotinylamino)benzoyl]phenyl}amino)propanoic acid (**11**), EDC, HOBt, DMF, DCM, RT, 15–36%; c) 4-benzoyl-*N*-biotinyl-L-phenylalanine (**12**), EDC, HOBt, DMF, DCM, RT, 20-46%; d) Fmoc-4-benzoyl-L-phenylalanine, EDC, HOBt, DMF, DCM, RT, 80%; e) piperidine, DCM, 0 °C, 75%; f) 5-hexynoic acid, DCC, DMAP, DCM, RT, 53%. DCC = *N*,*N*-dicyclohexylcarbodiimide; DCM = dichloromethane; DMAP = 4-dimethylaminopyridine; EDC = *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide; Fmoc = 9-fluorenylmethoxycarbonyl; HOBt = 1-hydroxybenzotriazole.

Results and Discussion

Design and synthesis of the probes

We reasoned that the probes suitable for chemoproteomic studies would need to have three general features: 1) a photoreactive group for ultraviolet (UV) light-induced cross-linking to target proteins such as benzophenone; 2) a biotin subunit, which enables enrichment and subsequent identification of bound proteins; and 3) a scaffold with affinity for the serotonin receptor under study that after introduction of the photo-cross-linking and biotin subunits would still keep the affinity for the receptor and, by extension, for the rest of the off-targets (or most of them). Alternatively, the possibility of a smaller tag such as a terminal alkyne for click chemistry was also considered. Taking into account these considerations, we designed two sets of probes starting from high affinity ligands of 5-HT_{1A} and 5-HT_6 receptors (Schemes 1 and 2, respectively). In the case of the 5-HT_{1A}R, ligand UCM-2550[12] (**1**, K_i = 2.4 nM, Scheme 1) was selected.

Previous structure–activity relationship (SAR) studies indicated that the 7a-position of the bicyclohydantoin could admit the introduction of a tag without dramatically affecting affinity.[13] Therefore, we explored the possibility of attaching the biotin moiety at the 7a-position and replacing the naphthalene ring by benzophenone (compound **2**, Scheme 1). Alternatively, we designed different tags that contain both benzophenone and biotin or benzophenone and a terminal alkyne, which can be introduced at the 7a-position of the bicyclohydantoin (compounds **3**–**7**, Scheme 1). These compounds were synthesized by condensation of the intermediate amines **8**–**10** with the corresponding carboxylic acids that contain the different tags (Scheme 1). With respect to the 5-HT_6R, we selected the ligand developed by SmithKline Beecham SB-271046[14] (**13**, K_i = 1.2 nM, Scheme 2) as starting point. In this case, we were able to successfully replace the benzothiophene ring by a benzophenone (compound **14**, K_i = 1.6 nM, Scheme 2), so we ex-



Scheme 2. Design and synthesis of probes for serotonin 5-HT$_6$R: a) 3-aminobenzophenone, pyridine, DCM, RT, 65%; b) 1 M aq. KOH, THF, RT, 76–98%; c) *tert*-butyl 6-bromohexylcarbamate, NaI, DMF, 80 °C, 67%; d) TFA, DCM, RT, 91–95%; e) biotin, EDC, HOBt, DMF, DCM, RT, 58–82%; f) 4-bromobutyne, NaI, DMF, 80 °C, 47%; g) *tert*-butyl 6-bromohexylcarbamate or *tert*-butyl 2-bromoethylcarbamate, Cs_2CO_3, DMF, RT, 74–81%; h) 5-hexynoic acid, DCC, DMAP, DCM, RT, 61%; i) *N*-[3-(3-aminobenzoyl)phenyl]biotinamide (**26**), pyridine, DMF, DCM, RT, 51%. TFA = trifluoroacetic acid.

plored the most suitable positions (*X*, *Y*, and *Z*) to introduce the biotin moiety or the terminal alkyne (compounds **15**–**19**, Scheme 2). The preparation of these compounds started with benzenesulfonyl chloride **20**, which was transformed into the benzophenone derivative **21** and subsequently alkylated in the nitrogen of the piperazine (compounds **15** and **19**) or in the nitrogen of the sulfonamide group (compounds **16** and **18**).

Alternatively, reaction of **20** with *N*-[3-(3-aminobenzoyl)phenyl]biotinamide (**26**) yielded, after deprotection of the trichloroacetyl group, final compound **17** (Scheme 2). All the final compounds were tested for affinity at the corresponding receptor by binding assays (Table 1). The results indicate that for 5-HT$_{1A}$R, the use of one tag that contains both groups (biotin and benzophenone) is more favorable in terms of affinity, as shown by the K_i values obtained for compounds **3**–**7** [K_i (5-HT$_{1A}$) = 1.1-5 nM] when compared to derivative **2** [K_i (5-HT$_{1A}$) = 76 nM]. In the case of 5-HT$_6$R, the introduction of the biotin moiety [K_i (**16**, 5-HT$_6$) = 94 nM] or the terminal alkyne [K_i (**18**, 5-HT$_6$) = 95 nM] in the sulfonamide allowed us to obtain probes with affinity for this receptor. Taking into account these data, probes **5** and **16**, with the best affinity values for 5-HT$_{1A}$ and 5-HT$_6$ receptors, respectively, were selected for the proteomic experiments.

Table 1. Binding affinities of synthesized probes **1**–**7**, **13**–**19**, **27**, and **28**.

Cpd	5-HT$_{1A}$ K_i (nM)[a]	Cpd	5-HT$_6$ K_i (nM)[a]
1	2.4[b]	**13**	1.2[c]
2	76 ± 5	**14**	1.6 ± 0.1
3	2.7 ± 0.3	**15**	> 1000
4	4 ± 1	**16**	94 ± 7
5	1.1 ± 0.5	**17**	> 1000
6	5 ± 2	**18**	95 ± 5
7	5 ± 1	**19**	380 ± 17
27	22 ± 6	**28**	89 ± 1

[a] K_i values are the mean ± standard error from three independent experiments performed in triplicate. [b] Value from reference [12]. [c] Value from reference [14].

Gel profiling of cell membranes

Before setting up the proteomic platform, it was necessary to confirm that the compounds were actually able to label proteins specifically, that is, in an irradiation- and dose-dependent manner. For this purpose we developed equivalent fluorescent probes to visualize the labeling using SDS/PAGE gel analysis. Hence, fluorescent compounds **27** and **28**, in which the fluorophore lissamine has been introduced in place of the biotin moiety (Figure 1 a), were prepared by following similar synthetic routes to the ones above mentioned (see Supporting Information for details) and the affinity for their corresponding receptors was determined (Table 1). As a representative example, Figure 1 b shows that probe **27** was able to label proteins in a UV irradiation-dependent manner indicating that these interactions reflect noncovalent binding events, as expected for GPCR ligands.

In addition, the intensity of the labeling was concentration-dependent (Figure 1 c) and this binding could be displaced in variable degrees by an excess of the high-affinity parent ligand **1** [K_i (5-HT$_{1A}$) = 2.4 nM)] (Figure 1 d). Similar results were obtained with probe **28** (data not shown). Furthermore, clickable probes **7** and **18** showed a similar capacity to label proteins in cell homogenates, although this capability was reduced in whole cells (Supporting Information, Figure S1). Together, these results prompted us to use probes **5** and **16** in a complex proteome to obtain information about their targets.

Mass spectrometry profiling of ligand-bound proteins

In order to characterize the ligand-bound proteins, we designed a chemical proteomic platform based on biotin-streptavidin affinity followed by MS analysis (Figure 2). We used homogenates of HEK-293 cells transfected with the receptor under study as the complex proteome.

Accordingly, membrane homogenates were treated with the corresponding probe and irradiated with UV light. In order to

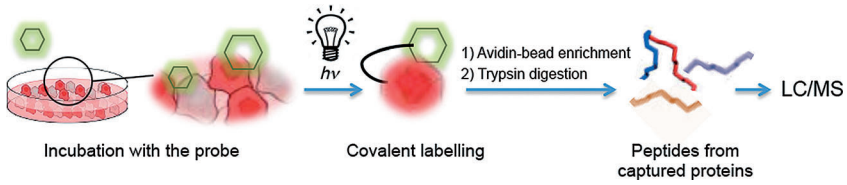


Figure 2. Scheme of the chemical proteomic platform.

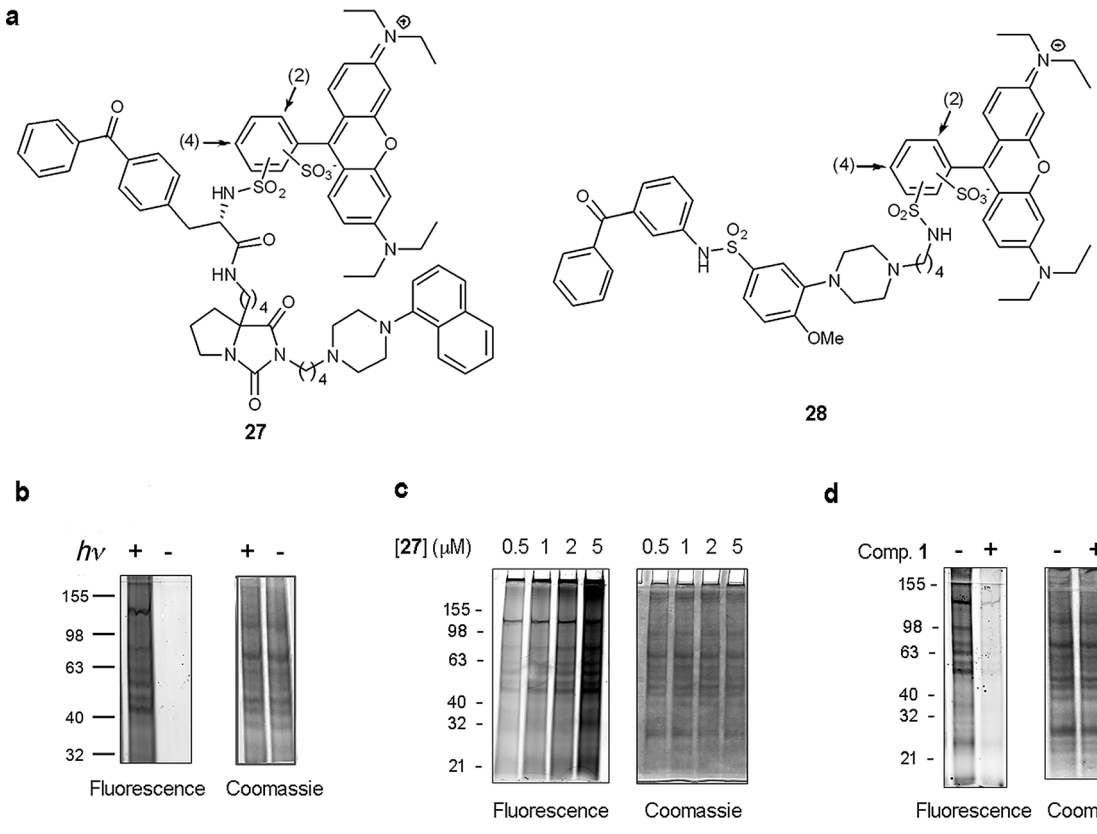


Figure 1. Fluorescent probes for serotonin 5-HT$_{1A}$ and 5-HT$_6$ receptors suitable for gel analysis. a) Structure of probes **27** and **28**. b) SDS-PAGE analysis of membranes treated with 5 μM of probe **27** with and without UV irradiation. c) Concentration-dependent labelling of membranes with probe **27** at the indicated concentrations. d) Labelling with probe **27** (5 μM) in the presence of an excess (500 μM) of the 5-HT$_{1A}$ high-affinity ligand **1**. In all cases, samples were irradiated at 360 nm for 1 h on ice.

distinguish the proteins that specifically interact with the probe, multiple control samples were carried out in parallel, including proteomes treated with the probe in the presence of an excess of a high-affinity ligand as competitor, and proteomes to which no probe was added. The probe-labeled proteins were enriched by binding to streptavidin-conjugated beads, subjected to on-bead trypsin digestion, and subsequently identified by multidimensional LC separation and MS–MS analysis (see the Supporting Information). Specific targets of the probes were identified by filtering the data for proteins that were present in the probe-treated samples and absent in the control samples (Supporting Information, Tables S1 and S2). Under these conditions, 30 proteins were identified with probe **5** and 12 with probe **16**. It is important to note that both probes **5** and **16** were able to capture their cognate primary receptors 5-HT_{1A} and 5-HT_6, respectively. This result supports the solidness of the chemoproteomic platform. We then performed an analysis based on gene ontology (GO) annotations (protein class, cellular localization, biological process, molecular function and signaling pathways) within our data set using the protein analysis through evolutionary relationships (PANTHER) classification system,[15] which includes both experimental data and bioinformatics algorithms. This analysis revealed that proteins targeted by probes **5** and **16** (black and white bars, respectively, Figure 3a) belong to a variety of functional classes. With respect to the cellular component, a notable enrichment in mitochondrial proteins was observed for both probes (Figure 3b). Searching for biological process and molecular function trends (Figure 3c and 3d, respectively), we discovered significant enrichments in proteins related to fatty

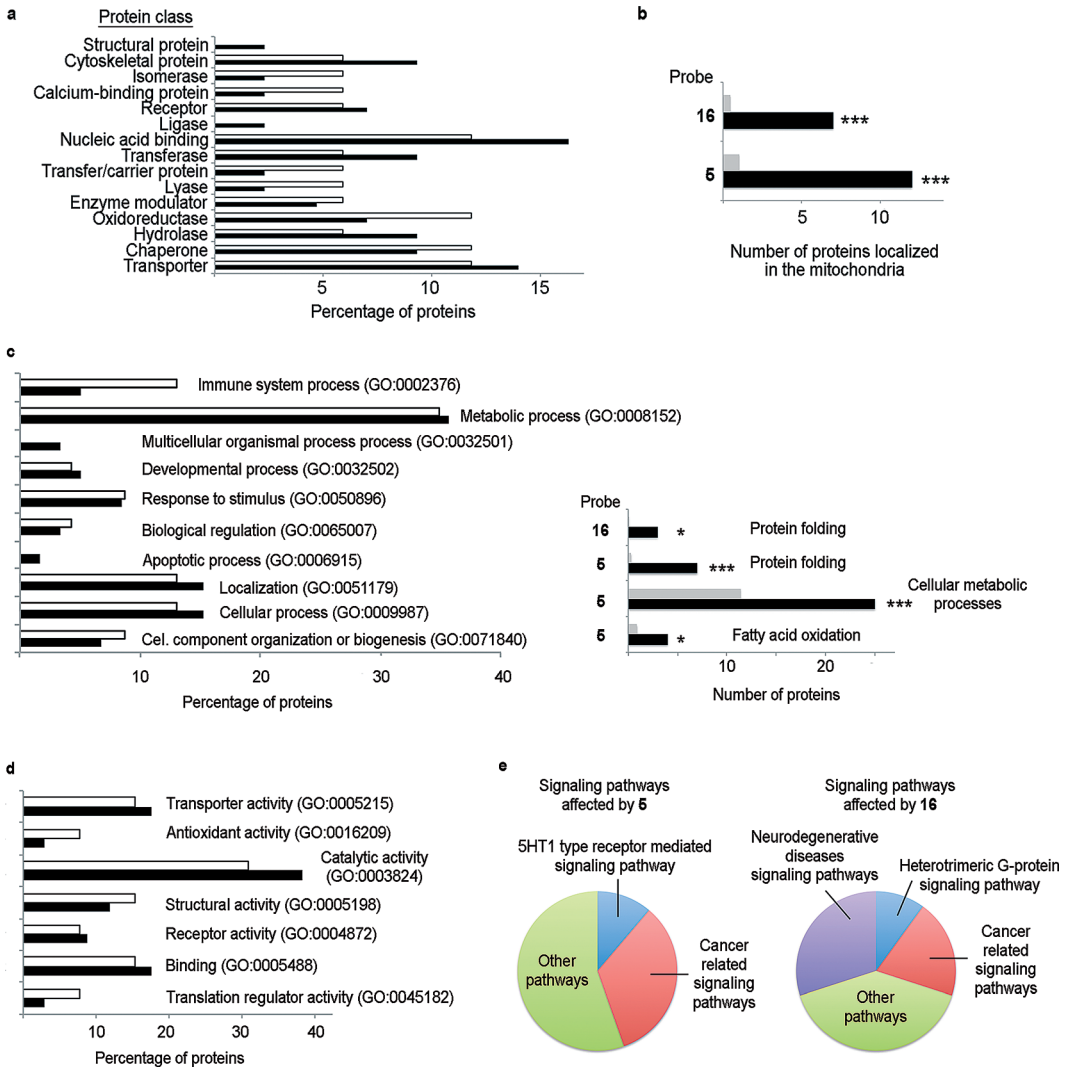


Figure 3. Analysis of gene ontology (GO) terms. a) Protein classes identified with probe **5** (in black) and **16** (white) expressed as percentage of the total identified proteins. b) Specific enrichment in mitochondrial proteins. Black bars represent the number of mitochondrial proteins identified and grey bars the expected number according to the PANTHER database (***, $p < 0.001$). c) Involvement of identified proteins in specific biological processes (in black are shown those identified with probe **5** and in white those identified with probe **16**). The right panel shows the enrichment in specific processes (black bars represent the number of identified proteins and grey bars the expected number according to the PANTHER database; *, $p < 0.05$; ***, $p < 0.001$). d) Classification of identified proteins based on their molecular function (in black are shown those identified with probe **5** and in white those identified with probe **16**). e) Involvement of identified proteins in specific signaling pathways.

acid oxidation, cellular metabolic processes, and protein folding for probe **5**, while for probe **16** protein folding is the biological process mainly affected (Figure 3c, right panel). This result is in agreement with the chaperones targeted by the two probes. In particular, HSP60, HSP70, HSP75, and HSP90, are captured with probe **5** (Supporting Information, see Table S1), and HSP70, HSP90, and prohibitin (PHB) with probe **16** (Supporting Information, Table S2). Then, we analyzed the signaling pathways affected by these two probes (Figure 3e). As expected, signaling pathways corresponding to the activation of the 5-HT_{1A} and 5-HT_6 GPCRs were identified (blue sectors in Figure 3e). In addition, signaling pathways related with cancer were also uncovered for both probes (red sectors). Remarkably, pathways related to Alzheimer (AD), Huntington (HD), and Parkinson (PD) diseases emerged for probe **16** (purple sector in Figure 3e, right chart). Taking a closer look at these results, and using the Database for Annotation, Visualization, and Integrated Discovery (DAVID)[16] and the implemented KEGG pathway maps,[17] we were able to further explore the identified proteins. This analysis revealed that some of the targeted mitochondrial proteins have been related to these neurodegenerative diseases. Substantial evidence supports the involvement of mitochondrial dysfunction in the pathogenesis of these disorders.[18] Specifically, heat-shock proteins (HSPs) have been proposed as therapeutic targets to reduce the neurodegeneration caused by protein aggregation[18a] and the voltage-dependent anion channel (VDAC) is currently under consideration as a therapeutic target for AD.[19] Moreover, ATP synthase and prohibitin have been linked to AD and PD.[20] In addition to the important role of these proteins for neurodegenerative diseases, the HSP family of proteins has received a growing attention given their potential as antitumor targets.[21]

Hit validation

Hence, at this point it was important to validate some of these hits, that is, to confirm whether the probe and the parent ligand recognize in a direct manner the identified proteins. Among them, HSP60 and prohibitin were chosen for further validation. Our results show that HSP60 is recognized by the fluorescent probe **27** (Figure 4a) and this binding is displaced by a 100-fold excess of the nonlabeled high-affinity ligand **1** (Figure 4b). In addition, to fully explore the versatility of the approach, clickable probe **7** was used and, after copper-catalyzed cycloaddition with the rhodamine–biotin–azide derivative ($TriN_3$,[22] see the Supporting Information for details), similar results were obtained (Figure 4c). Also, a specific recognition of HSP60 could be observed in the context of a complex proteome in which exogenous HSP60 is added (Figure 4d). We further extended our validation to prohibitin protein with probe **28** and with the alkyne-tagged compound **18**, as shown in Figure 4e,f. As expected, the recognition of HSP60 and prohibitin disappears if the proteins are denatured by heating prior to labeling (Supporting Information, Figure S2), a fact that supports the specificity of the binding.

Encouraged by these results, we further assessed that HSP60 was a bona fide target of the parent serotonin receptor ligand

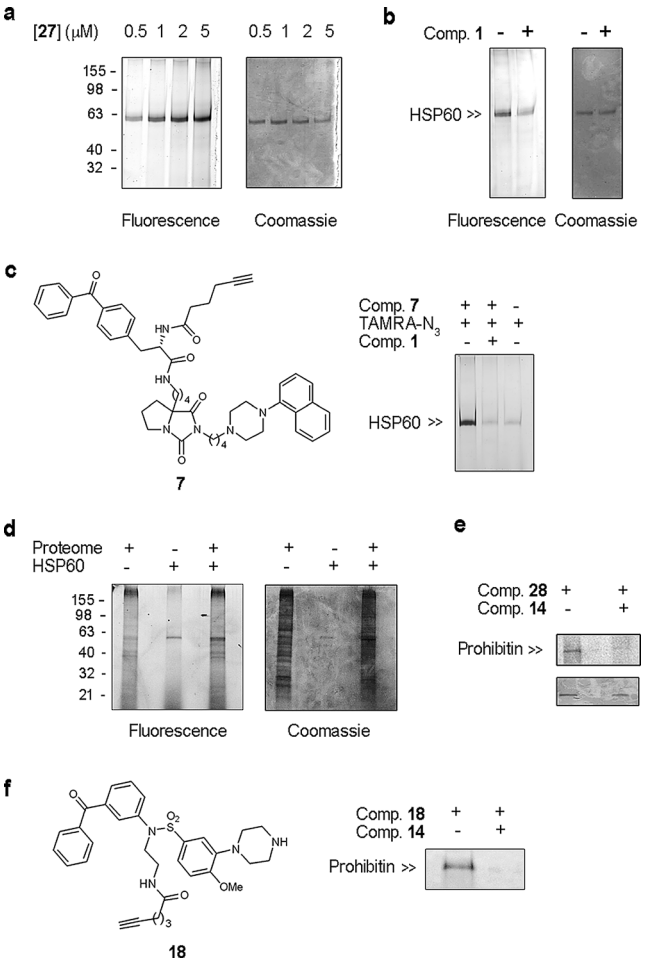


Figure 4. In-gel validation of HSP60 and prohibitin as hits of the 5-HT_{1A}- and of the 5-HT_6R-directed ligands, respectively. a) Probe **27** labels HSP60 in a dose-dependent manner. b) The labeling of HSP60 with probe **27** (2 μM) can be displaced with an excess (100-fold) of the 5-HT_{1A} high-affinity ligand **1**. c) Clickable probe **7** (10 μM) labels HSP60 in a specific manner. d) Probe **27** labels HSP60 in a complex proteome. e) Probe **28** labels prohibitin (fluorescence upper and coomassie bottom) at 10 μM. This labeling can be displaced with an excess (25-fold) of the 5-HT_6 high-affinity ligand **14**. f) Clickable probe **18** (10 μM) labels prohibitin and can be displaced with an excess of the 5-HT_6 high-affinity ligand **14**.

1. Indeed, a functional assay showed that this compound, at concentrations of 10 μM, reduced by 25% the ATPase activity of HSP60 (Figure 5a).■■ok?■■ This inhibition is dose-dependent (Supporting Information, Figure S3) with an IC_{50} value of 63 μM. We next sought to confirm these results in a cellular context. Towards this aim, we selected two cell lines that express HSP60 (Figure 5b) and whose survival is significantly regulated by this protein, the breast cancer cell lines MCF-7 and MDA-MB-231.[23] Remarkably, compound **1** induces cytotoxicity in both cell lines (Figure 5c) with an IC_{50} value of 12 μM in MCF-7 cells and 63 μM in MDA-MB-231 cells.

HSP60 is a chaperone protein that has been related to cancer and CNS disorders.[21,24] However, its potential as a drug target has not been widely explored because of a lack of inhibitors. They are basically restricted to two nucleoside derivatives, one natural product and one carboranyl derivative.[21,25,26]

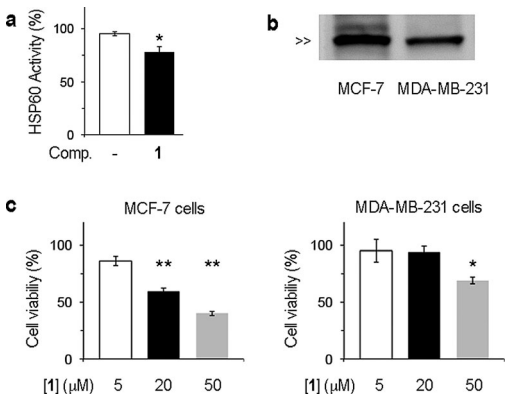


Figure 5. Enzyme and cellular effects of serotonin 5-HT$_{1A}$ ligand **1**. a) Inhibition of recombinant HSP60 ATPase activity by compound **1** (10 μM). b) Representative gel showing the expression of HSP60 in MCF-7 and MDA-MB-231 cells. Equal amounts of cell lysates (25 μg) were immunoblotted with a mouse anti-HSP60 and the corresponding band (60 kDa) is marked with the double-arrowhead. c) Compound **1** induces cell cytotoxicity in a dose-dependent manner. Data correspond to the average ± s.e.m. of two to three independent experiments performed in duplicate or triplicate. *, $P<0.05$; **, $P<0.01$ (vs. vehicle-treated samples) by the Student's *t* test.

Hence, the serotonin ligand **1** could represent a new structural scaffold for the development of novel HSP60 ligands. Together, these results support that the described probes are not only able to capture the GPCR they were originally designed for, but also can aid the identification of additional targets of the ligands, in this case other targets related to neurodegenerative diseases (VDAC, ATP synthase or chaperones as the HSPs or PHB) or cancer (such as HSPs, PHB or ANT2[27]; Figure 6).

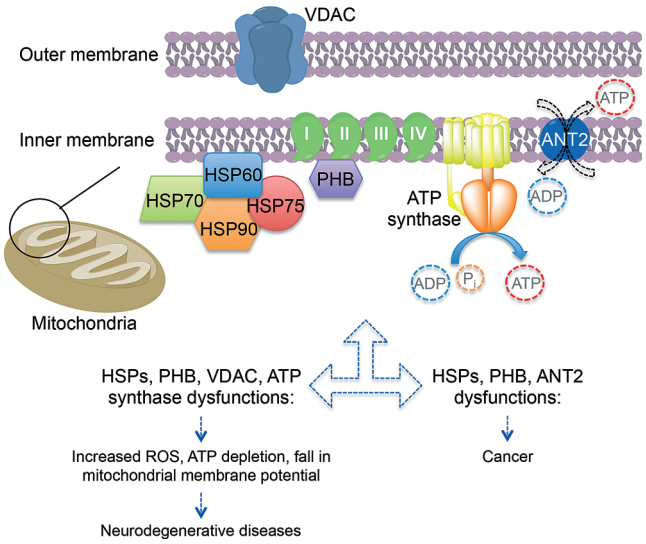


Figure 6. Schematic representation of the proteins targeted by probes **5** and **16** and their involvement in different pathologies.

Conclusions

In this work we have developed a new set of probes suitable for the nonbiased study of off-targets of 5-HT$_{1A}$ and 5-HT$_6$ receptor ligands. The protein profiling has revealed additional targets of these ligands some of which have been validated. Among the identified hits, a set of specific mitochondrial proteins related to neurodegenerative diseases and cancer stands out, as they could represent additional targets of the studied ligands. In particular, the chaperone HSP60 has been identified as a bona fide target of the studied 5-HT$_{1A}$ receptor ligand and the biological relevance of this binding has been validated in a cellular context. To the best of our knowledge, these probes are the first tools suitable for the study of serotonin GPCRs by chemoproteomic methods, and further insights on the identified hits are currently under investigation in our laboratory. This approach could be extended to other drugs of interest to study the proteome expressed in disease-relevant systems.

Experimental Section

Compound synthesis

Unless stated otherwise, starting materials, reagents, and solvents were purchased as high-grade commercial products from Sigma–Aldrich, Acros, ABCR, Bachem, Fluorochem, Scharlab, or Panreac, and were used without further purification. Dichloromethane was freshly distilled from calcium hydride. THF and diethyl ether were freshly distilled from sodium and benzophenone. All nonaqueous reactions were performed under an argon atmosphere in oven-dried glassware. Full details regarding the synthetic procedures and characterization data of all compounds are given in the Supporting Information. Spectroscopic data of all described compounds were consistent with the proposed structures. Elemental analyses (C, H, N, S) were within 0.5% of the calculated values, confirming a purity of at least 95% for all tested compounds.

Binding assays

Membranes from HEK-293-EBNA (5-HT$_{1A}$R) and HEK-293 (5-HT$_6$R) cells expressing the indicated human receptors were purchased from Perkin–Elmer and conserved at −80 °C in packaging buffer for subsequent use. Competitive inhibition assays were performed as previously described.[13,28] K_i values are expressed as the average and standard error obtained from three independent experiments carried out in triplicate.

Gel profiling of cell membranes

Membranes of cells expressing the corresponding human receptor (Perkin–Elmer) were homogenized in the appropriate buffer and incubated in the absence or presence of the probe in 96-well polystyrene plates (see the Supporting Information for full details). After incubation at 37 °C for 30 min, samples were irradiated at 360 nm using an UV lamp on ice for 1 h. Then, a 5% β-mercaptoethanol solution in Laemmli loading buffer (BioRad) was added and the samples were completely solubilized by heating at 80 °C for 10 min. Aliquots of each sample were loaded and resolved with 10% SDS-PAGE gels and fluorescence images were obtained with a Typhoon 9400 scanner.

Mass spectrometry profiling of ligand-binding proteins

Membrane homogenates were incubated in the absence or presence of the probe in a final volume of 600 μL of incubation buffer in a 24-well polystyrene plate (see the Supporting Information for full details). After incubation at 37 °C for 30 min, samples were irra-

diated as indicated above, and transferred to microtubes. Proteins were incubated with streptavidin-agarose beads (Pierce) for 12 h at 4 °C, transferred to falcon tubes, and the beads were processed as detailed in the Supporting Information. For MS analysis, proteins from pull-down samples were loaded onto SDS-PAGE and run in an unique band that concentrated all the proteins in the sample, stained with Coomassie Brilliant Blue G-250 and excised in 1 mm^3 small pieces, which were washed in ultrapure H_2O. Samples were subjected to reduction and alkylation, and in-gel digested overnight at 37 °C by adding modified porcine trypsin (Promega) at a final ratio of 1:20 (trypsin/protein). After digestion, peptides were vacuum-dried and finally dissolved in 0.1% formic acid for LCMS/MS analysis. The tryptic peptide mixtures were subjected to nanoliquid chromatography coupled to MS for protein identification as detailed in the Supporting Information.

In-gel hit validation

Pure HSP60 (2 μg) or prohibitin (1 μg) were incubated in the absence or presence of the corresponding probe and treated as described above. For click chemistry reactions followed by SDS/PAGE experiments, pure protein (2 μL of a 1 mg mL^{-1} in PBS) was incubated in the absence or presence of probe **7** (10 μM) or **18** (10 μM) in a final volume of 45 μL of PBS in a 96-well polystyrene plate (45 μL reactions were set up so that, once the cycloaddition reagents were added, the total reaction volume is 50 μL). After incubation at 37 °C for 30 min, samples were irradiated as described above, transferred to microtubes (eppendorf), and the click chemistry reaction was carried out using the reagents and concentrations detailed in the Supporting Information. Finally, aliquots of each sample were loaded and resolved with 10% SDS-PAGE gels. Fluorescence images were obtained with a Typhoon 9400 scanner.

HSP60 ATPase assay

Human recombinant HSP60 (Enzo Life Sciences) was incubated in the presence or absence of the compound under study using the conditions previously described[26] and ATP content was determined using the ATP assay kit from Abcam.

Cell culture, immunoblot analysis of HSP60 and cytotoxicity assays

Cells were routinely incubated at 37 °C with 5% CO_2 and grown in Dulbecco's modified Eagle's medium (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% sodium pyruvate, 50 U/mL penicillin, and 50 μg mL^{-1} streptomycin (Gibco). The expression of HSP60 was determined by immunoblot analysis using a mouse anti-HSP60 (BD Transduction) as the primary antibody following the conditions previously reported.[29,30] Cell viability was determined using a standard colorimetric assay based on the metabolism of the 3-(4,5-dimethyl-1,3-thiazol-2-yl)-2,5-diphenyl-2*H*-tetrazol-3-ium bromide (MTT) salt by the viable cells as previously described.[29–30]

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Chemical Probes

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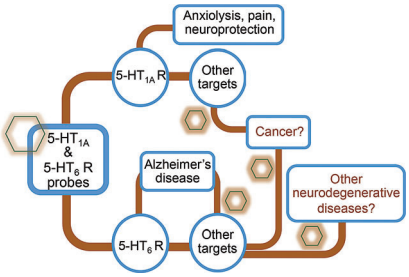

Chemoproteomic Approach to Explore the Target Profile of GPCR ligands: Application to 5-HT_{1A} and 5-HT_6 Receptors