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4-Biphenyl and 2-naphthyl substituted 6,7-dimethoxytetrahydroisoquinoline derivatives as potent P-gp modulators

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Abstract—Starting from lead compound **1** (EC_{50} = 1.64 μM), its non-basic nucleus has been conformationally restricted by 4-biphenyl and 2-naphthyl moieties. In each series we investigated if the presence of H-bond donor or acceptor substituents, the basicity and the lipophilicity (c log *P*) were correlated with the P-gp inhibiting activity of tested compounds. In the biphenyl series, derivative **4d** displayed the best results (EC_{50} = 0.05 μM). The corresponding amide **3d** was found less active (EC_{50} = 3.5 μM) ascertaining the importance of basicity in this series whilst the presence of hydroxy or methoxy substituents seems to be negligible. In the naphthyl series, both the basicity and the presence of H-bond donor or acceptor groups seem to be negligible. Moreover, the lipophilicity did not influence the P-gp inhibition activity of each series. Specific biological assays have been carried out to establish the P-gp interacting mechanism of tested compounds discriminating between substrates and inhibitors. Moreover, compound **4d** displayed a potent P-gp inhibition activity with good selectivity towards BCRP pump.

1. Introduction

Drug resistance is an obstacle that limits the efficacy of chemotherapeutic treatment. Some tumours are intrinsically resistant to pharmacological therapy, while others, initially sensitive to chemotherapy, become resistant during the treatment. Resistance to anticancer drugs is due to the contribution of several factors such as pharmacokinetic, tumour micro-environmental or cancer cell-specific factors.[1]

The acquisition of resistance to anticancer drugs differing in chemical structure and mechanism of action is defined MultiDrug Resistance (MDR). MDR decreases cellular accumulation of drugs causing their efflux out of cells by several ATP-dependent efflux pumps, known as ATP binding cassette (ABC) family, overexpressed in tumour cells.[2] Among ABC transporters, MRPs (1–6), P-glycoprotein (P-gp) and Breast Cancer Resistant Protein (BCRP) are mainly involved in MDR.[3]

Several strategies were suggested for reversing MDR and among them the co-administration of an efflux pump inhibitors was the most probed, although such reversal agents might actually increase the side effects of chemotherapy by blocking physiological anticancer drug efflux from normal cells.[4–7]

Many drugs have been tested for modulating the P-gp activity and among them the calcium channel blocker Verapamil[8] and the antisteroid Tamoxifen,[9] termed first generation P-gp inhibitors. Unfortunately, compounds belonging to this class displayed side effects such as interferences with several enzyme systems resulting in unpredictable pharmacokinetic interaction.

Then, compounds more potent and less toxic than first generation drugs, such as Valspodar[10] and Biricodar, have been developed.[11] These compounds, claimed modulators of second generation, although more potent and less toxic with respect to drugs of first generation,

Keywords: P-glycoprotein; BCRP; 4-Biphenyl-6,7-dimethoxytetrahydroisoquinoline; 2-Naphthyl-6,7-dimethoxytetrahydroisoquinoline.
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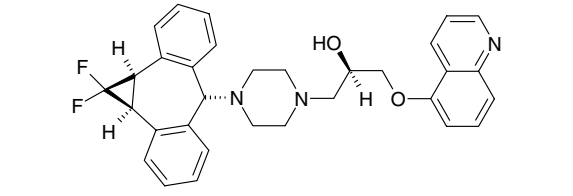
evidenced some limits because significantly inhibited the metabolism and excretion of cytotoxic agents.[12]

The most studied third generation P-gp modulators are Zosuquidar,[13] Elacridar[14,15] and Tariquidar[16–18] (Chart 1). These inhibitors displayed no pharmacokinetic interaction with chemotherapeutic drugs and showed high potency and specificity for P-gp. Although these compounds are in different phases of clinical trials, preliminary results are moderately satisfactory.[19]

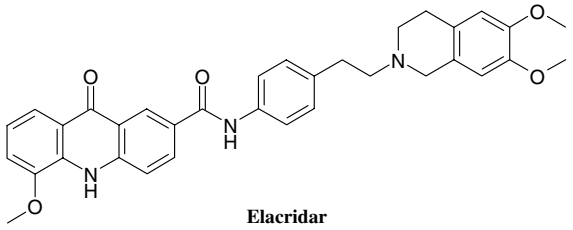
Starting from PB28, a sigma-2 receptor agonist displaying also a good P-gp modulating activity[20] (EC_{50} = 0.55 μM), we developed compound **1** as P-gp inhibitor[21] bearing two structural requirements: 6,7-dimethoxytetrahydroisoquinoline, the same of Elacridar and Tariquidar as basic moiety and alkyltetraline nucleus as non-basic fragment, the same of PB28 (Chart 2).

Although this compound displayed moderate P-gp inhibition activity (EC_{50} = 1.64 μM), SAR studies indicated that the conformational restriction of non-basic moiety is a requirement for improving P-gp inhibition activity. In order to restrict the conformation of non-basic nucleus, two different fragments, biphenyl and 2-naphthyl moieties, as depicted in Chart 2, were inserted.

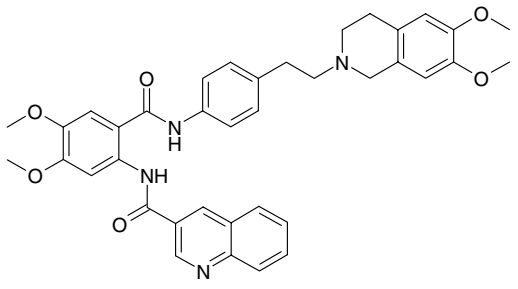
Moreover, P-gp interacting mechanism of tested compounds was investigated by combining three biological assays: Apparent Permeability (P_{app}) determination, [^{3}H]vinblastine transport inhibition and ATP-ase activation. From these assays can be hypothesised three P-gp interacting profiles: substrate, inhibitor, and non-transported substrate.[22,23] We summarized these categories in Table 1 considering the response to the three biological assays above mentioned. In Category I unambiguous substrates, non-substrates and inhibitors are reported. In Category II are reported substrates differing for the response in one of the three biological assays.



Zosuquidar



Elacridar



Tariquidar

Chart 1. Third generation P-gp inhibitors.

In order to better evaluate the profile of P-gp inhibitors, we preliminary determined both cytotoxic and antiproliferative effects for compounds **4c** and **4d**. The reason of this investigation is related to the following assay where we evaluated the antiproliferative effect of the chemotherapeutic agent in combination with our compounds in resistant tumour cells.

Moreover, for compound **4d** the selectivity towards BCRP transporter has been also investigated to establish the selectivity to inhibit P-gp pump because in Caco-2 cells also this pump is highly expressed.[24]

2. Chemistry

The preparation of amides and amines of biphenyl and 2-naphthyl series are reported in Scheme 1. The amides of biphenyl series **3a** and **3c–e** were synthesized by reaction between 6,7-dimethoxytetrahydroisoquinoline and the acylchlorides of the corresponding carboxylic acids **2a** and **2c–e**.[25,26] The obtained compounds were reduced to the corresponding amines **4a** and **4c–e** by $LiAlH_4$. Amine **4b** was prepared by alkylating the basic tetrahydroisoquinoline nucleus with bromoderivative **2b**. The final compounds of 2-naphthyl series **7f–h** were prepared by condensing appropriate 2-naphthyl carboxilic acids **5f–h** with isoquinoline moiety in the presence of carbonyldiimidazole.[27] By reducing amides **6f–h** with $LiAlH_4$ were obtained final compounds **7f–h**.

3. Results and discussion

3.1. Biphenyl derivatives 3c–e and 4a–e

The biphenyl (Table 2) and 2-naphthyl (Table 3) derivatives have been investigated for their P-gp inhibiting activity and selectivity towards BCRP transporter. The importance of the biphenyl linkage position (from 2 to 4 position, compounds **4a–c**) has been investigated.

The 4-biphenyl derivative **4c** displayed the best activity (EC_{50} = 0.10 μM) while 3-biphenyl **4b** and 2-biphenyl **4a** derivatives showed a strong decrease in P-gp inhibition activity (EC_{50} = 6.5 μM and 15 μM, respectively).

Moreover, the influence of hydroxy or methoxy substituent in 4′-position (compounds **4d** and **4e**, respectively) was studied in order to investigate the effect of hydrogen-bond donor or acceptor. Unfortunately, the methoxy derivative **4e** presented poor solubility in the experimental conditions so that it was not tested in the three biological assays.

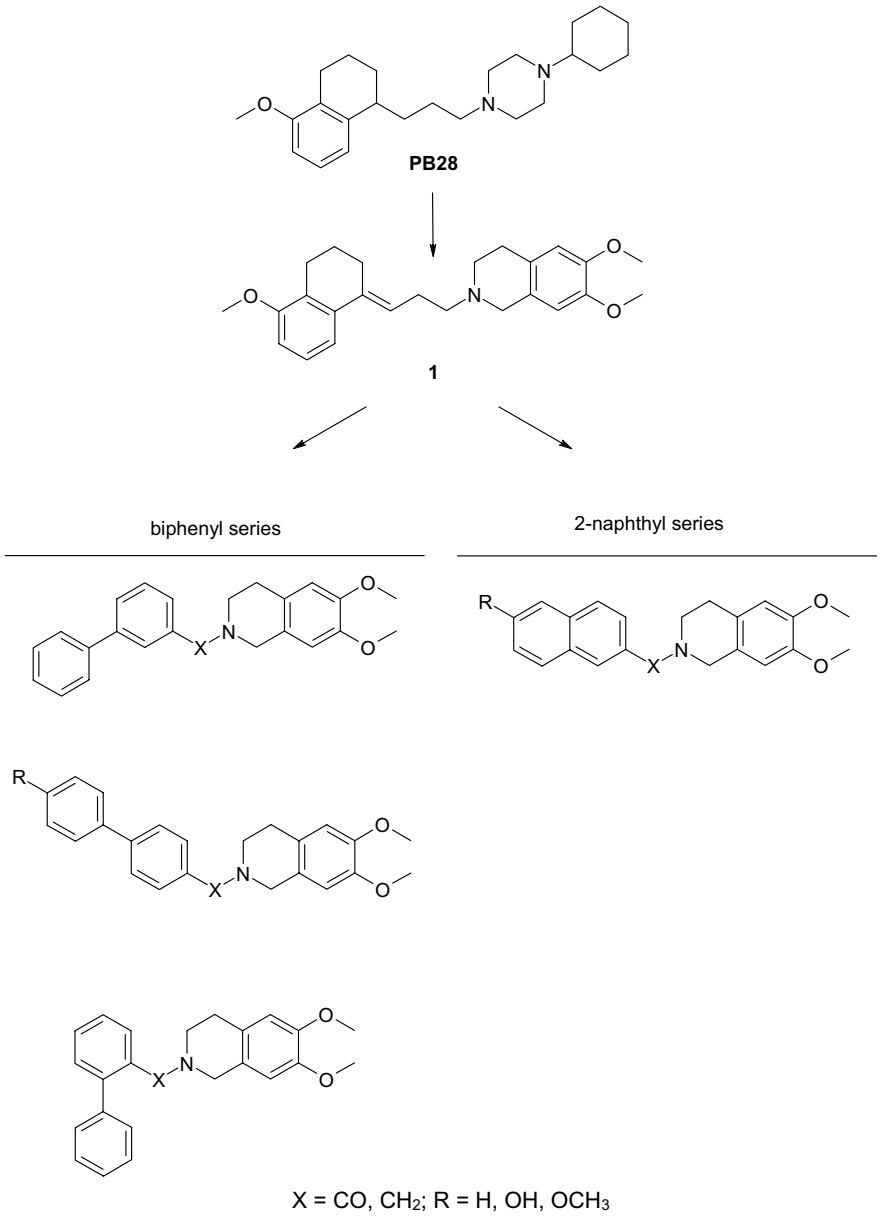


Chart 2. Strategy of lead compound modification.

The 4′-biphenyl-hydroxy derivative **4d** displayed a potent P-gp inhibition activity but with respect to the unsubstituted derivative **4c** lower difference in P-gp inhibition activity has been observed (EC_{50} = 0.05 μM vs 0.10 μM).

In order to establish the influence of the basicity for the P-gp inhibiting activity, compounds **3c**–**e** were prepared. Amides **3c** and **3d** were less active than the corresponding amines **4c** and **4d** (EC_{50} = 28 μM vs 0.10 μM and 3.5 μM vs 0.05 μM, respectively). Amide **3e** was not tested because of its poor solubility in the experimental conditions.

Regarding the mechanism involved in P-gp–drug interaction, three biological assays, reported in Table 2, have been carried out.

3.2. P-gp interacting mechanism of biphenyl derivatives 3c–e and 4a–e

The [^{3}H]vinblastine transport inhibition defined the competition for the P-gp site between the radiolabeled vinblastine (known P-gp substrate) and tested compound. Moreover, ATP cell depletion is linked to P-gp activity so that P-gp transported compounds decreased ATP in the cell. Finally, for each compound the Apparent Permeability (P_{app}) in Caco-2 cell monolayer has been determined both considering the flux from basolater to apical versus (B–A) and from apical to basolateral versus (A–B). Compounds displaying BA/AB ratio $\leqslant$2 are considered non-transported compounds whilst derivatives with BA/AB ratio >2 are effluxed by P-gp.

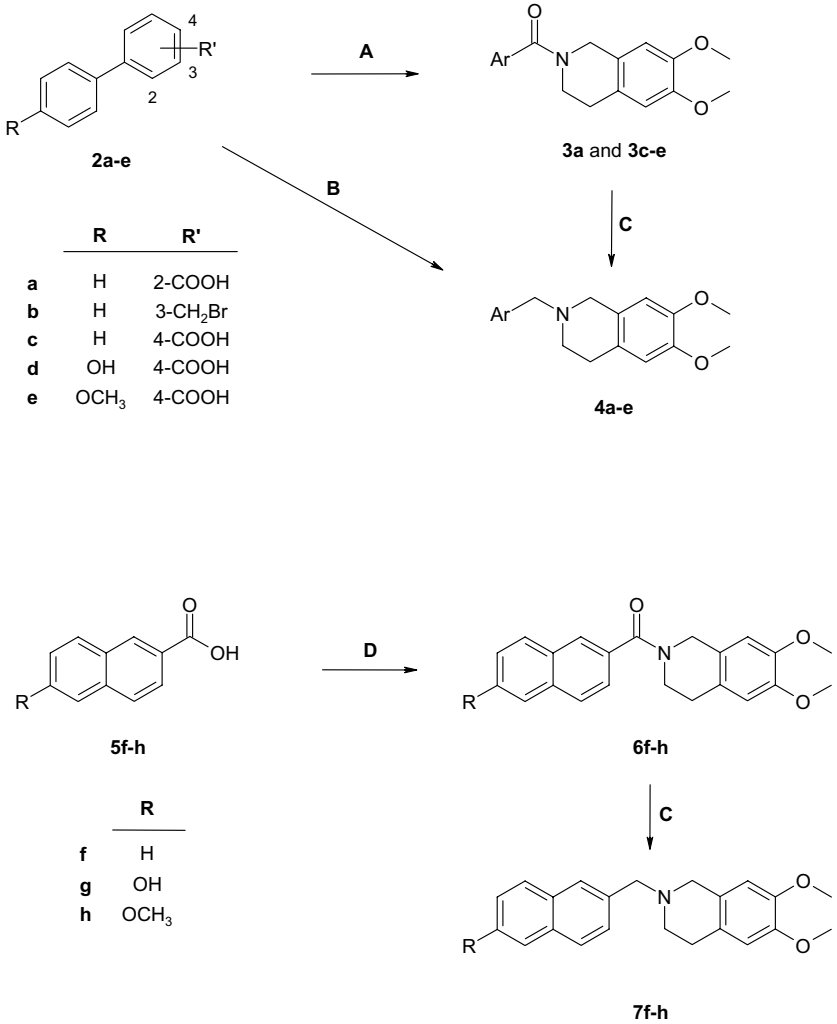
Table 1. Categories of P-gp ligands

	Biological assays in Caco-2 cell line		
	[^{3}H]Substrate transport inhibition	ATP-ase activation	Monolayer efflux
Category I			
Unambiguous non-substrates	No	No	No
Unambiguous substrates	Yes	Yes	Yes
Inhibitors Elacridar	Yes	No	No
Category II			
Non-trans. substrates (IIA) Verapamil	Yes	Yes	No
Transported substrates (IIB_1)	No	No	Yes
Transported substrates (IIB_2)	No	Yes	Yes
Transported substrates (IIB_3) Cyclosporin A	Yes	No	Yes

Compounds **4c** and **4d** displayed BA/AB ratio <2 (1.8 and 1.3, respectively) and were unable to activate ATP-ase so that they could be considered P-gp inhibitors Elacridar-like. Moreover, amide **3c** was an unambiguous P-gp substrate because it activated ATP-ase and had BA/AB ratio >2. The other compounds **3d**, **4a**, **4b** were unable to activate ATP-ase and displayed BA/AB ratio >2 so that they could be termed P-gp transported substrates Cyclosporin A-like.

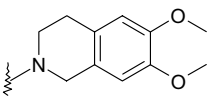
3.3. Naphthyl derivatives 6f–h and 7f–7h

In the 2-naphthyl series, amines **7f–h** were found more potent than the corresponding amides **6f–h** as displayed comparing **6f–7f** (EC_{50} = 20 μM vs 0.80 μM), **6g–7g** (EC_{50} = 0.65 μM vs 0.45 μM), and **6h–7h** (EC_{50} = 18 μM vs 0.30 μM). Also in this series the influence of hydrogen-bond donor or acceptor group was investigated. Comparing the unsubstituted amine **7f** (EC_{50} = 0.80 μM) to 6-hydroxy- or 6-methoxy-substituted derivatives (**7g** and **7h**, respectively) little differences were found



Scheme 1. Reagents: $SOCl_2$ and Et_3N; CH_2Cl_2 and 1.2% NaOH; 6,7-dimethoxytetrahydroisoquinoline (A); Na_2CO_3, DMF and 6,7-dimethoxytetrahydroisoquinoline for the preparation of compound **4b** (B); $LiAlH_4$, THF (C); carbonyldiimidazole, THF and 6,7-dimethoxytetrahydroisoquinoline (D).

Table 2. Biological P-gp modulating assays of biphenyl derivatives



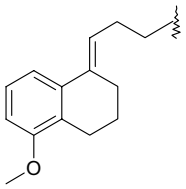
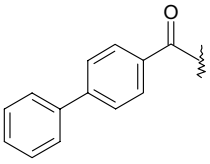
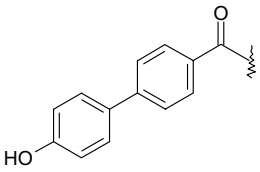
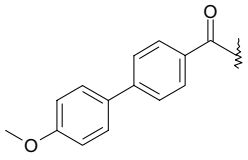
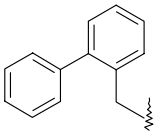
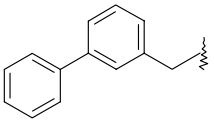
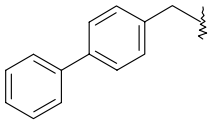
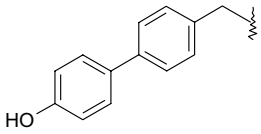
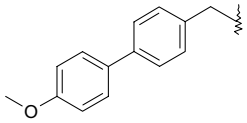
Compound	[^{3}H]Vinblastine transport inhibition EC_{50} ± SEM[a] (μM)	ATP-ase activation (%)[b]	P_{app} B–A[c] (nm/s)	P_{app} A–B[c] (nm/s)	BA/AB
1 	1.64 ± 0.20	N	386	237	1.6
3c 	28 ± 2.0	Y (35%)	2441	601	4
3d 	3.5 ± 0.40	N	2769	280	9.9
3e 	NT	NT	NT	NT	ND
4a 	15 ± 2.5	N	1971	216	9.1
4b 	6.5 ± 0.30	N	3659	301	12

Table 2 (*continued*)

Compound	[^{3}H]Vinblastine transport inhibition EC_{50} ± SEM[a] (μM)	ATP-ase activation (%)[b]	P_{app} B–A[c] (nm/s)	P_{app} A–B[c] (nm/s)	BA/AB
4c 	0.10 ± 0.025	N	1409	777	1.8
4d 	0.05 ± 0.001	N	609	476	1.3
4e 	NT	NT	NT	NT	ND
Elacridar	2.0[d]	N[d]			<2[d]
Verapamil	20[d]	Y (52%)[d]	687[d]	591[d]	1.2[d]
Cyclosporin A	80[d]	N[d]	153[d]	15.9[d]	9.6[d]

[a] Data are means of three-independent determinations, samples in triplicate.
[b] Effect measured at 100 μM. Data are means of three-independent determinations with samples in triplicate.
[c] Data are means of three-independent determinations (samples in triplicate).
[d] See Refs. 21,22.

(EC_{50} = 0.45 μM and 0.30 μM, respectively). In contrast, the presence of hydroxy group on amide **6g** (EC_{50} = 0.65 μM) strongly improved P-gp inhibition activity with respect to unsubstituted **6f** (EC_{50} = 20 μM) and methoxy-substituted **6h** (EC_{50} = 18 μM) derivatives. It is not possible to assert that the basicity is a requirement discriminating P-gp inhibition activity of tested compounds. In fact, amide **6g** displayed an inhibition activity similar to the best amines of this series.

3.4. P-gp interacting mechanism of naphthyl derivatives 6f–h and 7f–h

Regarding P-gp interacting mechanism, all derivatives displayed BA/AB ratio >2. Moreover, compounds **6f**, **6g** and **7g** could be defined as unambiguous P-gp substrates because of their ability to activate ATP-ase. Differently, **6h**, **7f** and **7h** were unable to activate ATP-ase so that they could be termed transported substrates Cyclosporin A-like.

4. Cytotoxic and antiproliferative effects

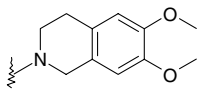
Among studied compounds, only biphenyl derivatives **4c** and **4d** could be defined P-gp inhibitors while all the other compounds could be considered P-gp modulators.

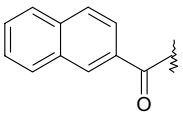
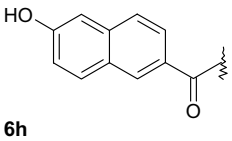
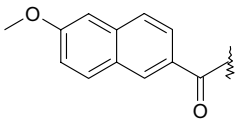
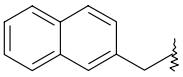
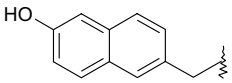
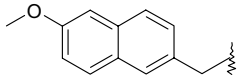
Compounds **4c** and **4d** were tested for evaluating their antiproliferative effect at 24 and 48 h and cytotoxic effect at 24 h in MCF-7/Adr cell line. The cytotoxicity was not determined at 48 h because at this time the antiproliferative effect could cause a reduction of total number of cells so that the cytotoxicity in the assay could be underestimated.

The results displayed that at 24 h compounds **4c** and **4d** were found not active in both assays while compound **4c** showed moderate antiproliferative effect at 48 h (40%, data not shown). The results of antiproliferative assay led us to exclude compound **4c** from further investigations. For this reason compound **4d** has been studied in biological assays where the antiproliferative activity of chemotherapeutic agent is evaluated both in the absence and in the presence of the P-gp inhibitor. These assays are carried out using a cell line displaying resistance towards the chemotherapeutic agent. As depicted in Figure 1, the antiproliferative effect at 48 h of compound **4d**, in MCF-7/Adr cell line, was negligible at 2 μM (Fig. 1D) and at 20 μM (Fig. 1F).

Starting from this finding, compound **4d** at 2 μM and at 20 μM has been incubated with 5 μM Doxorubicin (Fig. 1E and G, respectively). After wash out, Doxorubicin was added for 1 day. The antiproliferative effect of Doxorubicin after pre-treatment with compound **4d** (2 μM) increased from 28% to 42% (Fig. 1D vs E). After

Table 3. Biological P-gp modulating assays of 2-naphthyl derivatives



Compound	[^{3}H]Vinblastine transport inhibition EC_{50} ± SEM (μM)[a]	ATP-ase activation (%)[b]	P_{app} B–A[c] (nm/s)	P_{app} A–B[c] (nm/s)	BA/AB
6f 	20 ± 4.0	Y 10%	4150	324	13
6g 	0.65 ± 0.05	Y (40%)	1900	251	7.6
6h 	18 ± 1.5	N	1234	193	6.4
7f 	0.80 ± 0.02	N	1797	216	8.3
7g 	0.45 ± 0.02	Y (10%)	2351	353	6.7
7h 	0.30 ± 0.04	N	677	187	3.6

[a] Data are means of three-independent determinations, samples in triplicate.
[b] Effect measured at 100 μM. Data are means of three-independent determinations with samples in triplicate.
[c] Data are means of three-independent determinations (samples in triplicate).

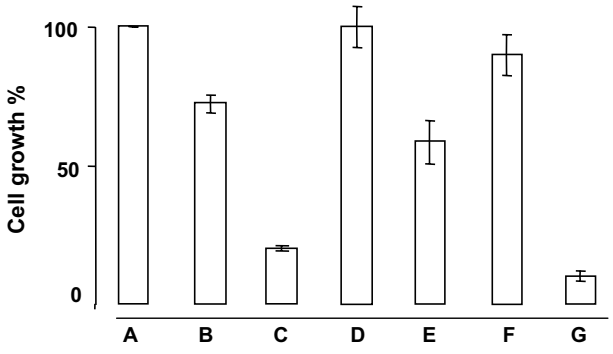


Figure 1. Antiproliferative activity of Doxorubicin (5 μM) in MCF-7/Adr cell line in the absence and in the presence of 2 μM and 20 μM **4d** or 20 μM Verapamil. (A) control (3 days); (B) Doxorubicin (3 days); (C) 20 μM Verapamil and Doxorubicin (2 days). After wash out Doxorubicin (1 day); (D) 2 μM **4d**; (E) 2 μM **4d** and Doxorubicin (2 days). After wash out Doxorubicin (1 day); (F) 20 μM **4d**; (G) 20 μM **4d** and Doxorubicin (2 days). After wash out Doxorubicin (1 day).

pre-treatment with 20 μM of compound **4d**, this effect markedly increased from 28% to 90% (Fig. 1F vs G).

In the same experimental condition, 20 μM Verapamil, improved Doxorubicin-induced cell growth inhibition from 28% to 75% (Fig. 1C). At 2 μM, Verapamil alone and in drug combination displayed poor activity (data not shown).

Comparing compound **4d** and Verapamil at 20 μM the same maximal effect has been obtained. This result seems to be an apparent discrepancy considering their P-gp inhibition values reported in Table 2 (EC_{50} = 0.05 μM and 20 μM, respectively). This finding could be explained considering that the biological assays have been carried out in different cell lines: Caco-2 cells for the permeability assay and MCF-7/Adr cells for the cytotoxicity assay. In Caco-2 cells it was reported a simultaneous overexpression of P-gp and BCRP transporters and the efflux activity of BCRP pump is recognized by [^{3}H]mitoxantrone.

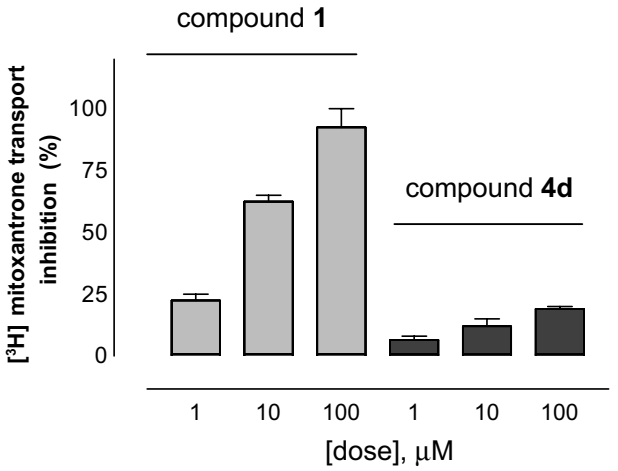


Figure 2. [^{3}H]Mitoxantrone (20 nM) transport inhibition in Caco-2 cells in the presence of compounds **1** or **4d**.

The effect on BCRP pump activity of the lead compound **1** and biphenyl compound **4d** has been evaluated. The results displayed that compound **1** inhibited BCRP pump in dose-dependent manner with maximal effect at 100 μM as depicted in Figure 2. In contrast, compound **4d** was unable to interact with BCRP pump (19% of inhibition at 100 μM). This finding could suggest that the fragment linked to 6,7-dimethoxytetrahydroisoquinoline nucleus is a potential requirement not only for the P-gp inhibition potency but also for the selectivity towards BCRP pump.

This finding is consistent with Elacridar clinical trials results where this compound was found as a potent BCRP modulating agent.[24]

5. Lipophilicity effect on P-gp inhibition activity

In order to verify a potential correlation between lipophylic properties of compounds (clog *P*) and their activities (pEC_{50}), we plotted these values for each series as reported in Figure 3. The results displayed that in both series there is not a correlation between activities and lipophilicity.

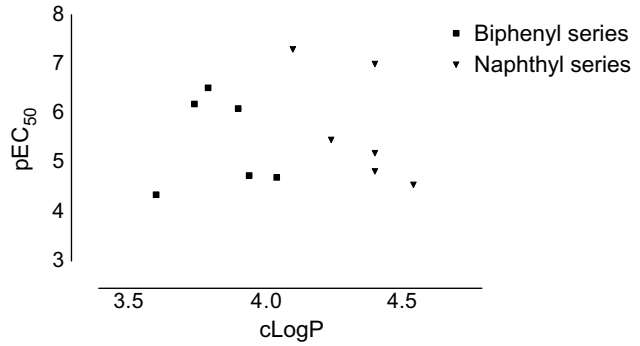


Figure 3. Correlation between lipophilicity and P-gp inhibition activity of tested compounds.

6. Conclusions

In present work two series of compounds bearing a simplified fragment linked to the basic nucleus have been evaluated.

Several elements such as the basicity, the lipophilicity, the presence of H-bond donor or acceptor groups have been investigated in both series. The basicity seems to be an important requirement in 4′-biphenyl series because amides are lower active than the corresponding amines. In this series the presence of hydroxy or methoxy substituents seems to be negligible.

Different conclusions could be carried out for the basicity in naphthyl series. In fact in this series amines and amides displayed little differences in P-gp activity. Moreover, the presence of H-bond donor or acceptor groups also in this series seems to be negligible. Furthermore, the lipophilicity did not influence the P-gp inhibition activity of each tested series.

Among studied compounds, **4d** displayed a potent P-gp inhibition activity with good selectivity towards BCRP pump.

In order to better establish for each series the effect of lipophilicity, basicity and H-bond on no-basic fragment and the selectivity towards other ABC transporters, other compounds could be evaluated.

7. Experimental

7.1. Biological method

7.1.1. Cell lines. The breast cancer cell line of human origin, MCF-7/Adr (resistant to Adriamycin or Doxorubicin), was routinely cultured in RPMI 1640 supplemented with 10% foetal 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. Caco-2 cells were grown in DMEM medium with 10% heat-inactivated foetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine.

7.1.2. Permeability experiments

7.1.2.1. Preparation of Caco-2 monolayer. This procedure has been previously reported.[28] Briefly, Caco-2 cells were harvested with trypsin–EDTA and seeded onto MultiScreen Caco-2 assay system at a density of 10,000 cells/well. The culture medium was replaced every 48 h for the first 6 days and every 24 h thereafter, and after 21 days in culture, the Caco-2 monolayer was utilized for the permeability experiments. The transepitelial electrical resistance (TEER) of the monolayers was measured daily before and after the experiment using a epithelial voltohommeter (Millicell®-ERS; Millipore, Billerica, MA). Generally, TEER values obtained are greater than 1000 Ω for a 21-day culture.

7.1.2.2. Drug transport experiment. Apical to basolateral (P_{app} A–B) and basolateral to apical (P_{app} B–A) permeability of drugs were measured at 120 min and

at various drugs concentrations (1–100 μM).[29] Drugs were dissolved in Hank's balanced salt solution (HBSS, pH 7.4) and sterile filtered. After 21 days of cell growth, the medium was removed from filter wells and from the receiver plate. The filter wells were filled with 75 μL of fresh HBSS buffer and the receiver plate with 250 μL per well of the same buffer. This procedure was repeated twice, and the plates were incubated at 37 °C for 30 min. After incubation time, the HBSS buffer was removed and drug solutions added to the filter well (75 μL). HBSS without the drug was added to the receiver plate (250 μL). The plates were incubated at 37 °C for 120 min. After incubation time, samples were removed from the apical (filter well) and basolateral (receiver plate) side of the monolayer and then were stored in a freezer (−20 °C) pending analysis.

The concentration of compounds was analyzed using UV–vis spectroscopy.

The apparent permeability (P_{app}), in units of nm/s, was calculated using the following equation:

$$P_{app} = \left(\frac{V_A}{\text{area} \times \text{time}}\right) \times \left(\frac{[\text{drug}]_{\text{acceptor}}}{[\text{drug}]_{\text{initial}}}\right)$$

where V_A is the volume (in mL) in the acceptor well; area is the surface area of the membrane (0.11 cm^2 of the well); time is the total transport time in seconds (7200 s); $[drug]_{acceptor}$ is the concentration of the drug measured by UV spectroscopy; $[drug]_{initial}$ is the initial drug concentration (1×10^{-4} M) in the apical or basolateral wells.

7.1.3. Cell ATP availability assay. This experiment was performed as reported in technical sheet of ATPlite Kit for luminescence ATP detection using Victor3, from PerkinElmer Life Sciences.[30] Caco-2 cells were seeded into 96-well microplate in 100 μL of complete medium at a density 2×10^4 cells/well. The plate was incubated overnight in a humidified atmosphere 5% CO_2 at 37 °C. The medium was removed and 100 μL of complete medium in the presence or absence of different concentrations (from 1 to 100 μM) of tested compounds was added. The plate was incubated for 2 h in a humidified atmosphere 5% CO_2 at 37 °C. Then, 50 μL of mammalian cell lysis solution was added to all wells and the plate stirred for 5 min in an orbital shaker. In all wells 50 μL of substrate solution was added, the plate stirred for 5 min as above reported. The plate was dark adapted for 10 min and the luminescence was measured in Victor3, from PerkinElmer Life Sciences.

7.1.4. [^{3}H]Substrate transport inhibition. Caco-2 cells were seeded onto MultiScreen Plates 10,000 cells/well for 21 days measuring the integrity of the cell monolayers by trans-epithelial electrical resistance (TEER, $\Omega \times cm^2$) with an epithelial voltohommeter. Mature Caco-2 cell monolayer exhibited a TEER $>800\ \Omega \times cm^2$ prior to use in transport experiments. Transport experiments for tested compounds were carried out as described by Taub et al.[31] In each well to basolateral (BL) compartment in the absence and in the presence of P-gp inhibitors (from 200 nM to 400 μM) was added 20 nM [^{3}H]vinblastine or 20 nM [^{3}H]mitoxantrone for 120 min at 37 °C and its appearance in the apical (AP) compartment was monitored. At 120 min a 20 μL sample was taken from donor compartment to determine the concentration of radioligand remaining in the donor chamber at the end of the experiment. Samples were analyzed using LS6500 Beckman–Counter. For each compound, [^{3}H]vinblastine transport inhibition or [^{3}H]mitoxantrone transport inhibition was calculated as radioactivity difference between radioligand in the presence and absence of compound. These differences were expressed as inhibition percentage at single drug concentration.

7.1.5. Cytotoxicity assay. The assay was performed using the CytoTox-One kit from the Promega Corp. (Madison, WI, USA) as reported in previous paper.[32] Cell death was determined as the release of lactate dehydrogenase (LDH) into the culture medium. The percentage of cytotoxicity was calculated relative to the LDH release from total lysis of cells in untreated control. It is assumed here that the drug-treated wells and the control wells contained the same total number of cells (dead plus alive cells) at the end of the treatment period. Therefore, the cytotoxic effect of tested compounds was unaffected by any underestimation of cytotoxicity that could occur because of decreased total number of cells in the treated samples compared to the untreated control. Cells were seeded into 96-well plates for optical performance in the fluorescent cell-based assay in 100 μL of complete medium in the presence or absence of different concentrations of test compounds. The plate was incubated for 24 h in a humidified atmosphere 5% CO_2 at 37 °C and then 100 μL of substrate mix in assay buffer was added. Ten microliters lysis solution was added to untreated wells in order to estimate total LDH. Plates were kept protected from light for 10 min at room temperature and 50 μL of stop solution was added to all wells. The fluorescence was recorded using a LS55 Luminescence Spectrometer PerkinElmer with a 560 nm excitation wavelength and a 590 nm emission wavelength. The cytotoxicity percentage was estimated as follows: 100 × (LDH in medium of treated cells − culture medium background)/(total LDH in untreated cells − culture medium background).

7.1.6. Evaluation of cell growth. Determination of cell growth was performed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay at 24 and 48 h.[20] On day one, 10,000 cells/well were plated in 96-well plates in a volume of 200 μL and on day two, the various drugs alone or in combination were added. In all the experiments, the various drug-solvents (ethanol, DMSO) were added in each control to evaluate a possible solvent cytotoxicity. After the established incubation time with drugs, 0.5 mg/mL MTT was added to each well and, after 1 h incubation at 37 °C, the supernatant was removed. The formazan crystals were solubilized by 100 μL of DMSO and the absorbance val-

ues at 570 and 630 nm were determined on the microplate reader SpectraCount (Packard-USA).

7.1.7. Effect of antiproliferative drug combination. In MCF7/Adr, compound **4d** and Verapamil were utilized at 2 μM and 20 μM; Doxorubicin at 5 μM (IC_{50} after 3 days drug exposure when the P-gp was not overexpressed); Verapamil was utilized as reference compound. The schedule of drugs administration was the P-gp inhibitors plus Doxorubicin for 2 days followed, after two wash steps with complete medium, by Doxorubicin for 1 day. The analysis of cell growth inhibition was performed using the MTT assay as reported above.[20]

7.2. Chemistry

Column chromathography was performed with 1:30 Merck silica gel 60 Å (63–200 μm) as the stationary phase. Melting points were determined in open capillaries on a Gallenkamp electrothermal apparatus. Elemental analyses (C, H, N) were performed on Eurovector Euro EA 3000 analyzer; the analytical results were within ±0.4% of the theoretical values for the formula given. ^{1}H NMR spectra were recorded in $CDCl_3$ at 300 MHz on a Varian Mercury-VX spectrometer. All spectra were recorded on the free bases. All chemical shift values are reported in ppm (δ). Recording of mass spectra was done on an HP6890-5973 MSD gas chromatograph/mass spectrometer; only significant *m*/*z* peaks, with their percentage of relative intensity in parentheses, are reported. All spectra were in accordance with the assigned structures. ESI-MS analyses were performed on an Agilent 1100 LC/MSD trap system VL. The UV–vis spectra of tested compounds and their corresponding calibration curves were recorded with LAMBDA BIO20 spectrophotometer PerkinElmer. The spectroscopic properties of each compound are reported in Section 7. Compound **3a** has not been characterized because of its instability.

7.2.1. 4′-Methoxy-biphenyl-4-carboxylic acid (2e). In 35 mL MeOH and 1 mL H_2SO_4 was dissolved 4′-hydroxy-biphenyl-4-carboxylic acid (1 mmol). The solution was heated under reflux for 10 h. The solvent was evaporated and the crude was washed with H_2O 2× 10 mL and dried. The obtained methyl ester was dissolved in CH_3CN (20 mL) in the presence of K_2CO_3 (2.0 mmol) and CH_3I (2.0 mmol). The mixture was refluxed for 6 h, cooled and the solid residue was removed by filtering. The solvent was evaporated and the crude was dissolved in 3 M NaOH (20 mL) and heated at 60 °C for 6 h. The mixture was cooled and acidified to pH 1.0 with 6 N HCl. The precipitate was filtered washed with Et_2O (2× 10 mL) and dried. The title compound was purified by silica gel chromatography (EtAc/petroleum ether 3:7) obtaining a white solid (46% yield).

7.3. General procedure for preparing amides 3a and 3c–e

A mixture of appropriate biphenyl carboxylic acid (2.0 mmol) and an excess of $SOCl_2$ (2.0 mL) and 0.1 mL triethylamine was stirred for 30 min at 70 °C. Then the excess of $SOCl_2$ was removed under vacuo and the crude was solubilized in CH_2Cl_2 (20 mL) and added to a solution containing 6,7-dimethoxytetrahydroisoquinoline (1.0 mmol) in CH_2Cl_2 (30 mL) and 1.2% in H_2O NaOH (30 mL). The resulting solution was stirred at room temperature for 4 h then the organic layer was separated and washed with 0.5 M Na_2CO_3 (3× 20 mL) and 3 N HCl (3× 20 mL). The organic layer was dried on anhydrous Na_2SO_4, the solvent was evaporated obtaining a crude solid. Compound **3a** was not characterized. Crude **3a** has been reduced affording compound **4a** as following reported.

7.3.1. Biphenyl-4-yl-(6,7-dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-methanone (3c). White solid, 55% yield from column chromatography ($CHCl_3$). Recrystallized from $CHCl_3$/petroleum ether. Mp 143–147 °C. Anal. ($C_{24}H_{23}NO_3 \cdot 0.25H_2O$) C, H, N. UV–vis λ_{max} = 266 nm (PBS) ε = 8240. ESI$^+$/MS *m*/*z* 374 (ESI M$^+$+1, 63), 282 (M$^+$ 100). ^{1}H NMR: δ 2.46–2.75 (m, 2H, NC*H*$_2$CH$_2$ isoquinoline), 3.60–3.68 (m, 8H, 2 of CH$_3$ NCH$_2$C*H*$_2$ isoquinoline), 4.52–4.66 (m, 2H, NCH$_2$ isoquinoline), 6.61–7.99 (m, 11 H, aromatic).

7.3.2. (6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-(4′-hydroxy-biphenyl-4-yl)-methanone (3d). White solid, 46% yield from column chromatography ($CHCl_3$/EtAc 4:1). Recrystallized from $CHCl_3$/petroleum ether. Mp 129–132 °C. Anal. ($C_{24}H_{23}NO_4 \cdot H_2O$) C, H, N. UV–vis λ_{max} = 280 nm (PBS) ε = 4420. ESI$^+$/MS *m*/*z* 390 (ESI$^+$+1, 100), 193 (M$^+$ 30). ^{1}H NMR: δ 2.49–2.75 (m, 2H, NC*H*$_2$CH$_2$ isoquinoline), 3.60–3.80 (m, 8H, 2 of CH$_3$, NCH$_2$C*H*$_2$ isoquinoline), 4.55–4.65 (m, 2H, NCH$_2$ isoquinoline), 6.72–7.66 (m, 10H, aromatic), 9.50–9.80 (br s, 1H, OH, D_2O exchanged).

7.3.3. (6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-(4′-methoxy-biphenyl-4-yl)-methanone (3e). White solid, 70% yield from column chromatography ($CHCl_3$/EtAc 9:1). Recrystallized from $CHCl_3$/Petroleum ether. Mp 158–160 °C. Anal. ($C_{25}H_{25}NO_4 \cdot 0.5 \cdot H_2O$) C, H, N. UV–vis λ_{max} = 280 nm (PBS) ε = 4420. ESI$^+$/MS *m*/*z* 404 (ESI$^+$+1, 100), 211 (M$^+$ 20). ^{1}H NMR: δ 2.68–2.86 (m, 2H, NC*H*$_2$CH$_2$ isoquinoline), 3.62–3.97 (m, 11H, 3 of CH$_3$, NCH$_2$C*H*$_2$ isoquinoline), 4.31–4.82 (m, 2H, NCH$_2$ isoquinoline), 6.43–7.62 (m, 10H, aromatic).

7.4. General procedure for preparing amines 4a–e and 7f–h

To a suspension of $LiAlH_4$ (2 mmol) in anhydrous THF (30 mL) under N_2 atmosphere was added dropwise a solution of appropriate amide in THF (10 mL). The mixture was refluxed for 2 h, cooled and H_2O (1 mL) was added dropwise to eliminate $LiAlH_4$ excess. Then, were added Et_2O (20 mL) and H_2O (20 mL) and the organic layer was separated and washed with 3 N HCl (3× 10 mL). The aqueous solution was separated and alkalinized with solid Na_2CO_3 and extracted with $CHCl_3$ (3× 20 mL). The organic layers were collected and dried on anhydrous Na_2SO_4. The solvent was evaporated and the crude was purified on silica gel column.

7.4.1. 2-Biphenyl-2-yl-methyl-6,7-dimethoxy-1,2,3,4-tetrahydro-isoquinoline (4a). White solid, 35% yield from column chromatography ($CHCl_3$). Recrystallized from

MeOH/Et_2O. Mp 207–209 °C. Anal. ($C_{24}H_{25}NO_2$·HCl·1.5H_2O) C, H, N. UV–vis λ_{max} = 280 nm (PBS) ε = 1600. ESI$^+$/MS *m/z* 360 (ESI M^++1, 100), 167 (M^+ 17). ^{1}H NMR: δ 2.65–2.77 (m, 4H, NCH_2CH_2 isoquinoline), 3.48 (s, 2H, NCH_2 biphenyl), 3.60 (s, 2H, NCH_2 isoquinoline), 3.80 and 3.83 (2s, 6H, 2 of CH_3), 6.45–7.67 (m, 11H, aromatic).

7.4.2. 2-Biphenyl-3-yl-methyl-6,7-dimethoxy-1,2,3,4-tetrahydro-isoquinoline (4b). Yellow oil, 61% yield from column chromatography ($CHCl_3$/EtAc 1:1). Recrystallized from MeOH/Et_2O. Mp 126–130 °C. Anal. ($C_{24}H_{25}NO_2$·HCl) C, H, N. GC–MS: 360 (M^++1, 7), 359 (M^+ 40), 358 (100), 192 (64). ^{1}H NMR: δ 2.66–2.85 (m, 4H, NCH_2CH_2isoquinoline), 3.50 (s, 2H, NCH_2 biphenyl), 3.63 (s, 2H, NCH_2 isoquinoline), 3.80–3.83 (s, 6H, 2 of CH_3), 6.44–7.69 (m, 11H, aromatic).

7.4.3. 2-Biphenyl-4-yl-methyl-6,7-dimethoxy-1,2,3,4-tetrahydro-isoquinoline (4c). A suspension containing bromoderivative **2b** (1.0 mmol) 6,7-dimethoxytetrahydroisoquinoline (2.0 mmol) Na_2CO_3 (2.0 mmol) in DMF (20 mL) was stirred for 6 h at 150 °C. Then, the solvent was evaporated and were added $CHCl_3$ (20 mL) and H_2O (20 mL). The organic layer was separated and dried on anhydrous Na_2SO_4. The solvent was evaporated obtaining crude **4b** as an oil that was purified on silica gel column. White solid, 87% yield from column chromatography ($CHCl_3$/EtAc 4:1). Recrystallized from MeOH/Et_2O. Mp 246–249 °C. Anal. ($C_{24}H_{25}NO_2$·HCl·0.75H_2O) C, H, N. UV–vis λ_{max} = 254 nm (PBS) ε = 8600. ESI$^+$/MS *m/z* 360 (ESI M^++1, 100), 167 (M^+ 42). ^{1}H NMR: δ 2.77–2.85 (m, 4H, NCH_2CH_2 isoquinoline), 3.60 (s, 2H NCH_2 isoquinoline), 3.74 (s, 2H, NCH_2 biphenyl), 3.81 and 3.84 (2s, 6H, 2 of CH_3), 6.50–7.64 (m, 11 H, aromatic).

7.4.4. 4′-(6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl-methyl)-biphenyl-4-ol (4d). White solid, 35% yield from column chromatography ($CHCl_3$/EtAc 4:1). Recrystallized from MeOH/Et_2O. Mp 265–268 °C. Anal. ($C_{24}H_{25}NO_3$·HCl·0.5H_2O) C, H, N. UV–vis λ_{max} = 270 nm (PBS) ε = 8240. ESI$^+$/MS *m/z* 376 (ESI M^++1, 100), 183 (M^+ 70). ^{1}H NMR: δ 2.65–2.71 (m, 4H, NCH_2CH_2 isoquinoline), 3.25–3.41 (m, 4H, NCH_2 isoquinoline and NCH_2 biphenyl), 3.63–3.67 (s, 6H, 2 of CH_3), 6.56–7.53 (m, 10H, aromatic), 9.55 (br s, 1H, OH, D_2O exchanged).

7.4.5. 6,7-Dimethoxy-2-(4′-methoxy-biphenyl-4-yl-methyl)-1,2,3,4-tetrahydro-isoquinoline (4e). White solid, 57% yield from column chromatography ($CHCl_3$/EtAc 9:1). Recrystallized from MeOH/Et_2O. Mp 238–241 °C. Anal. ($C_{25}H_{27}NO_3$·HCl·0.4H_2O) C, H, N. UV–vis λ_{max} = 270 nm (PBS) ε = 8240. ESI$^+$/MS *m/z* 390 (ESI M^++1, 100), 197 (M^+ 91). ^{1}H NMR: δ 2.77–2.85 (m, 4H, NCH_2CH_2 isoquinoline), 3.60 (s, 2H NCH_2 isoquinoline), 3.73 (s, 2H, NCH_2 biphenyl), 3.77–3.89 (m, 9H, 3 of CH_3), 6.50–7.56 (m, 10H, aromatic).

7.5. General procedure for preparing amides 6f–h

A mixture of appropriate 2-naphthalenylcarboxylic acid (1.0 mmol), carbonyldiimidazole (1.1 mmol) in THF (50 mL) was stirred overnight at room temperature under N_2 atmosphere. Then was added a solution of 6,7-dimethoxytetrahydroisoquinoline (1.0 mmol) in THF (20 mL). The resulting mixture was stirred at room temperature for 8 h. After this time were added ethylacetate (30 mL) and H_2O (30 mL) and the organic layer was separated and washed with Na_2CO_3 (s.s. 3 × 30 mL). The organic layer was dried on anhydrous Na_2SO_4 and the solvent was evaporated.

7.5.1. (6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-naphthalen-2-yl-methanone (6f). White solid, 80% yield from column chromatography ($CHCl_3$/EtAc 9:1). Recrystallized from $CHCl_3$/petroleum ether. Mp 136–139 °C. Anal. ($C_{22}H_{21}NO_3$) C, H, N. UV–vis λ_{max} = 280 nm (PBS) ε = 6920. ESI$^+$/MS *m/z* 348 (ESI M^++1, 100), 155 (M^+ 20). ^{1}H NMR: δ 2.83–2.91 (m, 2H, NCH_2CH_2 isoquinoline), 3.69–3.99 (m, 8H, 2 of CH_3 and NCH_2CH_2 isoquinoline), 4.57–4.87 (m, 2H, NCH_2 isoquinoline), 7.51–7.95 (m, 9H, aromatic).

7.5.2. (6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-(6-hydroxy-naphthalen-2-yl)-methanone (6g). White solid, 32% yield from column chromatography (CH_2Cl_2/AcEt 1:1). Recrystallized from $CHCl_3$/petroleum ether. Mp 116–120 °C. Anal. ($CC_{22}H_{21}NO_4$·0.8H_2O) C, H, N. UV–vis λ_{max} = 288 nm (PBS) ε = 5650. ESI$^+$/MS *m/z* 364 (ESI M^++1, 100), 171 (M^+ 18). ^{1}H NMR: δ 2.87–2.92 (m, 3H, NCH_2CH_2 isoquinoline and OH, D_2O exchanged), 3.62–4.02 (m, 8H, 2 of CH_3 and NCH_2CH_2 isoquinoline), 4.62–4.87 (m, 2H, NCH_2 isoquinoline), 6.64–7.95 (m, 8H, aromatic).

7.5.3. (6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-(6-methoxy-naphthalen-2-yl)-methanone (6h). White solid, 57% yield from column chromatography ($CHCl_3$/EtAc 9:1). Recrystallized from $CHCl_3$/petroleum ether. Mp 166–168 °C. Anal. ($C_{23}H_{23}NO_4$·0.5H_2O) C, H, N. UV–vis λ_{max} = 283 nm (PBS) ε = 6980. ESI$^+$/MS *m/z* 378 (ESI M^++1, 100), 185 (M^+ 17). ^{1}H NMR: δ 1.67–2.86 (m, 2H, NCH_2CH_2 isoquinoline), 3.62–3.94 (m, 11H, 3 of CH_3, NCH_2CH_2 isoquinoline), 4.62–4.84 (m, 2H, NCH_2 isoquinoline), 6.64–7.90 (m, 8H, aromatic).

7.5.4. 6,7-Dimethoxy-2-naphthalen-2-yl-methyl-1,2,3,4-tetrahydro-isoquinoline (7f). White solid, 26% yield from column chromatography ($CHCl_3$/EtAc 1:1). Recrystallized from MeOH/Et_2O. Mp 234–238 °C. Anal. ($C_{22}H_{23}NO_2$·HCl·0.5H_2O) C, H, N. UV–vis λ_{max} = 278 nm (PBS) ε = 6030. ESI$^+$/MS *m/z* 334 (ESI M^++1, 100), 141 (M^+ 17). ^{1}H NMR: δ 2.76–2.84 (m, 4H, NCH_2CH_2 isoquinoline and NCH_2 isoquinoline), 3.58 (s, 2H, NCH_2 biphenyl), 3.80–3.86 (m, 8H, 2 of CH_3 NCH_2CH_2 isoquinoline), 6.47–7.85 (m, 9H, aromatic).

7.5.5. 6-(6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-ylmethyl)-naphthalen-2-ol (7g). White solid, 42% yield from column chromatography (CH_2Cl_2/AcEt 1:1). Recrystallized from MeOH/Et_2O. Mp. 207–209 °C. Anal. ($C_{22}H_{23}NO_3$·0.25H_2O) C, H, N. UV–vis λ_{max} = 278 nm (PBS) ε = 7740. ESI$^+$/MS *m/z* 350 (ESI M^++1, 100), 194 (M^+ 68). ^{1}H NMR: δ 2.75–2.96 (m, 5H, NCH_2CH_2 isoquinoline and NCH_2 isoquinoline,

OH, D_2O exchanged), 3.62 (s, 2H NCH_2 naphthalene), 3.78–3.86 (m, 8H, NCH_2CH_2 isoquinoline 2 of CH_3), 7.00–7.70 (m, 8H aromatic).

7.5.6. 6,7-Dimethoxy-2-(6-methoxy-naphthalen-2-ylmethyl) -1,2,3,4-tetrahydro-isoquinoline (7h). White solid, 4% yield from column chromatography ($CHCl_3$). Recrystallized from MeOH/Et_2O. Mp 232–236 °C. Anal. ($C_{23}H_{25}NO_3 \cdot HCl \cdot 0.5H_2O$) C, H, N. UV–vis λ_{max} = 278 nm (PBS) ε = 5310. ESI^+/MS *m/z* 364 (ESI M^++1, 43), 171 (M^+ 100). ^1H NMR: δ 2.78–2.86 (m, 4H, NCH_2CH_2 and NCH_2 isoquinoline), 3.58 (s, 2H, NCH_2 naphthalene), 3.77–3.92 (m, 11H, 3 of CH_3 and NCH_2CH_2), 6.46–7.73 (m, 8H, aromatic).

7.6. Statistical analysis

The EC_{50} values of the compounds reported in Tables 1 and 2 were determined by non-linear curve fitting utilizing the GraphPad Prism program.[33]

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.01.055.

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