European Journal of Medicinal Chemistry 46 (2011) 4733–4741

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Preliminary communication

Arylamides hybrids of two high-affinity σ_2 receptor ligands as tools for the development of PET radiotracers

Carmen Abate*, Savina Ferorelli, Marialessandra Contino, Roberta Marottoli, Nicola Antonio Colabufo, Roberto Perrone, Francesco Berardi

Dipartimento Farmacochimico, Università degli Studi di Bari ALDO MORO, Via Orabona 4, I-70125 Bari, Italy

ARTICLE INFO

Article history:
Received 28 February 2011
Received in revised form
19 May 2011
Accepted 23 May 2011
Available online 30 May 2011

Keywords:
σ_2 Receptor
Cyclohexylpiperazine
6,7-Dimethoxytetrahydroisoquinoline
Dihydroisoquinolinone
P-Glycoprotein
PET

ABSTRACT

1-Cyclohexyl-4-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]piperazine **1** (PB28) and 2-Methoxy-5-methyl-*N*-[4-(6,7-dimethoxy-3,4-dihydro-1*H*-isoquinolin-2-yl)butyl]benzamide **2** (RHM-1) represent leads for tumor diagnosis, given their high affinity at σ_2 receptors. With the purpose of obtaining good candidates for σ_2 PET tracers development, hybrid structures between **1** and **2** were designed. Excellent σ_1/σ_2 selectivities were reached when 6,7-dimethoxytetrahydroisoquinoline was linked to an *o*-methoxy substituted arylamide (**11a**, **12a**, **15a**), and for these benzamides an intramolecular H-bond in the active conformation at the σ sites, was hypothesized. However these excellent σ_2 ligands were accompanied by interaction with P-gp, which may limit their use as σ_2 receptor PET agents when tumors overexpress P-gp. Compound **15a** whose P-gp interaction was just moderate represents an interesting tool for the development of σ_2 PET tracers useful in tumors overexpressing P-gp.

1. Introduction

After the discovery of sigma (σ) receptors in 1976 [1], initial interest was generated by the evidence that a number of antipsychotic agents displayed affinity for this protein and σ selective ligands might have served as novel antipsychotic drugs. However, the lack of selective σ agents and the concurrent use of different protocols led to contradictory findings for over a decade [2]. Therefore, interest in σ receptor research waned until the early 1990's, when the two subtypes of σ receptors, σ_1 and σ_2, were identified, renovating enthusiasm in these proteins [3]. Soon thereafter the σ_1 subtype was isolated and cloned showing its difference from any other known mammalian protein [4]. Located primarily at endoplasmic reticulum (ER) mitochondrion contact, σ_1 subtype has been classified as a chaperone of inositol(1,4,5) triphosphate receptor, ensuring proper ER-mitochondrial Ca^{2+} signaling and cell survival [5]. σ_1 Receptor ligands have been reported to modulate the release of a number of neurotrasmitters and are under evaluation for the treatment of several Central Nervous System (CNS) disorders [6–9] despite their mechanism of action is still unclear.

The σ_2 subtype is even more elusive. It has yet to be cloned and a recent attempt to characterize it using an affinity chromatography technique led to the isolation of possible histone proteins [10,11]. Such result was in disagreement with findings from fluorescence microscopy, which localizes σ_2 subtypes in several organelles except the nucleus [12]. Regardless of these ambiguities, the development of many high-affinity ligands [13,14] is improving the understanding of σ_2 receptor subtypes, and interest is on the increase since σ_2 receptors are overexpressed in a wide variety of human tumor cell lines [15]. Therefore, σ_2 receptors represent endogenous biomarkers for the diagnosis of tumors with non-invasive techniques such as Positron Emission Tomography (PET) or Single Photon Emission Computed Tomography (SPECT) [15]. In addition, σ_2 receptor density and the proliferative status of tumors proved to be highly correlated in a number of cell lines (e.g. σ_2 receptors density is about 10-fold higher in proliferative than quiescent cells), so that σ_2 receptor radiotracers may serve for the

Abbreviations: Calcein-AM, acetoxymethylester of Calcein; CNS, central nervous system; DMEM, dulbecco's modified eagle medium; DTG, 1,3-di-o-tolyl-guanidine; EMT-6, mouse mammary carcinoma; ER, endoplasmic reticulum; MDCK-MDR1, Madin Darby canine kidney cells transfected with the human MDR1 gene; MDR, multidrug resistance; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium-bromide; PET, positron emission tomography; P-gp, P-glycoprotein; SAfiR–affinity relationship, structure; SPECT, single photon emission computed tomography.

* Corresponding author. Tel.: +39 080 5442748; fax: +39 080 5442231.
E-mail address: abate@farmchim.uniba.it (C. Abate).

0223-5234/$ – see front matter © 2011 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2011.05.057

imaging of the proliferative status of tumors helping to select the tumor treatment [16]. Several are the σ_2 receptor radioligands which have been developed as PET or SPECT agents [15,17] with diverse results. 1-Cyclohexyl-4-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)-*n*-propyl]piperazine (**1**, PB28; Fig. 1), one of the highest affinity σ_2 receptor ligands known [13,18], was [^{11}C]-radiolabeled at the methoxy group, but its in vivo evaluation in mice failed for a high degree of non-specific binding in the brain, maybe related to a not appropriate lipophilicity of the compound [19]. On the other hand, the σ_2 receptor high-affinity ligand 5-methyl-2-[^{11}C]-methoxy-*N*-[4-(6,7-dimethoxy-3,4-dihydro-1*H*-isoquinolin-2-yl)butyl]benzamide (**2**, RHM-1; Fig. 1) [20] provided a clear image of murine breast tumors. Studies followed with the corresponding fluoroethoxy derivative [^{18}F]-radiolabeled which is reported to be currently under clinical study for imaging solid tumors with PET [16]. A correlation between the logP values of the σ_2-receptor ligands flexible benzamides and their tumor up-take was found out indicating that, in addition to receptor affinity, lipophilicity is an important property that must be considered when receptor-based tumor imaging agents are planned. Amide **2** showed the highest tumor up-take and its logD value (2.85) appeared as optimal for tumor imaging. With the aim of contributing to the research of σ_2 receptor PET probes we designed a series of hybrid structures combining the moieties of compound **1** (which displays subnanomolar σ_2 receptor affinity) and compound **2** (which displays a very high σ_2 selectivity and in vivo activity). The first hybrid was obtained through the replacement with 6,7-dimethoxytetrahydroisoquinoline nucleus (A) of 1-cyclohexylpiperazine basic moiety (B) present in compound **1**, generating compound **3** (Table 1) [21]. Such compound showed a noncompetitive binding at σ_2 receptor so that its structural template was no longer considered useful for our purposes. Therefore, moiety B, that was demonstrated to infer excellent σ affinities [13,22–27], was connected by a three-methylene linker to substituted arylamides which are moieties proper of compound **2** analogous structures. The three-methylene linker was selected for a better overlap of these new compounds with ligand **1**, also knowing from previous Structure Affinity Relationship (SAfiR) studies that high σ_2 receptor affinity was kept in shorter chain length analogous benzamides [16]. For each of these newly synthesized cyclohexylpiperazine derivatives, the corresponding 6,7-dimethoxytetrahydroisoquinoline derivatives were prepared in order to compare the impact of each of the two basic moieties in σ receptors binding. Preliminary binding results suggested that in the active conformation of these benzamides at the binding sites an hydrogen bond occurs between the amidic N-atom and the O-atom in the *o*-methoxy substituted compounds, and such hypothesis was verified through the synthesis of dihydroisoquinolinone derivatives (Fig. 2).

Since all of these novel compounds served to strengthen the development of σ_2 receptor imaging agents, we considered useful to evaluate their interaction at the P-glycoprotein (P-gp). Such protein, responsible of the multidrug resistance (MDR), is known to be overexpressed in several tumors [28,29], so that a tracer interacting with both σ_2 receptor and P-gp would lead to an ambiguous information about the tumor, as we recently speculated [24]. The data about the P-gp interaction appears particularly important if one considers that lead compound **1** interacts with P-gp as well as several other σ_2 receptor ligands do [24,25,30]. As for compound **2**, its promising results as PET agent were obtained from mice implanted with EMT-6 cells, where P-gp is not overexpressed [31]. The evaluation of the P-gp activity for the novel hybrid compounds add important pieces of information for the development of σ_2 receptor imaging agents reliable also in tumors overexpressing P-gp.

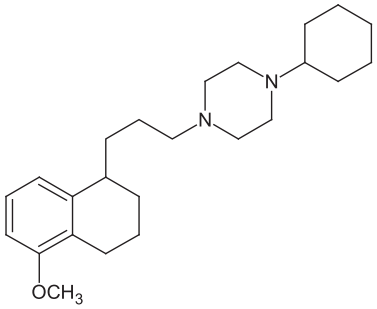
2. Chemistry

The synthesis of target compounds (**11a**,**b**–**15a**,**b**) reported herein is depicted in Schemes 1 and 2. The synthesis of the intermediate propylamines (**5a**,**b**) started from the alkylation of either 6,7-dimethoxytetrahydroisoquinoline (Scheme 1) or 1-cyclohexylpiperazine (Scheme 2) with 3-bromopropanenitrile to afford nitrile derivatives **4a**,**b** which were reduced to amine **5a**,**b** with borane dimethyl sulfide complex. Substituted benzoic acid (**6**) or 1-tetraline carboxylic [32] acids **7** were transformed into their corresponding acyl chloride, through the use of oxalyl chloride, and then reacted with propylamine intermediates (**5a**,**b**) to afford final amides (**11a**,**b**–**14a**,**b**, Scheme 1 and 2). The synthesis of intermediate mesylates **9a**,**b** was obtained through initial alkylation of either 6,7-dimethoxytetrahydroisoquinoline (Scheme 1) or 1-cyclohexypiperazine (Scheme 2) with 3-bromopropanol to alcohols **8a**,**b** followed by reaction with methanesulphonyl chloride. Alkylation of the already known 3,4-dihydroisoquinolin-1(2*H*)-one [33,34] with mesylate intermediates (**9a**,**b**) in the presence of NaH afforded final compounds **15a**,**b** (Scheme 1 and 2).

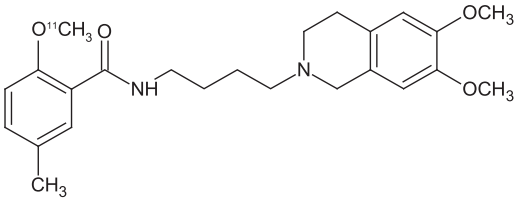
3. Biology

3.1. Receptor binding studies

The target compounds **2**, **3** and **11a**,**b**-**15a**,**b** were assayed as hydrochloride salts. All the compounds were evaluated for in vitro affinity at σ_1 and σ_2 receptors by radioligand binding assays. The specific radioligands and tissue sources were, respectively: (a) σ_1 receptor, (+)-[^{3}H]-pentazocine ((+)-[2*S*-(2*R*,6*R*,11*R*)]-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(3-methyl-2-butenyl)-2,6-methano-3-benzazocine-8-ol), guinea-pig brain membranes without cerebellum; (b) σ_2 receptor, [^{3}H]-DTG (1,3-di-2-tolyl-guanidine) in the presence of 1 μM (+)-pentazocine to mask σ_1 receptors, rat liver membranes. The following compounds were used to define the specific binding reported in parentheses: (a) (+)-pentazocine



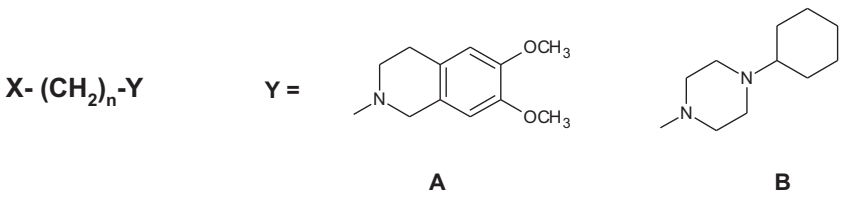
1, PB28

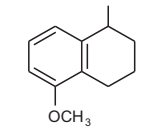
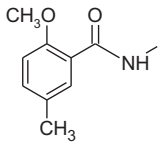
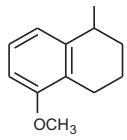
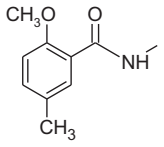
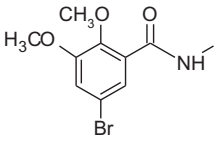
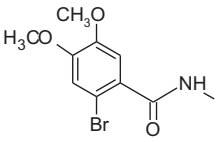
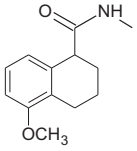
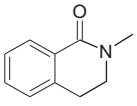


2, RHM-1

Fig. 1. Structures of High-Affinity σ_2 Receptors Ligands.

Table 1
σ Receptors Affinities, P-gp Activities and Lipophilicity.



				K_i nM		K_i ratio σ_1/σ_2	EC_{50} μM	$logD_{7.4}$[a]
Comp.	**X**	**n**	**Y**	σ_1	σ_2		P-gp[b]	
1		3	B	0.38 ± 0.10[c]	0.68 ± 0.20[c]		3.0 ± 0.2[c]	3.75[d]
2		4	A	2150 ± 50[e]	8.68 ± 0.4[e]	247	1.6 ± 0.3	2.83
3		3	A	151 ± 20	96%[f]		1.1 ± 0.2	4.33
11a		3	A	534 ± 104	2.63 ± 0.20	203	5.3 ± 1.3	2.7
11b		3	B	45.5 ± 5.1	26.1 ± 0.7	1.7	8.6 ± 1.5	1.94
12a		3	A	4400 ± 80	12.9 ± 1.7	341	2.5 ± 0.4	3.22
12b		3	B	25.8 ± 1.7	21.2 ± 2.0	1.2	2.7 ± 0.8	2.43
13a		3	A	2570 ± 1470	50.9 ± 14.8	50	45 ± 4	2.89
13b		3	B	268 ± 9	467 ± 3	0.6	45 ± 8	2.11
14a		3	A	146 ± 12	21.1 ± 6.9	7	2.7 ± 0.6	3.21
14b		3	B	35.1 ± 7.8	16.5 ± 7.9	2	8.6 ± 1.7	2.46
15a		3	A	709 ± 133	4.84 ± 0.74	147	12 ± 3	2.25
15b		3	B	24.7 ± 1.2	26.6 ± 4.1	1	20 ± 4	1.49
(+)-pentazocine				3.09 ± 0.26				
DTG					26.3 ± 0.7			
16							1.1 ± 0.3	

[a] Calculated for compounds at pH 7.4 [37].
[b] Transport Inhibition in MDCK-MDR1 cells with Calcein-AM (2.5 μM) as probe Ref. [43].
[c] From Ref. [13].
[d] $LogD_{7.4}$ experimental value was 3.99; Ref. [24].
[e] $K_i = 3078 \pm 87$ nM for σ_1; $K_i = 10.3 \pm 1.5$ nM for σ_2, Ref. [20].
[f] [^{3}H]-(+)-DTG binding inhibition percentage at 10^{-11} M of the compound.

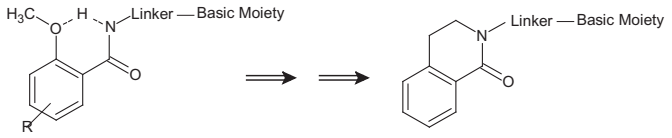


Fig. 2. Intramolecular Hydrogen Bond Formation in 2-Methoxybenzamide compounds in a sort of byciclic structure.

(76–85%), (b) DTG (85–93%). Concentrations required to inhibit 50% of radioligand specific binding (IC_{50}) were determined using six to nine different concentrations of the drug studied in two or three experiments with samples in duplicate and inhibition constants (K_i) values were determined by non-linear curve fitting, using the Prism v. 3.0, GraphPad software [35].

3.2. Calcein-AM experiment

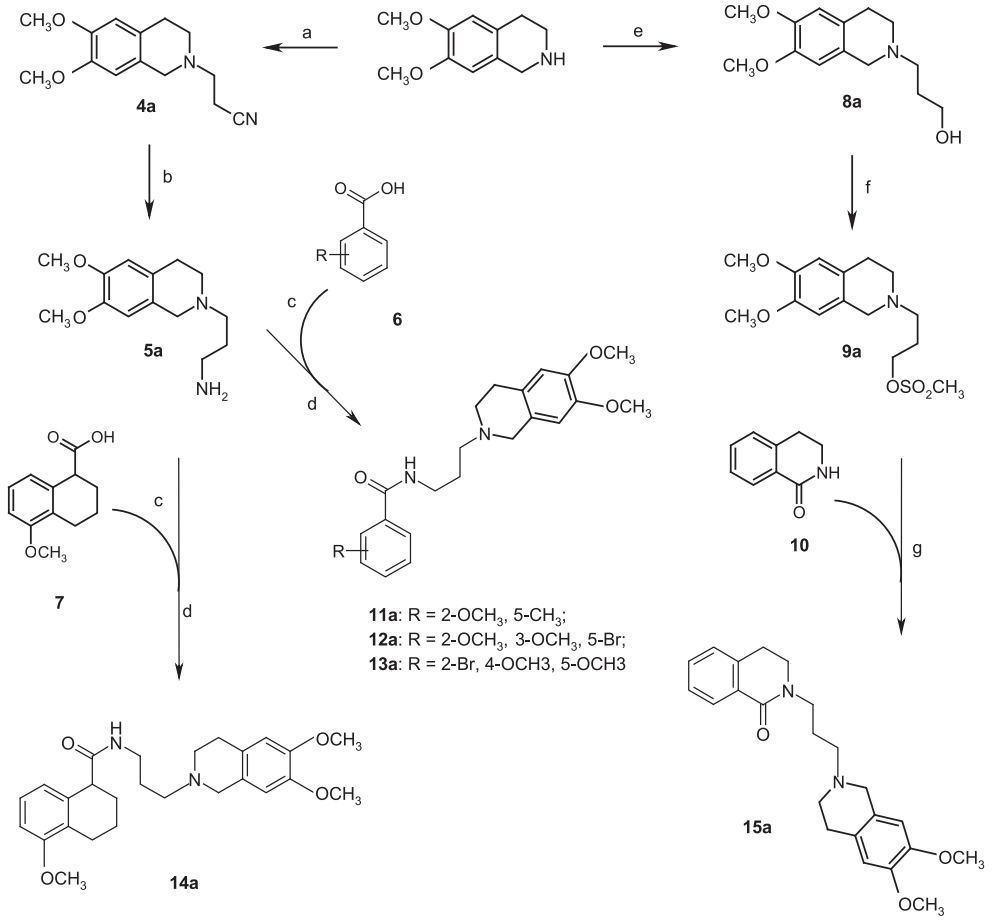
P-gp inhibiting activity of the compounds **2** and newly synthesized **11a,b**-**15a,b** and the reference compound 6,7-Dimethoxy-2-{3-[4-methoxy-3,4-dihydro-2H-naphthalen-(1*E*)-ylidene]propyl}-1,2,3,4-tetrahydroisoquinoline [21] (**16**) was determined by fluorescence measurement using Calcein-AM probe in MDCK-MDR1 cell line according to the published procedure [36]. These transfected canine kidney epithelial cells overexpress only P-gp among MDR transporters, so that the measured biological effect is ascribed essentially to the inhibition of this ATP-pump. The EC_{50} values were obtained from non-linear iterative curve fitting by Prism v. 3.0, GraphPad software [35].

4. Results and discussion

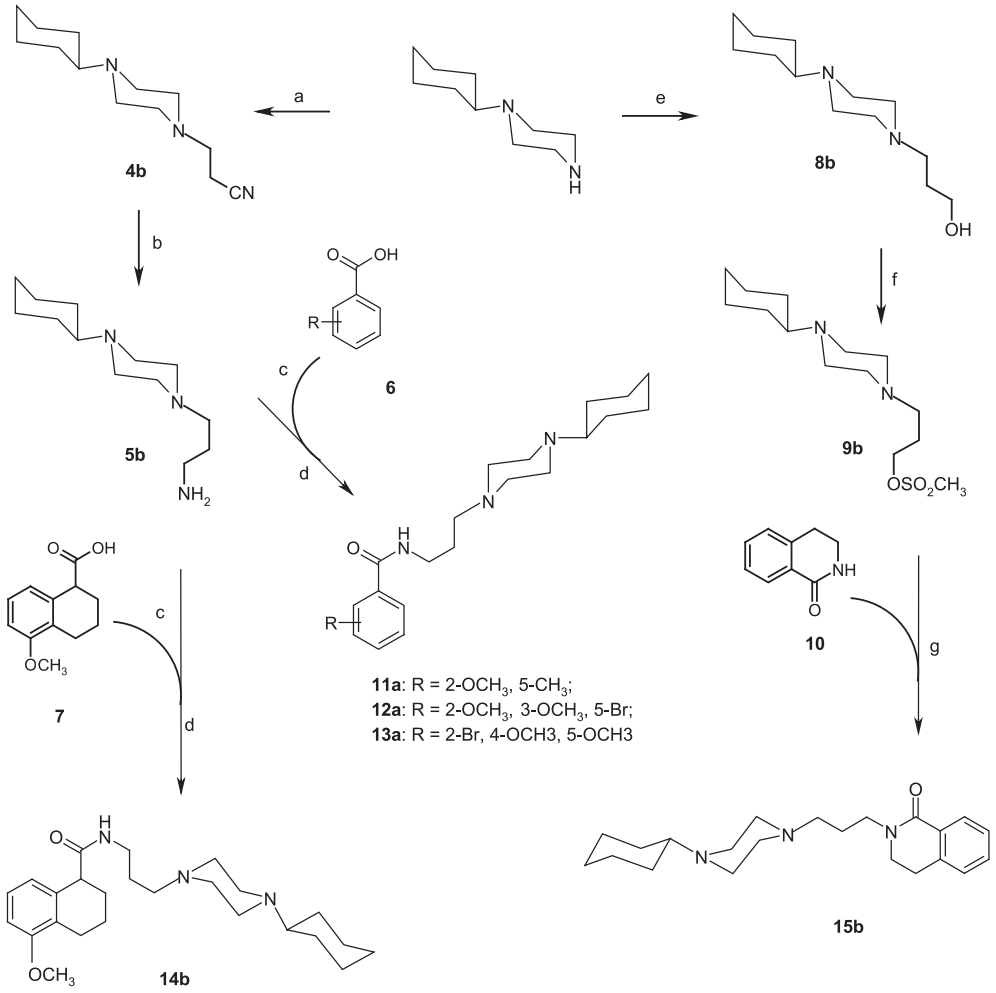
4.1. Structure–activity relationships studies and lipophilicity

σ_1 and σ_2 receptors affinities. Results from binding assays are expressed as inhibition constants (K_i values) in the Table 1. The K_i values at the σ_1 receptor subtype for the newly synthesized compounds ranged from 24.7 nM to 4400 nM. The highest K_i values were displayed by arylamides **11a**, **12a**, **13a** and **15a** bearing the 6,7-dimethoxytetrahydroisoquinoline moiety (A) (K_is from 534 nM to 4400 nM) in accordance to the result shown by the lead compound **2**. The replacement of the aryl ring with the 5-methoxytetralin one slightly improved σ_1 subtype affinity (compound **3** and **14a** $K_i = 151$ nM and $K_i = 146$ nM respectively). The presence of the cyclohexylpiperazine moiety (B) always improved σ_1 receptor affinity compared to moiety A (from 4- to 400-fold). The lowest affinity at the σ_1 receptor in the new cyclohexylpiperazine derivatives was displayed by **13b** ($K_i = 268$ nM), whereas for all the other cyclohexylpiperazine derivatives K_is were in a narrow lower range (from 24.7 nM to 45.5 nM, **15b** and **11b** respectively).

As for the σ_2 receptor affinity, differently from compound **1**, none of the compounds reached subnanomolar values, but most of them displayed results comparable to the σ_2 receptor affinity that we found for compound **2**. The replacement of moiety B with moiety A in the structure of lead compound **1**, surprisingly led to a noncompetitive binding at the σ_2 receptor (compound **3**) so that,



Scheme 1. Reagents: (a): $Br(CH_2)_2CN$, triethylamine; (b): $BH_3{\cdot}S(CH_3)_2$ (10M); (c): $(COCl)_2$; (d): triethylamine; (e) $Br(CH_2)_3OH$, K_2CO_3; (f): CH_3SO_2Cl, triethylamine; (g): NaH.



Scheme 2. Reagents: (a): $Br(CH_2)_2CN$, triethylamine; (b): $BH_3{\cdot}S(CH_3)_2$ (10M); (c): $(COCl)_2$; (d): triethylamine; (e) $Br(CH_2)_3OH$, K_2CO_3; (f): CH_3SO_2Cl, triethylamine; (g): NaH.

this scaffold appeared to be not appropriate for a specific σ_2 receptor interaction. Compound **2** inferior homolog **11a** displayed the highest σ_2 receptor affinity with a 4-fold improved K_i values at both σ_1 and σ_2 receptors ($K_i = 534$ nM for σ_1 and $K_i = 2.63$ nM for σ_2), so that the selectivity remained almost unchanged (about 200-fold). The highest σ_2 receptor selectivity was displayed by **12a** (about 340-fold) ($K_i = 4400$ nM at σ_1 and $K_i = 12.9$ nM at σ_2 receptors). The lowest affinity was shown by 2-bromobenzamide derivative **13b** ($K_i = 467$ nM), whereas its corresponding isomeric 3-bromobenzamide **12b** displayed a 20-fold higher affinity value ($K_i = 21.2$ nM). Such a difference suggested that an intramolecular hydrogen bond may occur in this kind of compounds between the amidic NH group and the O-atom in *o*-methoxy substituted derivatives, mimicking a byciclic ring. This suggestion is further supported by the difference in affinity between the two isomers **13a** ($K_i = 50.9$ nM) and **12a** ($K_i = 12.9$ nM) as well as by the high affinity shown by all of the amides where this intramolecular hydrogen bond is possible. Tetralin derivatives, which naturally contain a bicyclic structure, are endowed with a high affinity too. Results from final compounds **15a**,**b** (K_is = 4.84 nM and 26.6 nM at σ_2 receptor respectively), in which the presence of the above mentioned intramolecular H-bond was *frozen* in the dihydroisoquinolinone ring, strengthened the hypothesis of such an active conformation for these benzamides at the binding site (Fig. 2). Compound **15a** bearing moiety A, and mimicking the intramolecular H-bond resulted as a high affinity, highly selective σ_2 ligand (147-fold σ_2 relative to σ_1 receptor), showing a σ_2 receptor profile comparable to lead compound **2**. All of these results taken together confirmed that the presence of the basic moiety A is strongly detrimental for σ_1 receptor affinity when compared to basic moiety B, so that 6,7-dimethoxytetrahydroisoquinoline moiety is more appropriate than cyclohexylpiperazine to infer high selectivity for σ_2 receptors. As demonstrated in a previous work, the presence of the second N-atom is responsible of the high affinity for both the σ subtypes [23]. Finally, when the A moiety is linked to an arylamide *o*-methoxy substituted, as in lead compound **2**, the best compromise between affinity and high selectivity for excellent σ_2 receptor ligands is reached. In addition, the calculated $logD_{7.4}$ (Table 1, logD range 1.49–3.22) displayed by most of the novel amides (logD ~ 2.5 for compounds **11a**, **12b**, **13a**, **13b**, **14b**, **15a**) was close to the optimal value of the in vivo active compound **2** (logD 2.83) and therefore consistent with entry into tumor cells [37]. It has been shown that radiotracers with similar affinities at σ_2 receptors have different tumor up-take depending on their lipophilicity, suggesting that high receptor affinity and lipophilicity are both important for the design of receptor-based tumor imaging agents [16].

4.2. Calcein-AM experiment

Activity at the P-gp efflux pump, expressed as EC_{50} in Table 1, was determined for compound **2** and each of the target compounds.

The MDCK-MDR1 cells used in the assay overexpress the P-gp transporter, so that the measured biological effect can be ascribed to the interaction with this pump. All the compounds herein reported displayed activity at P-gp with EC_{50} values in a range from 1.1 μM to 45 μM. Apparently, P-gp interaction was independent from the nature of the basic moiety since A and their corresponding B counterparts displayed the same degree of activity (**1** and **3**, **11a** and **11b**, **12a** and **12b**, **13a** and **13b**, **14a** and **14b** and **15a** and **15b**).

Compound **2** proved to be a P-gp modulator (EC_{50} = 1.6 μM) together with compound **3** which showed the strongest P-gp activity (EC_{50} = 1.1 μM). In fact, in a previous work [38], compound [^{11}C]-**3** and [^{11}C]-**16** were successfully used to image P-gp in rats, therefore we reasonably hypothesize that compounds with P-gp activities similar to **3** and **16** (EC_{50} = 1.1 μM), will image this efflux pump as well. Furthermore, radiolabeled **3** and **16** proved an uptake in several periphery organs, so that an interference with the σ_2 receptor imaging of peripheral resistant tumors may occur with radiotracers displaying such P-gp activities [38]. Unfortunately compounds **11a** and **12a** which displayed the best σ_2 selectivity profiles, displayed also significant P-gp interactions (EC_{50} values = 5.3 μM and 2.5 μM respectively), only slightly lower than compound **2**. On the other hand, compound **15a** which displayed important σ_2 selectivity, showed a P-gp interaction (EC_{50} = 12 μM) 8-fold lower than lead **2**, appearing as a tool worthy of further investigation for the development of σ_2 receptor tracers.

4.3. Conclusions

All the results from σ receptors binding taken together show that the combination of 6,7-dimethoxytetrahydroisoquinoline linked to an *o*-methoxy substituted arylamide is responsible of excellent σ_1 relative to σ_2 selectivity, and an intramolecular hydrogen bond formation is suggested for the active conformation of such compounds. As shown by other authors, this class of σ_2 receptor ligands may be exploited for the imaging of σ_2 overexpressing tumors by PET analysis [15,16,39] through [^{11}C]- or [^{18}F]-radiolabeling. However, novel compounds with the best σ_1/σ_2 selectivity profile (**11a** and **12a**), as well as lead compound **2**, are also accompanied by an important interaction with P-gp which is overexpressed in several resistant tumors. Such interaction, which is comparable to the activity shown by P-gp reference compounds **3** and **16**, suggests that interference with the imaging of σ_2 receptors may occur, leading to misinterpretation, as already demonstrated for other classes of ligands which are characterized by P-gp activity besides a high affinity for their targets [40,41]. An interesting biological profile worthy of further investigation is shown by compound **15a**: σ_2 affinity and σ_1/σ_2 selectivity comparable to the in vivo active lead compound **2**, but a 8-fold lower interaction with P-gp. Therefore, compound **15a** which can potentially be developed as σ_2 radioligand, provides, above all, the template to drive future synthesis of optimal σ_2 receptor tumors tracers devoid of P-gp interaction.

5. Experimental

5.1. Chemistry

Both column chromatography and flash column chromatography were performed with 60 Å pore size silica gel as the stationary phase (1:30 w/w, 63–200 μm particle size, from ICN and 1:15 w/w, 15–40 μm particle size, from Merck respectively). Melting points were determined in open capillaries on a Gallenkamp electrothermal apparatus. Purity of tested compounds was established by combustion analysis, confirming a purity ≥ 95%. Elemental analyses (C, H, N) were performed on an Eurovector Euro EA 3000 analyzer; the analytical results were within ±0.4% of the theoretical values unless otherwise indicated. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz)spectra were 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 6890-5973 MSD gas chromatograph/mass spectrometer and 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. Infrared spectra were recorded on a Perkin–Elmer, Spectrum one, FTIR. Chemicals were from Aldrich and Across and were used without any further purification.

5.2. General procedure for the synthesis of nitrile derivatives (**4a**,**b**)

3-Bromopropionitrile (0.43 mL, 5.18 mmol) and triethylamine (1.4 mL, 10.4 mmol) were added to a solution of either 6,7-dimethoxytetrahydroisoquinoline or 1-cyclohexylpiperazine (5.2 mmol) in CH_2Cl_2. The solution was stirred at room temperature for 6 h and then the reaction mixture was washed with H_2O and dried over Na_2SO_4. The solvent was removed under reduced pressure and the crude product was purified by column chromatography using CH_2Cl_2/MeOH (95:5) as eluent.

N-[6,7-Dimethoxy-3,4-dihydro-isoquinolin-2(1*H*)-yl]propanenitrile (**4a**) was obtained as white solid (0.70 g, 55% yield); mp = 84–86 °C; ^{1}H NMR δ 2.64 (t, 2H, *J* = 6.9 Hz, CH_2CN), 2.82–2.92 [m, 6H, $NCCH_2CH_2N$, $N(CH_2)_2Ar$], 3.66 (s, 2H, NCH_2Ar), 3.82 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 6.51 (s, 1H, aromatic), 6.59 (s, 1H, aromatic); GC–MS *m*/*z* 246 (M^+, 50), 245 (52), 206 (100), 164 (80); IR cm^{-1}: 2248 (CN).

3-(4-cyclohexylpiperazin-1-yl)propanenitrile (**4b**) was obtained as a white solid (0.97 g, 85% yield); mp = 32–34 °C; ^{1}H NMR δ 1.05–1.28 [m, 5H, cyclohexyl ($CH)_5$], 1.61–1.87 [m, 5H, cyclohexyl ($CH)_5$], 2.18–2.25 (m, 1H, cyclohexyl CHN), 2.47–2.59 (m, 10H piperazine, and CH_2CN), 2.69 (t, 2H, *J* = 6.9 Hz, NCH_2CH_2CN); GC–MS *m*/*z* 222 (M^++1, 5), 221 (M^+, 33), 178 (100); IR cm^{-1}: 2250 (CN).

5.3. General procedure for the synthesis of amine derivatives (**5a**,**b**)

At the solution of **4a** or **4b** (1.00 mmol) in dry THF (10 mL), kept at 0 °C and under a stream of N_2, borane dimethyl sulfide 10 M (0.8 mL, 8.55 mmol) was added, in a dropwise manner. The reaction mixture was stirred under reflux for 4 h. Methanol and 30 mL of HCl 6 N were then added to the reaction mixture and the solution was refluxed for 1 h. After cooling the solvent was evaporated under reduced pressure. The acid aqueous layer was made basic with NaOH and extracted with CH_2Cl_2 (3 × 10 mL). The organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by flash column chromatography with $CHCl_3$/MeOH (95:5).

3-[3,4-dihydro-6,7-dimethoxyisoquinolin-2(1*H*)-yl]propan-1-amine (**5a**) was obtained as a white semi-solid (0.68 g, 96% yield); ^{1}H NMR δ 1.58–1.88 (m, 4H, $CH_2CH_2NH_2$, and NH_2, D_2O exchanged), 2.56 (t, 2H, $CH_2CH_2CH_2NH_2$), 2.62–2.96 (m, 6H, $ArCH_2CH_2N$, CH_2NH_2), 3.52–3.57 (br s, 2H, $ArCH_2N$), 3.78–3.88 (2s, 6H, 2 OCH_3), 6.51 (s, 1H, aromatic), 6.57 (s, 1H, aromatic); GC–MS *m*/*z* 250 (M^+, 1.6), 206 (20), 192 (100); IR cm^{-1}: 3303 (NH_2), 3020, 1613.

3-(4-cyclohexylpiperazin-1-yl)propan-1-amine (**5b**) was obtained as a yellow semi-solid (0.42 g, 66% yield); ^{1}H NMR δ 1.02–1.22 [m, 5H, cyclohexyl, ($CH)_5$], 1.55–1.86 [m, 7H, cyclohexyl, ($CH)_5$, and $CH_2CH_2NH_2$], 2.13–2.21 (m, 1H, CHN), 2.34–2.65 (m, 10H, piperazine, and $CH_2CH_2CH_2NH_2$), 2.68–2.85 (m, 2H, CH_2NH_2), 3.30 (br s, 2H, NH_2, D_2O exchanged); GC–MS *m*/*z*

225 (M^+, 3), 195 (32), 181 (94), 71 (100); IR cm^{-1}: 3281 (NH_2), 2930, 1646.

5.4. General procedure for the synthesis of alcohol **8a,b**

3-Bromo-1-propanol (0.22 mL, 2.5 mmol) and K_2CO_3 (0.41 g, 3.0 mmol) were added to a solution of either 6,7-dimethoxy-tetrahydroisoquinoline or 1-cyclohexylpiperazine (3.0 mmol) in CH_3CN (20 mL). The reaction mixture was stirred under reflux overnight. Then the solvent was removed under reduced pressure and, H_2O was added to the crude residue. The aqueous mixture was extracted with CH_2Cl_2 (3 × 10 mL) and 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 (95:5) as eluent.

3-[6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl]propan-1-ol (**8a**) was obtained as a yellow solid (0.43 g, 70% yield); mp = 70–72 °C; ^{1}H NMR δ 1.84–1.98 (m, 2H, CH_2CH$_2$OH), 2.88–2.97 (m, 6H, ArCH$_2$CH$_2$N and CH_2CH$_2$CH$_2$OH), 3.73–3.85 (m, 10H, 2 OCH_3, ArCH$_2$N and CH_2OH), 4.81 (br s, 1H, OH, D_2O exchanged), 6.52 (s, 1H, aromatic), 6.59 (s, 1H, aromatic); GC–MS *m*/*z* 251 (M^+, 18), 206 (100), 164 (48).

3-(4-cyclohexylpiperazin-1-yl)propan-1-ol (**8b**) was obtained as a brown semi-solid (0.48 g, 86% yield); ^{1}H NMR δ 1.04–1.28 [m, 5H, cyclohexyl, (CH*H*)$_5$], 1.59–1.87 [m, 7H, cyclohexyl, (C*H*H)$_5$, and CH_2CH$_2$OH], 2.19–2.27 (m, 1H, CHN), 2.58–2.62 (m, 10H, piperazine, and CH_2CH$_2$CH$_2$OH), 2.89 (br s, 1H, OH, D_2O exchanged), 3.76–3.80 (m, 2H, CH_2OH); GC–MS *m*/*z* 226 (M^+, 60), 183 (100), 181 (75), 111 (78).

5.5. General procedure for the synthesis of methanesulphonate (**9a,b**)

To a solution of the appropriate alcohol 8 (2.8 mmol) in dry CH_2Cl_2 (5 mL) kept at 0 °C, methanesulphonyl chloride (0.2 mL, 2.6 mmol) and triethylamine (1.0 mL, 7.3 mmol) were added. The mixture was stirred for 1 h. Then, H_2O was added to the mixture reaction and the aqueous solution was extracted with $CHCl_3$ (3 × 10 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by column chromatography with AcOEt/MeOH (9:1) as eluent.

3-[6,7-dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl]propan-1-methanesulphonate (**9a**) was obtained as a yellow oil (0.21 g; 25% yield); ^{1}H NMR δ 2.00–2.07 (m, 2H, CH_2CH$_2$O), 2.61–2.81 (m, 6H, ArCH$_2$CH$_2$N and CH_2CH$_2$CH$_2$O), 3.00 (s, 3H, CH_3SO_2), 3.83–3.86 (m, 8H, 2 OCH_3 and ArCH$_2$N), 4.35 (t, 2H, *J* = 6.3 Hz, CH$_2$O), 6.51 (s, 1H, aromatic), 6.59 (s, 1H, aromatic).

3-(4-cyclohexylpiperazin-1-yl)propan-1-methanesulphonate (**9b**) was obtained as a yellow semi-solid (0.35 g, 46% yield); ^{1}H NMR δ 1.05–1.28 [m, 5H, cyclohexyl, (CH*H*)$_5$], 1.59–1.94 [m, 7H, cyclohexyl, (C*H*H)$_5$, and CH_2CH$_2$O], 2.19–2.33 (m, 1H, CHN), 2.42–2.58 (m, 10H, piperazine CH$_2$, and CH_2CH$_2$CH$_2$O), 3.01 (s, 3H, CH_3SO_2), 4.28 (t, 2H, *J* = 6.3 Hz, CH$_2$O); LC–MS (ESI$^+$) *m*/*z* 305 [M + H]$^+$; LC–MS–MS 305: 209, 127.

5.6. General procedure for the synthesis of amides **11a,b**–**14a,b**

Oxalyl chloride (0.24 mL, 2.7 mmol) was added in a dropwise manner to a solution of the carboxylic acid **7** (0.30 g, 1.81 mmol) in dry CH_2Cl_2 (10 mL) added with dry DMF (0.2 mL). The mixture was stirred at room temperature for 1 h and then it was refluxed for 30 min. The solvent was removed under reduced pressure, and the crude residue was dissolved in dry CH_2Cl_2 and added to a solution of the appropriate amine (2.7 mmol) and triethylamine (3.6 mmol) in CH_2Cl_2 (10 mL), at 0 °C. The resulting mixture was heated under stirring to reflux for 1 h. Then, the solution was cooled and extracted with 3 N HCl. The aqueous layer was made basic with a solution of NaOH and extracted with CH_2Cl_2 (3 × 10 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated under reduced pressure to give a crude mixture which was purified by column chromatography to achieve the final product.

N-[3-[6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl]propyl]-2-methoxy-5-methylbenzamide (**11a**) was obtained as a yellow oil using AcOEt/CH_2Cl_2 (7:3) as eluent (0.095 g, 13% yield); ^{1}H NMR δ 1.86–1.90 (m, 2H, NHCH$_2$CH_2), 2.31 (s, 3H, CH_3), 2.58–2.75 (m, 6H, ArCH_2CH$_2$NCH$_2$), 3.51–3.62 (m, 4H, NHCH_2, ArCH$_2$N), 3.74–3.84 (m, 9 H, 3 OCH_3), 6.47 (s, 1H, aromatic), 6.57 (s, 1H, aromatic), 6.78 (d, 1H, *J* = 8.5 Hz, aromatic), 7.18–7.24 (m, 1H, aromatic), 7.98 (s, 1H, aromatic), 8.08–8.18 (br s, 1H, NH); ^{13}C NMR: 20.55; 27.08; 28.85; 38.65; 51.32; 56.01; 56.05; 56.10; 56.14; 56.55; 109.60; 111.39; 111.51; 121.58; 126.38; 126.79; 130.71; 132.65; 133.11; 147.41; 147.71; 155.58; 165.69; GC–MS *m*/*z* 398 (M^+, 0.1), 192 (100); Anal. $C_{23}H_{30}N_2O_4 \cdot HCl \cdot 0.5H_2O$ (C, H, N).

N-[3-(4-Cyclohexylpiperazin-1-yl)propyl]-2-methoxy-5-methylbenzamide (**11b**) was obtained as a yellow oil using CH_2Cl_2/MeOH (8:2) as eluent (0.20 g, 20% yield); ^{1}H NMR δ 1.09–1.37 [m, 5H, cyclohexyl (CH*H*)$_5$], 1.63–1.67 (m, 1H, cycloexyl), 1.78–1.85 [m, 4H, cyclohexyl (CH*H*)$_4$], 1.94–2.08 (m, 2H, NHCH$_2$CH_2), 2.31 (s, 3H, CH_3), 2.44–2.58 (m, 3H, CHN and NHCH$_2$CH$_2$CH_2), 2.75–2.84 (m, 8H, piperazine), 3.48–3.58 (m, 2H, CH_2NH), 3.92 (s, 3H, OCH_3), 6.86 (d, 1H, *J* = 8.3 Hz, aromatic), 7.22 (dd, 1H, *J* = 8.53 and *J*′ = 2.5 Hz, aromatic), 7.95 (d, 1H, *J* = 2.2 Hz, aromatic), 7.96–7.97 (br s, 1H, NH); GC–MS *m*/*z* 373 (M^+, 1.0), 235 (79), 149 (100); LC–MS (ESI$^+$) 374 [M + H]$^+$; LC–MS–MS 374: 206, 149. Anal. $C_{22}H_{35}N_3O_2 \cdot 2HCl \cdot 0.5H_2O$ (C, H, N).

N-[3-[6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl]propyl]-5-bromo-2,3-dimethoxybenzamide (**12a**) was obtained as colorless oil (0.13 g, 17%) with $CHCl_3$/MeOH (99:1) as eluent; ^{1}H NMR δ 1.85–1.89 (m, 2H, NHCH$_2$CH_2), 2.67–2.74 [m, 6H, Ar(CH_2)$_2$NCH_2(CH$_2$)$_2$], 3.53–3.60 (m, 4H, NHCH_2 and ArCH$_2$N), 3.80–3.85 (4s, 12H, 4 OCH_3), 6.45 (s, 1H aromatic), 6.47 (s, 1H aromatic), 7.11 (d, *J* = 2.5 Hz, 1H, aromatic), 7.72 (d, 1H, *J* = 2.5 Hz, aromatic), 8.38–8.58 (br s, 1H, NH); GC–MS *m*/*z* 492 (M^+, 0.2), 192 (100); LC–MS (ESI$^+$) *m*/*z* 493 [M + H]$^+$; LC–MS–MS 493: 302, 300, 245. Anal. $C_{23}H_{29}BrN_2O_5 \cdot (COOH)_2$ (C, H, N).

N-[3-(4-Cyclohexylpiperazin-1-yl)propyl]-5-bromo-2,3-dimethoxybenzamide (**12b**) was obtained as colorless oil using CH_2Cl_2/MeOH (9:1) as eluent (0.57 g, 78% yield); ^{1}H NMR δ 1.02–1.28 [m, 5H, cyclohexyl (CH*H*)$_5$], 1.55–1.68 (m, 1H, cyclohexyl), 1.68–1.92 (m, 6H, cyclohexyl (CH*H*)$_4$ and NHCH$_2$CH_2], 2.18–2.28 (m, 1H, CHN), 2.35–2.68 (m, 10H, piperazine and NHCH$_2$CH$_2$CH_2), 3.41–3.58 (m, 2H, NHCH$_2$), 3.85 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 7.11 (d, *J* = 2.5 Hz, 1H, aromatic), 7.72 (d, 1H, *J* = 2.5 Hz, aromatic), 8.38–8.58 (br s, 1H, NH); ^{13}C NMR: 26.14; 26.36; 26.54; 29.10; 39.07; 48.97; 53.98; 56.49; 56.90; 61.60; 63.68; 117.23; 118.22; 125.31; 129.28; 146.61; 153.56; 164.17; GC–MS *m*/*z* 469 (M^++2, 2), 468 (M^++1, 2), 467 (M^+, 4), 329 (72), 245 (100), 243 (93). Anal. $C_{22}H_{34}BrN_3O_3 \cdot 2HCl \cdot 0.5H_2O$ (C, H, N).

N-[3-[6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl]propyl]-2-bromo-4,5-dimethoxybenzamide (**13a**) was obtained as a yellow oil (0.14 g, 16% yield) with CH_2Cl_2/MeOH (98:2) as eluent; ^{1}H NMR δ 1.86–1.90 (m, 2H, NHCH$_2$CH_2), 2.66–2.73 [m, 6H, Ar(CH_2)$_2$NCH_2(CH$_2$)$_2$], 3.55–3.62 (m, 4H, NHCH_2 and ArCH$_2$N), 3.79–3.84 (4s, 12H, 4 OCH_3), 6.45 (s, 1H aromatic), 6.47 (s, 1H aromatic), 6.79 (s, 1H aromatic), 7.03 (s, 1H, aromatic), 7.78 (br s, 1H, NH); GC–MS *m*/*z* 492 (M^+, 0.8), 192 (100); LC–MS (ESI$^+$) *m*/*z* 493 [M + H]$^+$. Anal. $C_{23}H_{29}BrN_2O_5 \cdot HCl$ (C, H, N).

N-[3-(4-Cyclohexylpiperazin-1-yl)propyl]-2-bromo-4,5-dimethoxybenzamide (**13b**) was obtained as a colorless oil using

CH_2Cl_2/MeOH (9:1) as eluent (1.24 g, 49% yield); ^{1}H NMR δ 1.02–1.32 [m, 5H, cyclohexyl (CH*H*)$_5$], 1.58–1.68 (m, 1H, cyclohexyl), 1.69–1.85 (m, 6H, cyclohexyl (CH*H*)$_4$ and NHCH$_2$C*H*$_2$), 2.12–2.28 (m, 1H, CHN), 2.34–2.68 (m, 10H, piperazine and NHCH$_2$CH$_2$C*H*$_2$), 3.51–3.60 (m, 2H, NHC*H*$_2$), 3.87 (s, 3H, OCH$_3$), 3.88 (s, 3H, OCH$_3$), 6.98 (s, 1H, aromatic), 7.09 (s, 1H, aromatic), 7.95–8.02 (br s, 1H, NH); ^{13}C NMR: 24.83; 26.12; 26.51; 28.87; 40.62; 48.89; 53.72; 56.37; 57.90; 63.61; 109.86; 112.51; 115.62; 130.93; 148.56; 150.61; 167.32; GC–MS *m*/*z* 469 (M$^+$+2, 8), 468 (M$^+$+1, 5), 467 (M$^+$, 12), 331 (86), 329 (85), 245 (100), 243 (99); IR cm^{-1}: 3263 (NH), 1645 (CO). Anal. $C_{22}H_{34}BrN_3O_3\cdot 2HCl$ (C, H, N).

1,2,3,4-Tetrahydro-*N*-[3-[6,7-dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl]propyl]-5-methoxynaphthalene-1-carboxamide (**14a**) was obtained as a colorless semi-solid (1.0 g, 75%) with CH_2Cl_2/MeOH (9:1) as eluent; ^{1}H NMR δ 1.61–1.92 (m, 6H, NHCH$_2$C*H*$_2$ and CHC*H*$_2$C*H*$_2$), 2.42–2.79 (m, 8H, ArCH$_2$CH$_2$NCH$_2$ and CHCH$_2$CH$_2$C*H*$_2$), 3.28–3.38 (m, 2H, ArCH$_2$N), 3.48–3.65 (m, 2H, NHC*H*$_2$), 3.75–3.88 (m, 10H, 3 OCH$_3$ and CHCO), 6.47 (s, 1H aromatic, tetrahydroisoquinoline), 6.55 (s, 1H aromatic, tetrahydroisoquinoline), 6.68–6.78 (m, 2H, aromatic), 7.08 (t, 1H, J = 7.8 Hz, aromatic), 7.90 (br s, 1H, NH); GC–MS *m*/*z* 438 (M$^+$, 2), 206 (91), 192 (100); Anal. $C_{26}H_{34}N_2O_4\cdot HCl\cdot 0.25H_2O$ (C, H, N).

N-[3-(4-Cyclohexylpiperazin-1-yl)propyl]-1,2,3,4-tetrahydro-5-methoxynaphthalene-1-carboxamide (**14b**) was obtained as a colorless semi-solid (0.20 g, 27% yield) using CH_2Cl_2/MeOH (98:2) as eluent; ^{1}H NMR δ 1.10–1.30 [m, 5H, cyclohexyl (CH*H*)$_5$], 1.56–1.98 [m, 11H, cyclohexyl (C*H*H)$_5$, CHC*H*$_2$C*H*$_2$, NHCH$_2$C*H*$_2$], 2.11–2.23 (m, 11H, piperazine CH$_2$, CH$_2$N, CHN), 2.51–2.78 (m, 2H, benzyl CH$_2$), 3.24–3.34 (m, 2H, NHC*H*$_2$), 3.64 (t, 1H, J = 5.5 Hz, CHCO), 3.82 (s, 3H, OCH$_3$), 6.20 (br s, 1H, NH), 6.70–7.12 (m, 3H, aromatic); ^{13}C NMR: 20.26; 23.28; 25.86; 26.04; 26.50; 27.35; 29.27; 29.30; 39.04; 47.84; 49.14; 53.94; 55.46; 56.95; 63.57; 108.37; 122.01; 126.59; 126.96; 135.76; 157.71; 175.05; GC–MS *m*/*z* 413 (M$^+$, 7), 289 (57), 275 (97), 181 (66), 161 (100); Anal. $C_{25}H_{39}N_3O_2\cdot 2HCl$ (C, H, N).

5.7. General procedure for the synthesis of amide (**15a**,**b**)

To a solution of **10** (0.1g, 0.72 mmol) in dry toluene (5 mL), NaH 60% dispersion in mineral oil (0.07 g, 1.8 mmol) was added at 0 °C cautiously. Then, a solution of appropriate methansulphonate derivative **9ab** (1.15 mmol) in dry toluene (5 mL) was added in a dropwise manner. The resulting mixture was heated under stirring to reflux overnight. Then water was added to the reaction mixture which was extracted with AcOEt (3 × 5 mL). The collected organic layers were dried over Na_2SO_4 and evaporated under reduced pressure. The crude residue was purified by column chromatography with CH_2Cl_2/MeOH (95:5) as eluent.

2-[3-[6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl]propyl]-3,4-dihydroisoquinolin-1(2*H*)-one (**15a**) was obtained as a yellow solid (0.18 g, 65% yield); mp = 70–72 °C; ^{1}H NMR δ 1.90–2.00 (m, 2H, CH$_2$C*H*$_2$CH$_2$NCO), 2.56–3.00 [m, 8H, ArC*H*$_2$CH$_2$NCO(CH$_2$)$_2$C*H*$_2$, ArCH$_2$CH$_2$], 3.56–3.67 (m, 6H, ArCH$_2$C*H*$_2$NCOC*H*$_2$, ArCH$_2$N), 3.82 (s, 3H, OCH$_3$), 3.84 (s, 3H, OCH$_3$), 6.50 (s, 1H, aromatic), 6.58 (s, 1H, aromatic), 7.17–8.07 (m, 4H, aromatic); GC–MS *m*/*z* 380 (M$^+$, 1), 192 (100); LC–MS (ESI$^+$) *m*/*z* 381 [M + H]$^+$; LC–MS–MS 381: 188, 160. Anal. $C_{23}H_{28}N_2O_3\cdot HCl\cdot 0.75H_2O$ (C, H, N).

2-[3-(4-Cyclohexylpiperazin-1-yl)propyl]-3,4-dihydroisoquinolin-1(2*H*)-one (**15b**) was obtained as a yellow oil (0.11 g; 45% yield); ^{1}H NMR δ 1.11–1.31 [m, 5H, cyclohexyl, (CH*H*)$_5$], 1.58–2.04 [m, 7H, cyclohexyl (C*H*H)$_5$ and NCH$_2$C*H*$_2$CH$_2$NCO], 2.28–2.49 (m, 3H, NC*H*$_2$CH$_2$CH$_2$NCO and CHN), 2.52–2.82 (m, 8H, piperazine), 2.98 (t, 2H, J = 6.6 Hz, ArCH$_2$), 3.54–3.62 [m, 4H, (CH$_2$)$_2$NCO], 7.16–8.05 (m, 4H, aromatic); ^{13}C NMR: 25.47; 26.09; 26.51; 28.44; 29.14; 45.98; 46.45; 49.10; 53.88; 56.07; 63.71; 127.02; 127.23; 128.39; 129.83; 131.70; 138.17; 164.58; GC–MS *m*/*z* 355 (M$^+$, 2), 230 (39), 217 (100), 195 (47), 188 (62). Anal. $C_{22}H_{33}N_3O\cdot 2HCl\cdot 0.5H_2O$ (C, H, N).

5.8. Biological methods and materials: radioligand binding assays

All the procedures for the binding assays were previously described. σ_1 And σ_2 receptor binding experiments were carried out according to Matsumoto et al. [42]. [^{3}H]-DTG (30 Ci/mmol) and (+)-[^{3}H]-pentazocine (34 Ci/mmol) were purchased from Perkin–Elmer Life Sciences (Zavantem, Belgium). DTG was purchased from Tocris Cookson Ltd., U.K. (+)-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.

5.9. Cell culture

MDCK-MDR1 cell line was a gift from Prof. P. Borst, NKI-AVL Institute, Amsterdam, Nederland. MDCK-MDR1 cells were grown in DMEM high glucose supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, in a humidified incubator at 37 °C with a 5% CO_2 atmosphere. Cell culture reagents were purchased from Celbio s.r.l. (Milano, Italy). CulturePlate 96/wells plates were purchased from Perkin–Elmer Life Science; Calcein-AM, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) were obtained from Sigma–Aldrich (Milan, Italy).

5.10. Calcein-AM experiment

The experiments were carried out as described by Feng et al. with minor modifications [36]. Each cell line (50 000 cells per well) was seeded into black CulturePlate 96-well plates with 100 μL medium and allowed to become confluent overnight. Test compounds were dissolved in 100 μL culture medium and were added to the cell monolayers. The plates were then incubated at 37 °C for 30 min. Calcein-AM was added in 100 μL phosphate-buffered saline (PBS) to yield a final concentration of 2.5 μM, and plate incubation was continued for 30 min. Each well was washed three times with ice-cold PBS. Saline buffer was added to each well, and the plates were read with a Victor3 fluorimeter (Perkin–Elmer) at excitation and emission wavelengths of 485 nm and 535 nm, respectively. Under these experimental conditions, calcein cell accumulation in the absence and presence of tested compounds was evaluated, and basal-level fluorescence was estimated by untreated cell fluorescence. In treated wells, the increase in fluorescence was measured relative to the basal level. EC_{50} values were determined by fitting the percent fluorescence increase percentage versus log[dose] [35].

Contributors

CA and FB conceived the project and wrote the paper. MC and NAC conducted all the biological assays that provided the activity data. SF and RM synthesized all the compounds that were used in this study. R P planned and supervised the research.

Appendix. Supplementary material

The supplementary data associated with this article can be found in the on-line version at doi:10.1016/j.ejmech.2011.05.057.

References

[1] W.R. Martin, C.E. Eades, J.A. Thompson, R.E. Huppler, The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog, J. Pharmacol. Exp. Ther. 197 (1976) 517–532.

[2] R. Quirion, R. Chicheportiche, P.C. Contreras, K.M. Johnson, D. Lodge, S.W. Tam, J.H. Woods, S.R. Zukin, Classification and nomenclature of phencyclidine and sigma receptor sites, Trends Neurosci. 10 (1987) 444–446.

[3] R. Quirion, W.D. Bowen, Y. Itzhak, J.L. Junien, J.M. Musachio, R.B. Rothman, T.-P. Su, S.W. Tam, D.P. Taylor, A proposal for the classification of sigma binding sites, Trends Pharmacol. Sci. 13 (1992) 85–86.

[4] M. Hanner, F.F. Moebius, A. Flandorfer, H.G. Knaus, J.M. Striessnig, E. Kempner, H. Glossmann, Purification, molecular cloning, and expression of the mammalian sigma1-binding site, Proc. Natl. Acad. Sci. 93 (1996) 8072–8077.

[5] S.-Y. Tsai, T. Hayashi, T. Mori, T.-P. Su, Sigma-1 receptor chaperones and diseases, Cent. Nerv. Syst. Agents Med. Chem. 9 (2009) 184–189.

[6] E.J. Cobos, J.M. Entrena, F.R. Nieto, C.M. Cendan, E. Del Pozo, Pharmacology and therapeutic potential of sigma$_1$ receptor ligands, Curr. Neuropharmacol. 6 (2008) 344–366.

[7] G. Skuza, Potential antidepressant activity of sigma ligands, Pol. J. Pharm. 55 (2003) 923–934.

[8] G. Skuza, Z. Rogoz, Effect of BD 1047, a sigma1 receptor antagonist, in the animal models predictive of antipsychotic activity, Pharmacol. Rep. 58 (2006) 626–635.

[9] M. Mishina, M. Ohyama, K. Ishii, S. Kitamura, Y. Kimura, K. Oda, K. Kawamura, T. Sasaki, S. Kobayashi, Y. Katayama, K. Ishiwata, Low density of sigma1 receptors in early Alzheimer's disease, Ann. Nucl. Med. 22 (2008) 151–156.

[10] N.A. Colabufo, F. Berardi, C. Abate, M. Contino, M. Niso, R. Perrone, Is the σ_2 receptor a histone binding protein? J. Med. Chem. 49 (2006) 4153–4158.

[11] C. Abate, J. Elenewski, M. Niso, F. Berardi, N.A. Colabufo, A. Azzariti, R. Perrone, R.A. Glennon, Interaction of the σ_2 receptor ligand PB28 with the human nucleosome: computational and experimental probes of interaction with the H2A/H2B dimer, ChemMedChem. 5 (2010) 268–273.

[12] C. Zeng, S. Vangveravong, J. Xu, K.C. Chang, R.S. Hotchkiss, K.T. Wheeler, D. Shen, Z.-P. Zhuang, H.F. Kung, R.H. Mach, Subcellular localization of sigma-2 receptors in breast cancer cells using two-photon and confocal microscopy, Cancer Res. 67 (2007) 6708–6716.

[13] F. Berardi, C. Abate, S. Ferorelli, N.A. Colabufo, R. Perrone, 1-Cyclohexylpiperazine and 3,3-dimethylpiperidine derivatives as sigma-1 (σ_1) and sigma-2 (σ_2) receptor ligands: a review, Cent. Nerv. Syst. Agents Med. Chem. 9 (2009) 205–219.

[14] Z. Tu, J. Xu, L.A. Jones, S. Li, C. Dumsdorf, S. Vangveravong, D. Chen, K.T. Wheeler, M.J. Welch, R.H. Mach, Fluorine-18-labeled benzamide analogues for imaging the sigma2 receptor status of solid tumors with positron emission tomography, J. Med. Chem. 50 (2007) 3194–3204.

[15] A. van Waarde, A.A. Rybczynska, N. Ramakrishnan, K. Ishiwata, P.H. Elsinga, R.A. Dierckx, Sigma receptors in oncology: therapeutic and diagnostic applications of sigma ligands, Curr. Pharm. Des. 16 (2010) 3519–3537.

[16] R.H. Mach, K.T. Wheeler, Development of molecular probes for imaging sigma-2 receptors in vitro and in vivo, Cent. Nerv. Syst. Agents Med. Chem. 9 (2009) 230–245.

[17] K. Hashimoto, K. Ishiwata, Sigma receptor ligands: possible application as therapeutic drugs and as radiopharmaceuticals, Curr. Pharm. Des. 12 (2006) 3857–3876.

[18] F. Berardi, S. Ferorelli, C. Abate, N.A. Colabufo, M. Contino, R. Perrone, V. Tortorella, 4-(tetralin-1-yl)- and 4-(naphthalen-1-yl)alkyl derivatives of 1-cyclohexylpiperazine as sigma receptor ligands with agonist sigma2 activity, J. Med. Chem. 47 (2004) 2308–2317.

[19] M. Kassiou, F. Robert, R.F. Dannals, X. Liu, D.F. Wong, H.T. Ravert, U.A. Scheffel, Synthesis and in vivo evaluation of a new PET radioligand for studying sigma-2 receptors, Bioorg. Med. Chem. 13 (2005) 3623–3626.

[20] R.H. Mach, Y. Huang, R.A. Freeman, L. Wu, S. Vangveravong, R.R. Luedtke, Conformationally-flexible benzamide analogues as dopamine D3 and sigma-2 receptor ligands, Bioorg. Med. Chem. 14 (2004) 195–202.

[21] N.A. Colabufo, F. Berardi, M. Cantore, M.G. Perrone, M. Contino, C. Inglese, M. Niso, R. Perrone, A. Azzariti, L. Porcelli, G.M. Simone, A. Paradiso, Small P-gp modulating molecules: SAR studies on tetrahydroisoquinoline derivatives, Bioorg. Med. Chem. 16 (2008) 362–373.

[22] C. Abate, P.D. Mosier, F. Berardi, R.A. Glennon, A structure-affinity and comparative molecular field analysis of sigma-2 (sigma2) receptor ligands, Cent. Nerv. Syst. Agents Med. Chem. 9 (2009) 246–257.

[23] F. Berardi, C. Abate, S. Ferorelli, V. Uricchio, N.A. Colabufo, M. Niso, R. Perrone, Exploring the importance of piperazine N-atoms for σ_2 receptor affinity and activity in a series of analogs of 1-cyclohexyl-4-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]piperazine (PB28), J. Med. Chem. 52 (2009) 7817–7828.

[24] C. Abate, M. Niso, E. Lacivita, P.D. Mosier, A. Toscano, R. Perrone, Analogues of σ receptor ligand 1-cyclohexyl-4-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]piperazine (PB28) with added polar functionality and reduced lipophilicity for potential use as positron emission tomography radiotracers, J. Med. Chem. 54 (2011) 1022–1032.

[25] C. Abate, M. Niso, M. Contino, N.A. Colabufo, S. Ferorelli, R. Perrone, F. Berardi, 1-Cyclohexyl-4-(4-arylcyclohexyl)piperazines: mixed σ and human Δ(8)–Δ(7) sterol isomerase ligands with antiproliferative and P-glycoprotein inhibitory activity, ChemMedChem. 6 (2011) 73–80.

[26] J.H. Poupaert, R.R. Matsumoto, C.R. McCurdy, Conversion of a highly selective sigma-1 receptor-ligand to sigma-2 receptor preferring ligands with anti-cocaine activity, J. Med. Chem. 51 (2008) 1482–1486.

[27] Y.T. Xu, N. Kaushal, J. Shaikh, L.L. Wilson, C. Mesangeau, C.R. McCurdy, R.R. Matsumoto, A novel substituted piperazine, CM156, attenuates the stimulant and toxic effects of cocaine in mice, J. Pharmacol. Exp. Ther. 333 (2010) 491–500.

[28] R. Perez-Tomas, Multidrug resistance: retrospect and prospects in anti-cancer drug treatment, Curr. Med. Chem. 13 (2006) 1859–1876.

[29] E. Teodori, S. Dei, S. Martelli, F. Scapecchi, F. Gualtieri, The functions and structure of ABC transporters: implications for the design of new inhibitors of Pgp and MRP1 to control multidrug resistance (MDR), Curr. Drug Targets 7 (2006) 893–909.

[30] A. Azzariti, N.A. Colabufo, F. Berardi, L. Porcelli, M. Niso, M.G. Simone, R. Perrone, A. Paradiso, Cyclohexylpiperazine derivative PB28, a sigma2 agonist and sigma1 antagonist receptor, inhibits cell growth, modulates P-glycoprotein, and synergizes with anthracyclines in breast cancer, Mol. Cancer Ther. 5 (2006) 1807–1816.

[31] P.R. Twentyman, K.A. Wright, N.E. Fox, Characterisation of a mouse tumour cell line with in vitro derived resistance to verapamil, Br. J. Cancer 61 (1990) 279–284.

[32] R. Perrone, F. Berardi, M. Leopoldo, V. Tortorella, M.G. Fornaretto, C. Caccia, R.A. McArthur, 1-aryl-4-[(1-tetralinyl)alkyl]piperazines: alkylamido and alkylamino derivatives. Synthesis, 5-HT1A receptor affinity, and selectivity. III, J. Med. Chem. 39 (1996) 3195–3202.

[33] W. Ren, M. Yamane, Palladium-catalyzed carbamoylation of aryl halides by tungsten carbonyl amine complex, J. Org. Chem. 74 (2009) 8332–8335.

[34] U.R. Mach, A.E. Hackling, S. Perachon, S. Ferry, C.G. Wermuth, J.-C. Schwartz, P. Sokoloff, H. Stark, Development of novel 1,2,3,4-tetrahydroisoquinoline derivatives and closely related compounds as potent and selective dopamine D3 receptor ligands, ChemBioChem. 5 (2004) 508–518.

[35] Prism Software, Version 3.0 for Windows. GraphPad Software, Inc., San Diego, CA, 1998.

[36] B. Feng, J.B. Mills, R.E. Davidson, R.J. Mireles, J.S. Janiszewski, M.D. Troutman, S.M. de Morais, In vitro P-glycoprotein assays to predict the in vivo interactions of P-glycoprotein with drugs in the central nervous system, Drug Metab. Dispos. 36 (2008) 268–275.

[37] ACD Labs, Version 7.07. Advanced Chemistry Development Inc., Toronto, Canada, 2003.

[38] A. van Waarde, N.K. Ramakrishnan, A.A. Rybczynska, P.H. Elsinga, F. Berardi, J.R. de Jong, C. Kwizera, R. Perrone, M. Cantore, J.W. Sijbesma, R.A. Dierckx, N.A. Colabufo, Synthesis and preclinical evaluation of novel PET probes for P-glycoprotein function and expression, J. Med. Chem. 52 (2009) 4524–4532.

[39] Phase I Clinical Trial. ClinicalTrials.gov Identifier: NCT00968656. Assessment of Cellular Proliferation in Tumors by Positron Emission Tomography (PET) Using [^{18}F]ISO-1 (FISO PET/CT).

[40] P.H. Elsinga, N.H. Hendrikse, J. Bart, A. van Waarde, W. Vaalburg, Positron emission tomography studies on binding of central nervous system drugs and P-glycoprotein function in the rodent brain, Mol. Imaging Biol. 7 (2005) 37–44.

[41] V. Sharma, J.L. Prior, M.G. Belinsky, G.D. Kruh, D. Piwnica-Worms, Characterization of a ^{67}Ga/^{68}Ga radiopharmaceutical for SPECT and PET of MDR1 P-glycoprotein transport activity in vivo: validation in multidrug-resistant tumors and at the blood-brain barrier, J. Nucl. Med. 46 (2005) 354–364.

[42] R.R. Matsumoto, W.D. Bowen, M.A. Tom, D.D. Truong, B.R. De Costa, Characterization of two novel sigma receptor ligands: antidystonic effects in rats suggest sigma receptor antagonism, Eur. J. Pharmacol. 280 (1995) 301–310.

[43] N.A. Colabufo, F. Berardi, M. Cantore, M.G. Perrone, M. Contino, C. Inglese, M. Niso, R. Perrone, Multi-drug-resistance-reverting agents: 2-aryloxazole and 2-arylthiazole derivatives as potent BCRP or MRP1 inhibitors, ChemMedChem. 4 (2009) 188–195.