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Synthesis, σ_1, σ_2-receptors binding affinity and antiproliferative action of new C1-substituted adamantanes

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ABSTRACT

The synthesis of *N*-{4-[*a*-(1-adamantyl)benzyl]phenyl}piperazines **2a–e** is described. The in vitro antiproliferative activity of most compounds against main cancer cell lines is significant. The σ_1, σ_2-receptors and sodium channels binding affinity of compounds **2** were investigated. One of the most active analogs, **2a**, had an interesting in vivo anticancer profile against the BxPC-3 and Mia-Paca-2 pancreas cancer cell lines with caspase-3 activation, which was associated with an anagelsic activity against the neuropathic pain.

1. Introduction

Sigma(σ)-receptors are expressed to a greater degree in tumours than they are in the surrounding normal tissue.[1–3] Although they have been considered as opioid receptors, the sigma receptors have been classified as distinct pharmacological entity and their function shown to be unrelated to the function of the opioid receptors.[4–6] Based on the ligand selectivity in the binding assays, two subtypes, sigma-1 (σ_1) and sigma-2 (σ_2) receptors, were identified.[5–7] Furthermore, σ_1-receptors have been shown to be involved in programmed cell death (apoptosis), with σ_1-agonists (activators according to their molecular function)[8] being antiapoptotic and neuroprotective, with putative anti-neurodegenerative activity.[6,9,10] σ_1-Antagonists (deactivators) or σ_2-agonists are proapoptotic and can, because of the high expression of σ_1 and σ_2-receptors in the rapidly proliferating cancer cells[11–14] act as putative anticancer drugs by inducing cell death via apoptosis.[15–18] Presently, there is considerable evidence of antiproliferative and cytotoxic activity for σ_1-antagonists,[15,16] σ_2 putative agonists,[17–20] mixed σ_1/σ_2 ligands[15,19,21] and even one σ_1 agonist.[22] More specifically, Spruce et al.[16] have shown that σ_1-antagonists induce caspase-dependent apoptosis, which is in accord with recent observations that σ_1 agonists prevent caspase-3 activation.[9,10] On the other hand, σ_2-agonists have been shown to activate a caspase-independent apoptotic pathway and have been proposed as putative anti-cancer drugs.[17,18] Recent data have also suggested the importance of the σ_1-receptor modulated ion channels (Na^+, K^+, Cl^-, Ca^{2+})[23–27] and σ_1-receptor binding of cholesterol in lipid rafts[28] concerning the proliferation of cancer cells. The Na^+ channels are, in particular, modulated by σ_1 receptors and implicated in the adhesion, migration and apoptosis of cancer cells.[25–27] These investigations provide new areas in which to elucidate the mechanism of action of σ-ligands and their putative role as therapeutic anticancer agents. Classical SAR studies indicated that the presence of a cycloalkyl or aryl group attached to the cationic amine center via a linker of 3 to 5-membered chain (including a heteroatom), was essential for affinity at the σ-receptors.[29,30]

In the course of our program directed towards the design and synthesis of new pharmacological active adamantane amino

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derivatives, the above structural requirement for σ-receptor affinity prompted us to investigate the synthesis of the adamantane piperazines **2** and **3**. In the piperazine **2**, one benzene ring is linked to the second piperazine nitrogen by three atoms (N, 2C), while in **3**, two benzene rings are linked to the second piperazine nitrogen by a chain of 4 atoms (C, N, 2C). Our synthetic route gave mainly compounds of type **2** with, in some cases, compounds of type **3** as by-products. Compounds **2** were investigated for their binding affinity at both σ_1 and σ_2-receptors and we carried out an in vitro study of their antiproliferative and cytotoxic activity. Finally, the in vivo effect of **2a** on pancreas cancer cells xenografts was investigated in parallel with a prototypical study of its effect in the formalin putative neuropathic pain amplified by the widely used anticancer drug Paclitaxel.

2. Results and discussion

2.1. Chemistry

Reaction of excess 1-substituted piperazine with α-(1-adamantyl)benzydryl chloride (**1**)[31,32] gave **2** as the main product with only minor amounts of **3**. In the case of the reactions of 1-methyl and 1-benzylpiperazine could these minor products be isolated (**3a**,**d**). The main product **2** could be readily distinguished from the minor product **3** in the ^{1}H NMR spectrum as it had only nine aromatic hydrogens compared with the ten required for **3**, with the first compound showing the characteristic methine proton on Cα. The structure of **2** was further confirmed by the ^{13}C NMR spectra.

For comparative purposes, derivative **4** (60%) was prepared by the reaction of **1** with morpholine, the isomer **5** again being obtained as the minor product (13%) (Scheme 1). The compounds **3a**,**d** and **5** are unstable in the presence of acids and protic solvents.

A plausible pathway for the conversion of **1** to analogues **2**, is outlined in Scheme 2. According to the proposed mechanism, the nucleophilic reagents (piperazines) attack preferentially the *para*-position of one of the benzene rings, as the tertiary carbocation site is sterically hindered by the bulky adamantane and phenyl substituents.

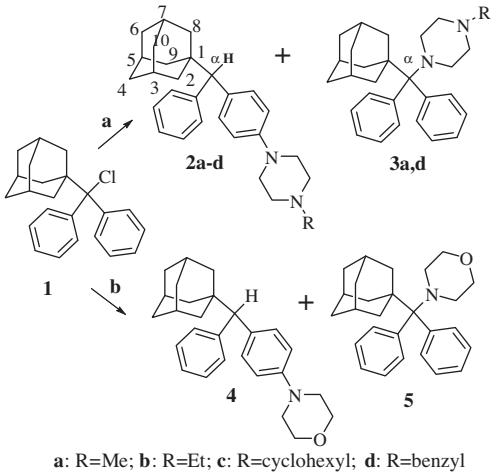
Reductive debenzylation of the benzyl derivative **2d** with ammonium formate and Pd on charcoal gave the adduct **2e** (Scheme 3).

2.2. Biology

The results for the in vitro antiproliferative activity of the derivatives **2a–e** and **4** are shown in Tables 1a, 1b. Also, their affinities for both the σ_1 and σ_2-receptors and site 2 of Na^+ channels are summarised in Table 2.

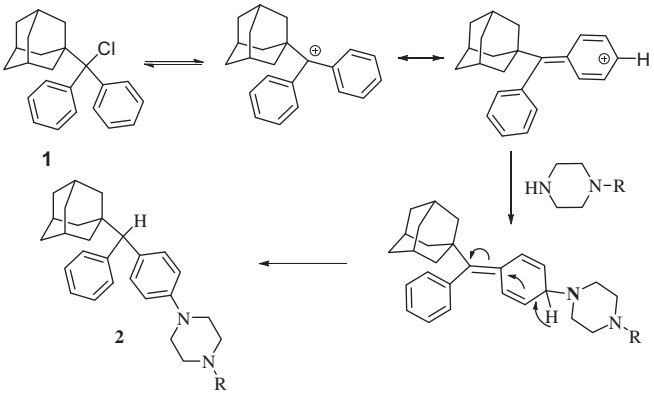
As is clear from Table 2, the piperazine analogues **2a–c** and **2e** have a notable binding affinity for the σ_1 and σ_2-receptors and also site 2 of the Na^+ channels. The benzyl derivative **2d** shows a much lower affinity for σ_1-receptors and the Na^+ channels. None of these interactions are observed in the case of the morpholine analogue **4**. This difference in the activity of **2a–e** and **4** may be attributed to the presence of the second piperazine nitrogen atom in **2a–e** (Schemes 1 and 3). Analogue **2e** seems to have moderate binding activity at the σ_1-receptors, whilst it appears inactive at the σ_2-receptors.

These results, in conjunction with the in vitro antiproliferative and cytotoxic action of the new adamantane piperazines **2** (Table 1a and 1b), imply that the new molecules act as mixed σ_1/σ_2 ligands, with a notable selectivity for σ_1- receptors. As the binding data do not classify the ligands as agonists or antagonists and thus continuing attempts to define the characteristics of adamantane ligands required for receptor activation, subtype

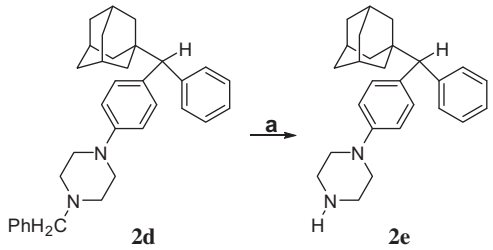


a: R=Me; **b**: R=Et; **c**: R=cyclohexyl; **d**: R=benzyl

Scheme 1. Reagents and conditions: (a) Monosubstituted piperazine, 130 °C, 12 h; (b) morpholine, 110 °C, 14 h.



Scheme 2. Plausible mechanism for the formation of piperazines **2a–d**.



Scheme 3. Reagents and conditions: (a) Pd/C, HCO_2NH_4, MeOH, reflux, 3 h.

selectivity and especially mode of action (agonistic or antagonistic) are needed. However, it must be considered that, in fact, no validated in vitro isolated organ tests exist for the functional characterization of σ ligands as agonists or antagonists.[33] It is worth noting that in the derivatives **2**, the introduction of a bulky substituent on the second piperazine nitrogen (**2c**, **d**) causes a reduction of cytotoxic and antiproliferative activity, which is accompanied by a decrease of the σ_1 affinity of **2d.** However, the high affinity of **2c**, for the σ_1 receptors (more than 10-fold, compared to **2a**), could indicate that a decisive factor, in the expression of the cytotoxic activity of these ligands may be also the intrinsic activity of these ligands on σ_1 and σ_2 receptors, that is, the type of activity (agonist or antagonist), its efficacy (full or partial) and the interaction between σ_1 and σ_2 intrinsic activities. In conclusion, a rational SAR

Table 1a
Summary results from the in vitro screening of antiproliferative activity of tested derivatives on colon, prostate, breast, ovarian and central nervous system cancer cell lines

Compound	Values	Colon		Prostate		Breast	Ovarian		CNS		
		HCT-116	HCT-15	DU145	PC3	MCF7	IGROV-1	OVCAR-5	SF268	SF295	U251
2a	LC_{50}	100	100	100	100	3.17	100	94.5	100	85.22	9.14
	TGI	9.368	12.85	65.52	32.9	16.93	58.49	41.9	61.64	48.12	6.57
	GI_{50}	4.92	5.54	6.43	6.8	11.56	8.83	8	18.88	11.03	4
2b	LC_{50}	100	100	99.57	100	100	100	77.8	100	82.21	11.95
	TGI	32.93	41.21	58.69	65.61	59	49	24.5	53.46	50.9	7.42
	GI_{50}	6.39	6.63	17.81	9.01	9.23	8	6	8.24	19.6	4.23
2c	LC_{50}	100				100		100	100		
	TGI	100				55		98.2	99.76		
	GI_{50}	22.19				8.7		55.37	53.07		
2d	LC_{50}	100				100		100	100		
	TGI	100				99.36		100	100		
	GI_{50}	11.95				22.29		84.3	100		
2e	LC_{50}	9.41	8.9			8.68	8.89	10.42	8.76		
	TGI	6.54	6.15			5.59	6.1	7.333	6.11		
	GI_{50}	3.67	3.4			2.49	3.32	4.267	3.45		
4	LC_{50}	100				100		100	100		
	TGI	100				100		100	100		
	GI_{50}	100				100		100	100		
5FU	LC_{50}	100	100	100	100	100	100	100		100	100
	TGI	100	100	100	100	100	100	100		100	100
	GI_{50}	9.9	21	9.37	21.0	9.9	9.1	20		76.0	82

For experimental agent: LC_{50} is the concentration of drug resulting in 50% reduction in measured protein, TGI the concentration resulting in total growth inhibition, GI_{50} the concentration resulting in growth inhibition of 50% (see also pharmacological protocol).

Table 1b
Summary results from the in vitro screening of antiproliferative activity of tested derivatives on lung, leukemia, pancreas, melanoma cancer cell lines and on normal cell lines (HUVEC: Human Umbilical Vein Endothelial Cells, hMSC: Human Mesenchymal Stem Cells and NHDF: Normal Human Dermal Fibroblasts)

Compound	Values	Non small cell lung	Small lung			Leukemia	Pancreas		Liver	Melanoma	Normal cell lines		
		NCI-H460	DMS 114	NCI-H69	H69AR	HL-60 (TB)	MiaPaca2	BX-PC3	SKHep1	LOX-IMVI	HUVEC	hMSC	NHDF
2a	LC_{50}	100	100	100	90.47	78.51		16.61	77.64	8.41	10.34	91.56	100
	TGI	7.66	9.17	100	18.43	9.63		6.96	28.42	5.88	7.43	54.21	9.6
	GI_{50}	4.41	5.43	7.09	6.04	5.04		3.84	6.05	3.36	4.52	16.85	5.3
2b	LC_{50}	100	20.33	100	100	100		>100	29.81	8.40	9.88	100	100
	TGI	9.44	11.58	100	7.82	100		41.62	7.24	6.09	6.72	72	100
	GI_{50}	5.77	6.05	5.54	4.75	6.39		7.07	3.98	3.79	3.56	19.5	7.43
2c	LC_{50}	100					100	100					
	TGI	100					66.13	76.75					
	GI_{50}	47.3					20.22	8.58					
2d	LC_{50}	100					100	100	100		100		
	TGI	100					100	52.07	100		100		
	GI_{50}	54.74					14.88	7.51	23.46		76.76		
2e	LC_{50}	9.7	9.05			100		9.05	9.04	8.11	100	9.33	9.49
	TGI	6.72	6.51			9.92		6.33	6.48	5.45	7.62	7.1	6.23
	GI_{50}	3.74	3.97			5.97		3.61	3.91	2.79	4.8	4.87	2.97
4	LC_{50}	100						100					
	TGI	100						100					
	GI_{50}	100						100					
5FU	LC_{50}	100	100	100	100	100	100	100	100		100	100	
	TGI	100	100	100	100	100	100	100	100		100	86.06	
	GI_{50}	6.5	100	100	100	100	30	8.2	6.3		9.72	8.92	
Gemcitabine	LC_{50}						100	8.71					
	TGI						100	9.05					
	GI_{50}						35.83	3.64					

For each experimental agent: LC_{50} is the concentration of drug resulting in 50% reduction in measured protein, TGI the concentration resulting in total growth inhibition, GI_{50} the concentration resulting in growth inhibition of 50% (see also pharmacological protocol).

study of sigma ligands could not be possible without validated functional tests for the sigma receptors.

Compound **2a** was initially screened in vitro on colon (colon 205), breast (MCF-7) and prostate (LNCap) cancer cells, by the trypan blue methodology, where significant apoptotic, anti proliferative and cytotoxic effects were observed. Then, apoptosis was more extensively confirmed with the Annexin V (high throughput cytometry) methodology[34,35] on MCF-7 breast cancer cells, by obtaining close to 30% of total apoptosis at 5 μM and more than 80% of total apoptosis at 50 μM for **2a**,[40] in agreement with the above (trypan blue) in vitro screening and with the sulforhodamine B (SRB) staining[36–38] (Tables 1a,b). Considering the in vitro results (Tables 1a,b) of antiproliferative activity of derivatives **2** on cancer cell lines (colon, prostate, breast, ovarian, central nervous system, lung, leukemia, pancreas, melanoma) and on normal cell lines (HUVEC: Human Umbilical Vein Endothelial Cells, hMSC:

Table 2
Affinities of the adamantane piperazines **2** for the σ_1 and σ_2-receptors

Compound	$\sigma 1$ IC_{50} ± SEM (nM)	IC_{50} ± SEM (nM)	σ_1/σ_2	Na^+ Channels IC_{50} ± SEM (μM)
2a	3.2 ± 0.7	37.6 ± 9.3	11.8	1.2 ± 0.15
2b	2.9 ± 0.6	4.0 ± 2.8	1.4	0.7 ± 0.1
2c	0.27 ± 0.2	97.0 ± 36.0	360	0.7 ± 0.1
2d	63.3 ± 18.9	119.0 ± 44.0	1.9	1.6 ± 0.5
2e	12.4 ± 2.3	>1000		1.2 ± 0.2
4	>100	>1000		>10.0

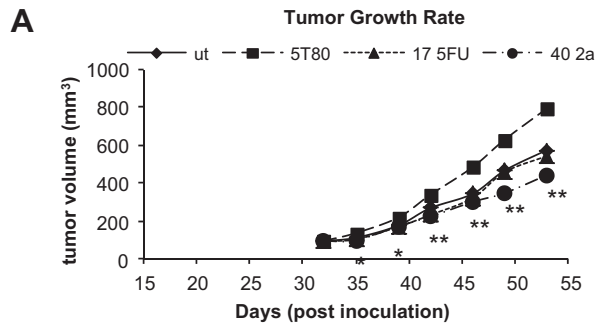
Human Mesenchymal Stem Cells and NHDF: Normal Human Dermal Fibroblasts) (Tables 1a,b), it appears that **2a** exhibited the most selective activity against melanoma and pancreas, given that the cytotoxic effect of **2a** on the HUVEC cells could, in vivo, also be an antiangiogenic factor against the tumors.However, the in vivo anti-tumor activity of **2a**, in melanoma LOX-IMVI xenografts on SCID mice, was not comparative to the reference drug Dacarbazine (data not shown). In contrast, interesting results were obtained with **2a** in the BxPC-3 and Mia-Paca-2 pancreas cancer xenografts (described in Section 4.7[38,39]). Anti-tumor activity of **2a** was in most of the aforementioned xenografts, superior to those of 5-fluorouracile (5-FU) and similar to those of Gemcitabine, with **2a** exerting interesting synergies with both of these reference drugs (Fig. 1).

The pharmacological profile of compound **2a** was further extended by toxicological experiments. Toxicological studies were performed on CD1 male and female mice and, for xenografts, on mice with severe combined immune deficiency (SCID). Compound **2a** was well tolerated at 40 mg/kg (ip) in chronic 3–4 weeks treatments on CD1 and SCID mice. There were no dead animals or important loss of weight in SCID mice during the xenografts experiments. Concerning the mechanistic effects induced by **2a** on the BxPC-3, IGROV-1 and PC-3 cell cycle and apoptosis (Table 3), significant increases of sub-G1 and, for BxPC-3 and PC-3 at 15 μM, also of G1 populations were observed in cells treated with **2a**, compared to vehicle treated group, combined with a decrease of populations engaged in S phase, indicative of the apoptotic activity of **2a**. Indeed, **2a** exhibited apoptotic plasmatic membrane modifications, from 5 μM for BxPC-3, associated, at 5 μM for IGROV-1 and 50 μM for Bx-PC-3 or PC-3, with caspasse-3 activation (Table 3). Results of in vitro assays concerning the effect of **2a** on cell cycle and apoptosis of BxPC3, IGROV-1 and PC3 cancer cells are summarised in Table 3.

Finally, **2a** was tested in a neuropathic pain model (described in Section 4.7[40]). Compound **2a** exhibited a notable analgesic effect in the formalin test[41,42] operated on mice on which pain sensitizations was obtained by previous (2 weeks) administration of Paclitaxel[40] as shown in Fig 2.

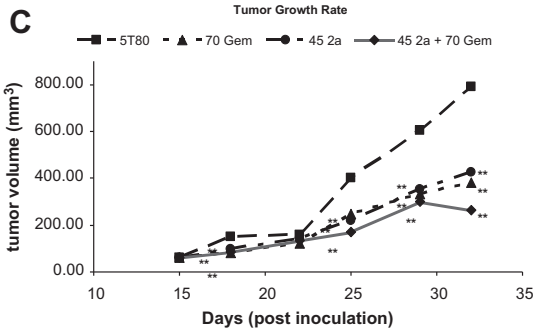
3. Conclusion

The in vitro and in vivo toxicological and xenograft screening studies showed that piperazine **2a**, exhibited an acceptable toxicological profile associated with a notable anti-tumor activity, in good agreement with its in vitro results, was particularly prominent in pancreas. It is noteworthy that this anti-tumor activity of **2a** is associated with a putative antagonism of the neuropathic pain induced by the clinically used anti-cancer drugs (particularly, taxanes and platines). Given the fatal prognosis of pancreatic cancer, due to its high metastatic potential, compound **2a** is currently undergoing extensive investigations, with particular concern to its putative anti-metastatic activity.



B

	Post mortem weight Average weight ±SEM(mg)	% reduction
untreated	723±45	
5T80	1109±92	
17 5FU	700±37	3.25
40 2a	690±70	37.78**



D

	Post mortem weight Average weight ±SEM(mg)	% reduction
5T80	1225±308	
70 Gem	536.8±164	56.2**
45 2a	642.5±208	47.5**
45 2a + 70 Gem	370.45±119	69.7**

Figure 1. Growth of BX-PC3 (pancreas) and MiaPaca2 (pancreas) tumours in SCID mice treated with fluorouracil (5FU) (reference drug for pancreas) and Gemcitabine (Gemzar) (reference drug for pancreas) and **2a**. (A): tumour size (in mm^3) of each mouse group (19-20 mice per group that is 38–40 tumours per group), (B) table showing % reduction of tumours on day of termination of experiment.for BX-PC3 xenograft, (C) tumour size (in mm^3) of each mouse group (12 mice per group that is 24 tumours per group), (D) table showing % reduction of tumours on day of termination of experiment for MiaPaca2 xenograft. **Ut**, untreated animals; **5T80**, animals treated with 5%Tween80; **17 5FU**, animals treated with 17 mg/kg fluorouracil (dissolved in water for injection) twice a week, **40 2a**, animals treated with 40 mg/Kg **2a** (dissolved in 5% Tween 80) for 5 consecutive days (two cycles of treatment) and 2 administrations for one more week (for BX-PC3). **5T80**, animals treated with 5%Tween80; **70 Gem**, animals treated with 70 mg/kg Gemcitabine (dissolved in 5% Tween 80) twice a week; **45 2a**, animals treated with 45 mg/Kg **2a** (dissolved in 5% Tween 80) for 3 consecutive days (three cycles of treatments); **45 2a + 70 Gem**, co-administration of 45 mg/Kg **2a** and 70 mg/kg Gemcitabine (three cycles of treatment) for MiaPaca2 xenograft. Statistical evaluation was assessed with a two-tailed Students' *t*-test. Points with $p \leqslant 0.05$ are indicated by one asterisk (*), $p \leqslant 0.001$ by two asterisks (**).

Table 3
Summary results of the cell cycle and apoptotic in vitro assays performed with compound **2a** at 0.5, 5, 15 and 50 μM on BxPC-3, PC-3 and IGROV-1 cancer cell lines

Cancer cell lines	Treatment (μM)	Cell cycle analysis (%)					Cleaved Caspase-3 containing cells (%)	Annexin V binding/7-AAD incorporation (%)			
		<G1	G_1	S	G_2	>G2		Healthy cells Annexin V^- 7-AAD^-	Early apoptosis Annexin V^+ 7-AAD^-	Late apoptosis Annexin V^+ 7-AAD^+	Total apoptotic death
BxPC-3 (Pancreas)	0	9	41	33	12	6	10	77	11	9	20
	0.5	11	37	34	12	8	3	84	10	6	16
	5	8	43	30	12	8	5	65	17	17	33
	15	8	51	19	16	5	11	60	12	16	28
	50	25	33	24	13	5	16	26	15	33	48
IGR-OV-1 (Ovary)	0	4	27	29	19	21	4	86	9	4	13
	0.5	3	26	28	20	20	5	89	8	3	10
	5	5	25	29	19	21	7	88	8	3	11
	15	11	21	27	21	20	30	62	18	15	33
	50	NA	NA	NA	NA	NA	68	31	46	16	62
PC-3 (Prostate)	0	4	36	42	13	6	3	92	5	2	7
	0.5	9	33	40	14	4	1	95	3	1	5
	5	9	34	42	13	5	2	92	5	2	7
	15	8	49	33	9	5	3	89	6	3	9
	50	10	35	34	10	11	18	61	24	9	33

In cell cycle analysis, the percentage of cells was estimated for each phase of cell cycle (G0/G1, S and G2/M) according to their DNA content. Cells having less than n chromosomes (<G0/G1) or more than 2n chromosomes (>G2/M) were also reported. The high toxicity of compound **2a** at 50 μM in IGROV-1 cell line prevented correct cell cycle analysis (NA, non applicable). In Caspase-3 assay, the percentage of cells showing cleaved/activated caspase-3 is indicated. In Annexin V/7-AAD assay, the percentage of healthy, early apoptotic and late apoptotic cells was estimated according to their affinity to Annexin V-FITC and 7-AAD intercalating agent.

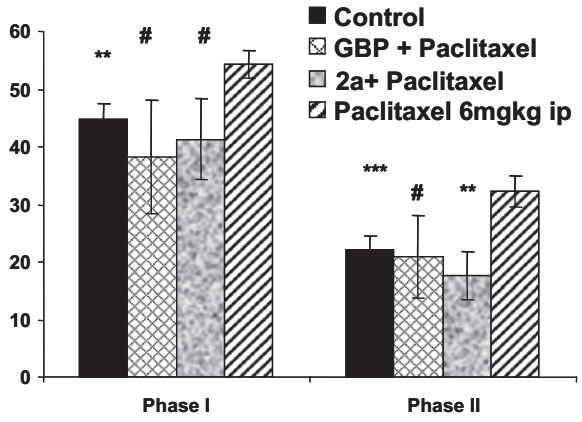


Figure 2. Effects of Orally Administered (po) **2a** at the dose of100 mg/kg on Paclitaxel treated mice in the Formalin test, compared to the reference drug Gabapentin (GBP) at 100 mg/kg (ip). GBP was administered ip since oral administration of the compound exerts no analgesic effect in the formalin test, (data not shown). One way Anova followed by LSD post hoc test confirmed the expected hyperalgia of Paclitaxel treated animals compared to Vehicle treated animals in both time intervals examined ($^{**}p$ <0.01 and $^{***}p$ <0.001, respectively). GBP administration in Paclitaxel pretreated animals was found to exert a mild analgesic effect in both time intervals, as indicated by their lower licking, of the administered paw, times compared to Paclitaxel treated animals ($^{\#}$ p<0.1 in both intervals). Administration of **2a**, however, exerted a stronger analgesic effect in Paclitaxel treated animals. **2a** Paclitaxel treated animals displayed lower licking times compared to Paclitaxel treated animals both at 0–5 min ($^{\#}p = 0.06$) and 35–40 min ($^{**}p$ <0.01) after formalin injection.

4. Experimental

4.1. Binding studies

Binding studies were carried out by CEREP (France) with the σ_1 binding assay performed in triplicate. Affinities of the adamantane piperazines **2** for the σ_1 and σ_2-receptors measured by displacement of [^{3}H](+)pentazocine[43] and [^{3}H]1,3-di-*o*-tolylguanidine,[44] respectively. The σ_1 binding assay was performed, according to Ganapathy et al.,[43] by incubating Jurkat cell membranes (10–20 mg protein per tube) with [^{3}H](+)pentazocine (8 nM) and a range of concentrations of the compounds at 22 °C for 2 h in 5 nM Tris/HCl buffer, pH 7.4. The σ_2 binding assay was performed, according to Bowen et al. (1993),[44] by incubating rat cerebral cortex membranes (10–20 mg protein per tube) with [^{3}H](+)1-DTG (3-di-*o*-tolylguanidine; 5 nM) in the presence of (+)pentazocine (300 nM), to saturate σ_1 site binding, and a range of concentrations of the compounds at 22 °C for 2 h in 5 nM Tris/HCl buffer, pH 7.4. The final assay volume was 0.5 ml. Nonspecific binding was defined, in both assays, as that remaining in the presence of 10 μM haloperidol. Affinities, for site 2 of Na^+ channel were measured by displacement of [^{3}H]batrachotoxin benzoate (^{3}H-BTX-B).[45] Binding reactions were initiated by addition of 150 μl of vesicular preparation containing 150–500 μg protein to a solution in standard incubation buffer of ^{3}H-BTX-B, 50 μg *Leiurus quinquestriatus* scorpion venom and various un labeled effectors as indicated. The concentration of ^{3}H-BTX-B was generally 20–25 nM, and the total assay volume was 335 μl. Standard incubation buffer contained 130 mM choline chloride, 50 mM HEPES buffer adjusted to pH 7.4 with Tris base, 5.5 mM glucose, 0.8 mM $MgSO_4$, 5.4 mM KCl, and 1 mg/ml BSA. Incubations were carried out for 60 min at the indicated temperature and were then terminated by addition of 3 ml ice-cold wash buffer. The tissue was immediately collected on Whatman GF/C glass fiber filters and washed 3 more times with 3 ml cold wash buffer. The wash buffer contained 163 mM choline chloride, 5 mM HEPES (pH 7.4) 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 1 mg/ml BSA. Radioactivity associated with the tissue was determined by liquid-scintillation spectroscopy of the filters suspended in 10 ml scintillation cocktail (3a70B;RPI). Nonspecific binding was determined from parallel incubations containing 250 μM veratridine and has been subtracted from the data. Specific binding measured in this way was nominally 75% of the total binding.

4.2. In vitro anti-proliferative and cytotoxic activity

All human cancer cell lines were obtained from the National Cancer Institute, NIH (Bethesda, MD, USA) with the exception of BX-PC3 and the normal cells Hs888Lu, CCD18Co that were obtained from ATCC, the hMSCs, NHF and HUVECs that were purchased from Lonza. All cell lines were adapted to propagate in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum, 2 mM L-glutamine and antibiotics. The cultures were

grown in a humidified 37 °C-incubator with 5% CO_2 atmosphere. Cell viability was assessed at the beginning of each experiment by the trypan blue dye exclusion method, and was always greater than 95%. Cells were seeded into 96-well microtiter plates in 100 μL of medium at a the corresponding density (3500–30,000 cells per well) and subsequently, the plates were incubated at standard conditions for 24 h to allow the cells to resume exponential growth prior to addition of the agents to be tested. Then in order to measure the cell population, cells in one plate were fixed in situ with trichloroacetic acid (TCA) followed by sulforhodamine B solution (SRB) staining, as described elsewhere.[41–43] To determine the activity, each compound was dissolved in dimethyl sulfoxide (DMSO) and then was added at 10-fold dilutions (from 100 to 0.01 μM) and incubation continued for an additional period of 48 h. The assay was terminated by addition of cold TCA followed by SRB staining and absorbance measurement at 540 nm, in an DAS plate reader, to determine the GI_{50}, that is, the concentration required in the cell culture to inhibit cell growth by 50%, TGI, the concentration that is required to completely inhibit cell growth and the LC_{50}, the concentration that is needed in culture to kill 50% of the cellular population as described.[41–43]

4.3. In vivo antitumor activity

SCID (NOD.CB17 Prkdcscid) mice were purchased from Jackson Laboratories/Charles River Laboratories (L'Arbresle, France). The mouse colony was maintained under restricted flora conditions in a pathogen-free environment in type IIL-cages. Males or females mice, 7–9 weeks old, were injected subcutaneously according to the British practice of bilateral trocar implants at the axillary region. Each inoculum contained 106 cells exponentially growing at the time of harvesting. The mice were subsequently randomly divided into groups of 6 to 20 animals per group when the average tumor volume had reached about 100 mm^3. Treatments started at that point. Tumor volume was calculated as described elsewhere.[43,44] All administrations were intraperitoneal. Treated animals received a single injection daily for 5 days per week throughout the experiments. Tumor volume was measured with a calliper twice per week. In addition to tumor volume, we calculated the parameter, $\%\Delta T/\Delta C$, where $\Delta T = T - Do$ and $\Delta C = C - Do$ (Do is the average tumor volume at the beginning of the treatment; T and C are the volumes of treated and untreated tumors, respectively, at a specified day). Concurrently, we scored the number of tumor-free animals, number of drug-related deaths, and average number of days required to reach a defined tumor volume. Optimal $\Delta T/\Delta C$ value was used as a measure of drug activity. Losses of weight, neurological disorders and behavioral and dietary changes were also recorded as indicators of toxicity (side effects). The experiment was terminated when tumor size in untreated animals reached a volume of about 10–11% of animals' weight.

4.4. In vitro cell cycle modifications and apoptotic activity

Supplying PC-3 (human prostatic adenocarcinoma), BxPC-3 (human pancreatic adenocarcinoma) and IGROV-1 (human ovarian carcinoma) cancer cells, as well as, cell cycle and apoptosis assays were carried out by Oncodesign S.A. (France). Cells were plated 24 h before treatment at the appropriate seeding density in 6-well plates or 25 cm^2 flasks according to the assay. Compound **2a** was dissolved at 10 mM in 100% DMSO then diluted in cascade to obtain concentrations of 0.1, 1 and 3 mM in 100% DMSO. These dilutions were further diluted at 1:10th with RPMI 1640 medium. The last dilution was performed on cells at 1:20th to reach the appropriate concentrations of 0.5, 5, 15 or 50 μM. The final concentration of DMSO was 0.5% in cell culture medium. The treatment period was 24 h. The effect of compound **2a** on cell cycle was evaluated by quantification of propidium iodide (PI) incorporation into genomic DNA. After incubation, cells were detached from the well by trypsinisation, transferred in tubes, washed and resuspended in 500 μl ice cold PBS before being fixed with 1.5 ml dropwise of 100% cold ethanol for 3 h at 4 °C . Then, the cell suspensions were centrifuged at 1,500 rpm for 5 min and pellets were resuspended in a mix of 100 μl of 200 μg/ml RNase and 100 μl of 1 mg/ml PI. Cells were incubated for 45 min at room temperature in the dark. The preparation was centrifuged 5 min at 1,500 rpm then the pellets were resuspended in PBS for FACS analysis.

The apoptotic membrane modifications induced by compound **2a** were evaluated by Annexin V binding at the end of the treatment period. 7-AAD, a fluorescent agent incorporated into DNA, was also used to differentiate early (no membrane disruption) and late (membrane disruption) apoptosis. After incubation, cells were detached from the well by gentle scraping, transferred to FACS tubes and labelled according to Annexin V-FITC/7-AAD Kit (Beckman Coulter). Briefly, after being washed, cells were incubated in ice-cold binding buffer. Then 10 μl of Annexin V-FITC solution and 20 μl of 7-AAD viability dye were added to 100 μl of cells suspension and incubated 15 min on ice in the dark. After incubation, 800 μl of binding buffer were added. Cell preparations were analyzed by FACS within 1 h.

The activation of caspase pathway by compound **2a** was evaluated by measuring the level of activated caspase-3 by FACS. After incubation, cells were detached from the culture flask by trypsinisation, transferred to FACS tubes and labeled according to PE Active Caspase-3 Apoptosis Kit (Pharmingen). Briefly, after being washed, 106 cells were fixed and permeabilized in ice-cold BD Cytofix/CytopermTM buffer for 20 min. Cells were then pelleted and washed with BD Perm/WashTM buffer. Incubation with antibody included in the kit was performed in 100 μl of same buffer completed with 20 μl of antibody for 30 min. Cell preparations were analyzed by FACS within 1 h.

For all assays staining cells were analyzed with a CyFlow® space flow cytometer (Partec) using a 488 nm wavelength laser excitation. The acquisition was stopped after a total of 10,000 FSC/SSC-gated cells were collected for each sample.

4.5. Formalin test

CD1 male mice weighing 34–40 g were used. They were kept in a room maintained at 21–22 °C with free access to standard laboratory diet and tap water. Paclitaxel (Brystol Myers Squibb Company) was diluted in saline and administered at one ip injection (6 mg/kg) on day 0, as adapted from Matsumoto et al.[40] On day 14, 1 h after oral administration (po) of the compound **2a** (100 mg/kg) the formalin test, as a tonic and persistent pain model of nociception, was performed. Injection of formalin into the hind paw is followed by two phases of behavior.[41,42] The first phase consists of intense licking and biting of the injected paw for the first 5 min followed by a period of little activity. The second phase spans from 15 to 40 min after the formalin injection and involves periods of licking and biting of the injected paw. The first phase is thought to be a model of acute chemical pain, whereas the second phase reflects a state of central sensitization driven by the presumed first phase.[41,42] The amount of time spent licking and biting the injected paw and leg was recorded in 5-min intervals for 0–5 and 35–40 min after the formalin injection.

4.6. Statistical analysis

Significant difference in tumor volume was determined by the Student's t-test using the SPSS for Windows (release 11.0.0, SPSS Inc., USA) software package. A difference was considered significant if $p < 0.05$.

4.7. Materials and methods

Melting points were determined using a Büchi capillary apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 833 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker MSL 400 spectrometer using $CDCl_3$ as solvent and TMS as internal standard. Carbon multiplicities were established by DEPT experiments. The 2D NMR experiments (HMQC, COSY and NOESY) were performed for the elucidation of the structures of the new compounds. Microanalyses were carried out by the Service Central de Microanalyse (CNRS) France, and the results obtained had a maximum deviation of ±0.4% from the theoretical value.

4.7.1. General procedure for the preparation of analogues 2a–d and 3a–d

A stirred mixture of α-(1-adamantyl)benzhydryl chloride (**1**)[31,32] (1.7 g, 5 mmol) and 1-substituted piperazine (72 mmol) was heated at 130 °C, under argon, for 12 h. After cooling, water was added and the mixture was extracted with dichloromethane (3 × 50 ml). The combined organic layers were washed with water, dried over sodium sulfate and evaporated in vacuo. The residue was purified by flash column chromatography.

4.7.1.1. 1-Methyl-4-{4-[α-(1-tricyclo[3.3.1.1^{3,7}]decyl)phenyl methyl]phenyl}piperazine (2a). This compound was obtained in 51% yield following the general method. Purification by flash column chromatography using a mixture of Et_2O/MeOH (97:3) as eluent. Mp 171–173 °C (Et_2O); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.50–1.61 (m, 12H, 2,4,6,8,9,10-H), 1.91 (br s, 3H, 3,5,7-H), 2.30 (s, 3H, CH_3), 2.50–2.53 (t, 4H, A_2X_2, J_{AX} ∼4.8 Hz, 2,6-Hp), 3.13–3.16 (t, 4H, A_2X_2, J_{AX} ∼4.8 Hz, 3,5-Hp), 3.39 (s, 1H, α-H), 6.81–6.83 (∼d, 2H, AA′BB′, J_{AB} ∼8.6 Hz, 3,5-Har), 7.11–7.15 (∼t, 1H, 4′-Har), 7.20–7.24 (∼t, 2H, 3′,5′-Har), 7.29–7.32 (∼d, 2H, AA′BB′, $J_{AB} = J_{A'B'}$ = 8.6 Hz, 2,6-Har), 7.38–7.40 (∼d, 2H, 2′,6′-Har). ^{13}C NMR (50 MHz, $CDCl_3$): δ 28.7 (3,5,7-C), 29.5 (1-C), 36.8 (2,8,9-C), 41.0 (4,6,10-C), 46.0 (CH_3), 48.8 (3,5-Cp), 55.0 (2,6-Cp), 65.4 (*a*-C), 115.2 (3,5-Car), 125.6 (4′-Car), 127.6 (2′,6′-Car), 129.8 (3′,5′-Car), 130.5 (2,6-Car), 133.2 (1-Car), 142.5 (4-Car), 149.1 (1-Car). Anal. Calcd for $C_{28}H_{36}N_2$ (%): C, 83.95; H, 9.06; found (%) C, 83.75; H, 9.01. Monohydrochloride, mp 277–279 °C (dec) (acetone–Et_2O); IR ($C_{28}H_{37}ClN_2 \times ½H_2O$, nujol) ν_{max} 3420 cm^{-1}; Anal. Calcd for $C_{28}H_{37}ClN_2 \times ½H_2O$ (%): C, 75.39; H, 8.59; Cl, 7.95; N, 6.28; found (%): C, 75.65; H, 8.29; Cl, 8.03; N, 6.32.

4.7.1.2. 1-Methyl-4-[α,*a*-diphenyl-(1-tricyclo[3.3.1.1^{3,7}] decyl)-methyl]piperazine (3a). Compound **3a** was isolated in 15% yield as a less polar extract during the purification of **2a**. Crystalline solid. Mp 156–158 °C (Et_2O/*n*-pentane); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.48–1.52 (m, 6H, 2,8,9-H), 1.87 (br s, 6H, 4,6,10-H), 1.90 (br s, 3H, 3,5,7-H) 2.42 (s, 3H, CH_3), 2.43 (br s, 4H, 2,6-Hp), 2.80 (br s, 4H, 3,5-Hp), 7.16–7.26 (m, 6H, 2,4,6-Har), 7.54–7.56 (∼d, 4H, *J* ∼7.5 Hz, 3,5 -Har). ^{13}C NMR (100 MHz, $CDCl_3$): δ 29.6 (3,5,7-C), 36.8 (2,8,9-C), 40.0 (4,6,10-C), 43.4 (1-C), 45.8 (CH_3), 50.6 (3,5-Cp), 56.4 (2,6-Cp), 78.8 (*a*-C), 126.0 (4-Car), 126.3 (2,6-Car), 131.7 (3,5-Car), 141.85 (1-Car); Anal. Calcd for $C_{28}H_{36}N_2$ (%): C, 83.95; H, 9.06; N, 6.99; found (%): C, 83.65; H, 9.26; N, 5.04.

4.7.1.3. 1-Ethyl-4-{4-[α-(1-tricyclo[3.3.1.1^{3,7}]decyl)phenylmethyl]phenyl}piperazine (2b). Piperazine **2b** was obtained in 41% yield as a waxy material from the reaction of benzhydryl chloride **1** with 1-ethylpiperazine following the procedure used for **2a**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.04–1.07 (t, 3H, A_3X_2, J_{AX} = 7 Hz, CH_3CH_2), 1.45–1.56 (m, 12H, 2,4,6, 8,9,10 -H), 1.85 (br s, 3H, 3,5,7 -H), 2.37–2.43 (q, 2H, A_3X_2, J_{AX} = 7 Hz, CH_2CH_3), 2.51–2.54 (∼t, 4H, A_2X_2, J_{AX} = 4.5 Hz, 2,6-Hp), 3.10–3.13 (∼t, 4H, A_2X_2, J_{AX} = 4.5 Hz, 3,5-H p), 3.32 (s, 1H, α-H), 6.75–6.77 (∼d, 2H, AA′BB′, $J_{AB} = J_{A'B'}$ = 8.5 Hz, 3,5-Har), 7.06–7.1 (∼t, 1H, 4′-Har), 7.17–7.18 (∼t, 2H, 3′,5′-Har), 7.23–7.25 (∼d, 2H, AA′BB′, $J_{AB} = J_{A'B'}$ = 8.5 Hz, 2,6-Har), 7.32–7.33 (∼d, 2H, 2′,6′-H ar). ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.9 (CH_3CH_2), 28.8 (3,5,7 -C), 29.7 (1-C), 36.9 (2,8,9-C), 41.1 (4,6,10-C), 48.9 (3,5-Cp), 52.4 (CH_2CH_3), 52.9 (2,6-Cp), 66.4 (α-C), 115.3 (3,5-Car), 125.6 (4′-Car), 127.7 (3′,5′-Car), 129.9 (2,6-Car), 130.6 (2′,6′-Car), 133.3 (1-Car), 142.6 (4′-Car), 149.2 (1′-Car). Monohydrochloride, mp 250 °C (EtOH–Et_2O); IR ($C_{29}H_{39}ClN_2 \times ½H_2O$, nujol) ν_{max} 3415 cm^{-1}; Anal. Calcd for $C_{29}H_{39}ClN_2 \times ½H_2O$ (%): C, 75.70; H, 8.76; N, 6.09; found (%): C, 75.88; H, 8.70; N, 5.95.

4.7.1.4. 1-Cyclohexyl-4-{4-[α-(1-tricyclo[3.3.1.1^{3,7}]decyl) phenylmethyl]phenyl}piperazine (2c). Piperazine **2c** was obtained as a waxy material in 31% yield from the reaction of benzhydryl chloride **1** with 1-cyclohexylpiperazine following the procedure used for **2a**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.17–1.25 (m, 11H, 2,8,9-H, 2,3,4,5,6-Hax,cycl), 1.48–1.59 (m, 9H, 4,6,10-H, 3,4,5-Hax,cycl), 1.76 (br s, 2H, 2,6-Heq,cycl), 1.88 (br s, 3H, 3,5,7-H), 2.28 (br s, 1H, 1-Hax,cycl), 2.68–2.70 (t, 4H, A_2X_2, J_{AX} = 4.5 Hz, 2,6-Hp), 3.12–3.14 (t, 4H, A_2X_2, J_{AX} = 4.5 Hz, 3,5-Hp), 3.36 (s, 1H, α-H), 6.78–6.80 (d, 2H, AA′BB′, J_{AB} = 8 Hz, 3,5-Har), 7.07–7.13 (∼t, 1H, 4′-Har), 7.18–7.22 (∼t, 2H, 3′,5′-Har), 7.25–7.27(d, 2H, AA′BB′, $J_{AB} = J_{A'B'}$ = 8 Hz, 2,6-Har), 7.35–7.37 (d, 2H, 2′,6′-Har). ^{13}C NMR (100 MHz, $CDCl_3$): δ 25.8 (3,5-Ccycl), 28.8 (2,6-Ccycl), 28.8 (3,5,7-C), 29.7 (1-C), 36.9 (2,8,9-C), 41.1 (4,6,10-C), 49.0 (3,5-Cp), 49.3 (2,6-Cp), 63.5 (1-Ccycl), 65.4 (α-C), 115.3 (3,5-Car), 125.7 (4′-Car), 127.7 (3′,5′-Car), 129.9 (2,6-Car), 130.6 (2′,6′-Car), 133.2 (1-Car), 142.6 (4-Car), 149.3 (1′-Car). Monohydrochloride, mp 178–181 °C (EtOH–Et_2O); IR ($C_{33}H_{45}ClN_2 \times 1H_2O$, nujol) ν_{max} 3430 cm^{-1}; Anal. Calcd for $C_{33}H_{45}ClN_2 \times 1H_2O$ (%): C, 75.76; H, 9.05; N, 5.35; found (%): C, 75.65; H, 9.26; N, 5.08.

4.7.1.5. 1-Phenylmethyl-4-{4-[α-(1-tricyclo[3.3.1.1^{3,7}]decyl) phenyl methyl]phenyl} piperazine (2d). Benzyl derivative **2d** was prepared from benzhydryl chloride **1** and 1-benzylpiperazine following the procedure used for **2a**. The residue obtained was purified by flash column chromatography using a mixture of ether/*n*-hexane (1:2) as eluent to give the title compound in 29% yield as an off-white solid. Mp 78–80 °C (*n*-pentane); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.51–1.63 (m, 12H, 2,4,6,8,9,10-H), 1.91 (br s, 3H, 3,5,7-H), 2.56–2.58 (t, 4H, A_2X_2, J_{AX} ∼5 Hz, 2,6-Hp), 3.13–3.15 (t, 4H, A_2X_2, J_{AX} ∼5 Hz, 3,5-Hp), 3.38 (s, 1H, *a*-H), 3.54 (s, 2H, PhCH_2), 6.80–6.82 (d, 2H, AA′BB′, $J_{AB} = J_{A'B'}$ = 8.5 Hz, 3,5-Har), 7.06–7.10 (t, 1H, 4′-Har), 7.25–7.32 (complex m, 9H, 2,6,3′,5′, 2″, 3″, 4″, 5″, 6″-Har), 7.38–7.40 (d, 2H, 2′,6′-Har). ^{13}C NMR (100 MHz, $CDCl_3$): δ 28.8 (3,5,7-C), 29.9 (1-C), 36.9 (2,8,9-C), 41.1 (4,6,10-C), 49.0 (3,5-Cp), 53.2 (2,6-Cp), 63.0 (PhCH_2), 65.4 (*a*-C), 115.3 (3,5-C), 125.7 (4′-C), 127.1 (4″-Car), 128.20 (3″,5″-Car), 129.2 (2″,6″-Car), 129.9 (3′,5′-Car), 130.6 (2,6-Car), 133.2 (1-Car), 138.1 (1″-Car), 142.6 (4-Car), 149.3 (1′-Car). Monohydrochloride, mp 241–242 °C (EtOH–Et_2O); Anal. Calcd for $C_{34}H_{41}ClN_2 \times H_2O$ (%): C, 75.76; H, 9.05; N, 5.35; found (%) C, 75.65; H, 9.26; N, 5.08. Monopicrate, mp 194–196 °C (acetone–Et_2O); Anal. Calcd for $C_{40}H_{43}N_5O_7 \times CH_3OH$ (%): C, 66.74; H, 6.42; N, 9.49; found (%): C, 66.41, H, 6.06; N, 9.34.

4.7.1.6. 1-Phenylmethyl-4-[α,*a*-diphenyl-(1-tricyclo[3.3.1.1^{3,7}]-decyl)methyl]piperazine (3d). Compound **3d** was isolated in 20% yield as a less polar extract during the purification of **2d**. Off-white solid. Mp 126–128 °C (ether/*n*-pentane); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.48–1.62 (m, 6H, 2,8,9-H), 1.88 (br s, 6H, 4,6,10-H), 1.91 (br s, 3H, 3,5,7 -H), 2.46 (br s, 4H, 2,6-Hp), 2.77 (br s, 4H, 3,5-Hp), 3.49 (s, 2H, PhCH_2), 7.23–7.89 (m, 15H, Har). ^{13}C NMR (100 MHz, $CDCl_3$): δ 29.6 (3,5,7-C), 36.9 (2,8,9-C), 40.0

(4,6,10-C), 43.4 (1-C), 50.8 (3,5-Cp), 54.6 (2,6-Cp), 63.4 ($PhCH_2$), 71.1 (*a*-C), 125.9, 126.2, 128.1, 129.3, 131.8, 132.4 (2,3,4,5,6,2′,3′,4′,5′,6′ -Car), 140.0 (1′-Car), 141.8 (1-Car); Anal. Calcd for $C_{33}H_{38}N_2$ (%): C, 85.67; H, 8.28; N, 6.05; found (%): C, 85.65; H, 8.26; N, 6.04.

4.7.2. 1-{4-[α-(1-Tricyclo[3.3.1.1^{3,7}]decyl)phenylmethyl]-phenyl}piperazine (2e)

To a stirred suspension of *N*-benzyl derivative **2d** (950 mg, 2 mmol) and 10% palladium on charcoal (950 mg) in methanol (20 mL), ammonium formate (630 mg, 10 mmol) was added all at once. The reaction mixture was refluxed under argon for 3 h and after cooling the catalyst was removed by filtration and washed with chloroform (20 mL). The filtrate was concentrated in vacuo and the residue was purified by flash column chromatography using a mixture of DCM/MeOH (9:1) as eluent. Piperazine derivative **2e** was obtained in 74% yield as a white solid. Mp 145–147 °C (Et_2O); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.48–1.56 (m, 12H, 2,4,6,8,9,10-H), 1.78 (br s, 1H, NH), 1.85 (br s, 3H, 3,5,7-H), 2.93–2.95 (m, 4H, 3,5-Hp), 3.02–3.04 (m, 4H, 2,6-Hp), 3.33 (s, 1H, α-H), 6.75–6.77 (∼d, 2H, AA′BB′, $J_{AB} = J_{A'B'} = 8.9$ Hz, 3,5-Har), 7.06–7.10 (m, 1H, 4′-Har), 7.15–7.19 (m, 2H, 3′,5′-Har), 7.23–7.25 (∼d, 2H, AA′BB′, $J_{AB} = J_{A'B'}$ =8.9 Hz, 2,6-Har), 7.32–7.34 (∼d, 2H, 2′,6′-Har). ^{13}C NMR (100 MHz, $CDCl_3$): δ 28.8 (3,5,7-C), 36.9 (2,8,9-C), 41.1 (4,6,10-C), 46.2 (3,5-Cp), 50.3 (2,6-Cp), 52.8 (1-C), 65.5 (α-C), 115.4 (3,5-Car), 125.8 (4′-Car), 127.8 (3′,5′-Car), 129.9 (2,6-Car), 130.6 (2′,6′-Car), 133.4 (1-Car), 142.6 (4-Car), 149.8 (1′-Car). Dihydrochloride, mp 215–216 °C (dec) (EtOH–Et_2O); Anal. Calcd for $C_{27}H_{36}Cl_2N_2$ (%): C, 70.58; H, 7.90; found (%): C, 70.19; H, 8.09.

4.7.3. 4-{4-[α-(1-Tricyclo[3.3.1.1^{3,7}]decyl)phenylmethyl]phenyl} morpholine (4)

Morpholine derivative **4** was prepared following the procedure used for piperazine **2a**. The residue obtained was purified by flash column chromatography using a mixture of Et_2O/*n*-hexane (1:2) as eluent to give the title compound as a crystalline solid in 60% yield. Mp 147–149 °C (Et_2O); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.45–1.56 (m, 12H, 2,4,6,8,9,10-H), 1.85 (br s, 3H, 3,5,7-H), 3.02–3.05 (t, 4H, A_2X_2, J_{AX} ∼5 Hz, 3,5-Hm), 3.33 (s, 1H, α-H), 3.75–3.77 (t, 4H, A_2X_2, J_{AX} ∼5 Hz, 2,6-Hm), 6.73–6.75 (∼d, 2H, AA′BB′, $J_{AB} = J_{A'B'} = 9.0$ Hz, 3,5-Har), 7.07–7.09 (m, 1H, 4′-Har), 7.10–7.19 (m, 2H, 3′,5′-Har), 7.24–7.27 (∼d, 2H, AA′BB′, $J_{AB} = J_{A'B'} = 9.0$ Hz, 3,5- Har), 7.31–7.34 (∼d, 2H, 2′,6′-Har). ^{13}C NMR (100 MHz, $CDCl_3$): δ 28.8 (3,5,7-C), 36.9 (2,8,9-C), 38.8 (1-C), 41.1 (4,6,10-C), 49.3 (3,5- Cm), 65.4 (*a*-C), 66.9 (2,6-Cm), 115.1 (3,5-Car), 125.8 (4′-Car), 127.8 (3′,5′-Car), 129.9 (2,6-Car), 130.7 (2′,6′-Car), 133.8 (1-Car), 142.6 (4-Car), 149.2 (1′-Car). Fumarate, mp 178–181 °C (EtOH–Et_2O); Anal. Calcd for $C_{31}H_{37}NO_5$ (%): C, 83.67; H, 8.58; found (%): C, 83.43; H, 8.62.

4.7.4. 4-[*a*,α-Diphenyl-(1-tricyclo[3.3.1.1^{3,7}]decyl)methyl]morpholine (5)

Compound **6** was isolated in 13% yield as a less polar extract during the purification of **5**. Crystalline solid. Mp 137–139 °C (Et_2O/*n*-pentane); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.45–1.54 (m, 6H, 2,8,9-H), 1.80–1.86 (m, 9H, 3,4,5,6,7,10-H), 2.66–2.68 (t, 4H, A_2X_2, $J_{AX} = 4$ Hz, 3,5-Hm), 3.61–3.64 (t, 4H, A_2X_2, $J_{AX} = 4$ Hz, 2,6-Hm), 7.13–7.23 (m, 6H, 2,4,6-Har), 7.47–7.49 (∼d, 4H, J ∼9 Hz, 3,5-Har). ^{13}C NMR (100 MHz, $CDCl_3$): δ 29.3 (3,5,7-C), 36.6 (2,8,9-C), 39.9 (4,6,10-C), 42.9 (1-C), 51.9 (3,5-Cm), 68.1 (2,6-Cm), 70.9 (α-C), 125.9 (4-Car), 126.1 (2,6-Car), 131.9 (3,5-Car), 140.82 (1-Car); Anal. Calcd for $C_{27}H_{33}NO$ (%): C, 83.68; H, 8.58; N, 3.61; found (%): C, 83.65; H, 8.68; N, 3.64.

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