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Original article

SAR study on arylmethyloxyphenyl scaffold: Looking for a P-gp nanomolar affinity

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ABSTRACT

Starting from the previously developed P-gp ligands **1a** and **1b** (EC_{50} = 0.25 μM and 0.65 μM, respectively), new arylmethyloxyphenyl derivatives have been synthesized as P-gp modulators in order to investigate: (*i*) the effect of small electron-donor groups (OMe) (**5**–**11**), (*ii*) the effect of the replacement of methoxy groups with an electron-withdrawal substituent (Cl) on C-ring (**13**) (*iii*) the effect induced by the replacement of C-ring with heteroaromatic cycles such as thiophene and pyrimidine (**13**, **15**, **16**), (*iv*) the effect induced by molecular constriction on C ring (**14**, **17**, **18**) on P-gp modulating activity. The results demonstrated that P-gp inhibition potency is strongly correlated to the number of methoxy groups in the A-ring whereas the methoxylation of C-ring seems to poorly affect P-gp activity. The best result was found for compound **10** that displays a nanomolar affinity (EC_{50} = 7.1 nM) towards P-gp pump and, in the meantime lacks of activity against MRP1 pump.

1. Introduction

Over the last two decades a new research field emerged with a focus on ATP-binding cassette (ABC) transporters at the Blood–Brain Barrier (BBB) and in other cells of the Central Nervous System (CNS). ABC transporters utilize ATP to move substrates across membranes of organelles, cells, and tissues. The substrates of ABC transporters include lipids, peptides, metabolites, and xenobiotics as well as several therapeutic drugs. Thus, ABC transporters play an important role in maintaining the body's homeostasis by extruding metabolites and limiting uptake of xenobiotics. In addition to the well-known detrimental role of ABC-transporters in the multidrug resistance (MDR) phenomenon in cancer cells [1,2], recent evidences suggest that ABC transporters are also involved in the etiology and the progression of some neurological disorders such as Alzheimer's and Parkinson's diseases [3,4]. Within ABC transporters, such a pivotal role seems to be played by P-gp, MRP1 (Multidrug Resistance associated Protein), and BCRP (Breast Cancer Resistance Protein) transporters.

The activity of P-gp at BBB level prevents xenobiotics entry into the CNS [5,6] by active efflux across the apical membrane of the capillary endothelial cells in brain vasculature. However, the P-gp is shown to be overexpressed in response to cellular stress triggered by drugs, toxins, thus leading to MDR, an important gap for the pharmacological treatment of several neurological disorders as well as cerebral cancer.

The knowledge of the crucial role of P-gp in limiting the absorption and preventing the distribution of drugs into vital organs such as brain, has forced scientists to design and synthesize new molecules for a better comprehension of P-gp involvement in pharmacokinetic processes.

The search for new suitable molecules which could interact with P-gp and appropriately regulate its activity led us to design and synthesize new arylmethyloxyphenyl-derivatives which showed both an appreciable P-gp modulating activity and a high degree of P-gp selectivity [7–9]. In particular, the best activity in terms of potency and selectivity against other efflux pumps, has been showed by compounds **1a** and **1b** (0.25 μM and 0.65 μM) which bear a methoxy group in 3-position on A-ring and two methoxy groups in 2,3 and 3,4 positions of C-ring, respectively (Fig. 1).

Starting from these results and aiming both to optimize the activity of lead compounds **1a**,**b** and to study the Structure–

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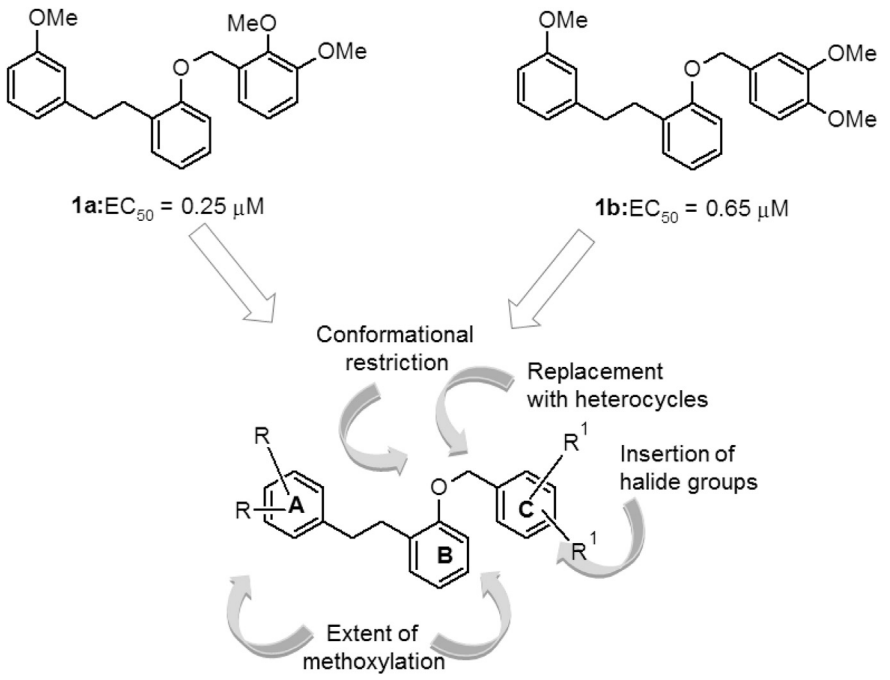


Fig. 1. Medicinal Chemistry Optimization of arylmethyloxyphenyl derivatives.

Activity Relationships of this new class of compounds, new arylmethyloxyphenyl derivatives have been synthesized. In particular, compounds **5**–**18** (Tables 1 and 2) were synthesized and evaluated for their P-gp activity in order to investigate: (*i*) the influence of the addition of small electron-donor groups (OMe) on the A-ring (**6**,**7**), C-ring (4) (**5**) or both (**8**–**11**), (*ii*) the effect of the replacement of methoxy groups with an electron-withdrawal substituent (Cl) on C-ring (**13**) (*iii*) the effect induced by the replacement of C-ring with heteroaromatic cycles such as thiophene and pyrimidine (**12**, **15**, **16**), (*iv*) the effect induced by molecular constriction on C ring (**14**, **17**, **18**). All the chemical manipulations described above could allow us to get important indications about the critical (or essential) structural requirements to attest a good P-gp modulating activity to compounds with an arylmethyloxyphenyl scaffold.

The P-gp interacting mechanism for each compound has been studied by three combined biological assays: (*i*) Calcein-AM transport inhibition [10], (ii) ATP cell depletion [11], and (*iii*) apparent permeability (P_{app}) determination in Caco-2 cells monolayer [12]. Moreover, the best P-gp modulators have been tested for cytotoxicity and their MRP1 activity.

2. Chemistry

Compounds **5**–**15** were prepared by alkylation of phenols **4a**–**d** with the appropriate arylmethylchloride in the presence of KOH, as reported in Scheme 1. The phenols **4a**–**d** were obtained as previously described [7]. Briefly, the Wittig reaction of the appropriate phosphonium salt **2a**–**d** and salicylaldehyde afforded derivative **3a**–**d** as a mixture of *cis* and *trans* isomers which was submitted to a catalytic hydrogenation in the presence of 10% Pd/C to give phenol derivative **4a**–**d**. Derivatives **16**–**18** were synthesized starting from the aniline derivative **19**. [8] The pyridine derivative **16** was obtained by reaction of **19** with the pyridine-4-carbaldehyde and subsequent reduction with $NaBH_4$, while the amides **17**,**18** were obtained by the reaction of the aniline derivative **19** with the appropriate aryl-carbonylchloride in the presence of TEA (Scheme 2).

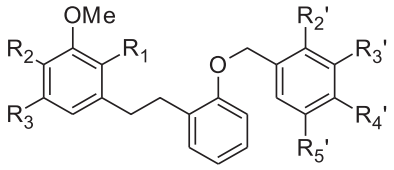
3. Results and discussion

The new compounds synthesized (**5**–**18**) were evaluated for their ability to modulate P-gp pump. Results are reported in Tables 1 and 2, together with those already obtained for the previously described analogs (**1a**,**b** and **20**–**23**) and the reference drugs (tariquidar and verapamil).

In particular, Table 1 lists the P-gp modulatory potency of compounds in which the extension of methoxylation on A- and/or C-rings has been carried out. Starting from compound **1b** (EC_{50} = 0.65 μM) previously synthesized [8] the presence of an additional methoxy group in *para*-position on A-ring (**6**) reduce the activity causing a 3-fold reduced P-gp potency (0.65 μM vs. 2.1 μM), while the tri-substitution on A-ring (**9**) proved to influence the P-gp inhibition activity only slightly with respect to the reference compound **1b** (0.65 μM vs. 0.9 μM). In the previous paper [8] we observed that as concerns the mono-substituted derivatives on C-ring the *para*-substitution determined the best activity toward P-gp. Taking that into account, we planned to further explore the effect of an additional methoxy group on *para*-position of C-ring (compounds **5** and **7**). Both compounds showed to be active with an EC_{50} value ranging from 3 μM for **5** to 0.3 μM for **7**. These results displayed that the extension of methoxylation on A-ring could cause an increased potency for **7** of one order of magnitude respect to the analog **5** mono-substituted on A-ring. For this purpose the effect of the substitution of the A-ring with additional methoxy groups has been investigated. Within this small series of compounds (**8**–**11**) derivative **10** showed to be the most potent P-gp ligand displaying nanomolar activity (EC_{50} = 7.1 nM).

These data highlight that P-gp inhibition potency is strongly correlated to the number of methoxy groups in the A-ring (**5**; **7**; **10**; EC_{50} = 3.1. 0.3 and 0.0071 μM, respectively). On the contrary the methoxylation of C-ring seems to poorly affect the activity. In

Table 1
Biological results of the arylmethyloxyphenyl derivatives **5**–**11**.



Compds	R_1	R_2	R_3	R_2'	R_3'	R_4'	R_5'	Calcein–am transport inhibition $EC_{50} \pm SEM$ (μM)[a]	ATP-ase activation[b]	P_{app} (BA) P_{app} (AB)[m]
5	H	H	H	H	OMe	OMe	OMe	3.1 ± 0.60	N	3.14
6	H	OMe	H	H	OMe	OMe	H	2.1 ± 0.32	N	2.77
7	OMe	H	H	H	OMe	OMe	OMe	0.3 ± 0.03	Y	3.23
8	H	OMe	OMe	OMe	OMe	H	H	3.2 ± 0.60	Y	3.45
9	H	OMe	OMe	H	OMe	OMe	H	0.9 ± 0.10	Y	3.80
10	H	OMe	OMe	H	OMe	OMe	OMe	0.0071 ± 0.1	Y	3.96
11	H	OMe	OMe	H	OMe	H	OMe	1.3 ± 0.16	N	nd
1a[d]	H	H	H	OMe	OMe	H	H	0.25 ± 0.05	Y	7.8
1b[d]	H	H	H	H	OMe	OMe	H	0.65 ± 0.08	N	5.1
20[e]	H	H	H	H	OMe	H	H	17.2 ± 0.5	N	13
verapamil								20 ± 1.0	Y	>2[f]
tariquidar								0.044[f]	N	22.0[f]

(Y) ATPase activation; (N) no ATPase activation; nd: not determined.
[a] Data are the mean of three independent determinations, samples in triplicate.
[b] Effect measured at 100 μM. Data are the mean of three independent determinations with samples in triplicate.
[c] Data are the mean of three independent determinations (samples in triplicate).
[d] See Ref. [8].
[e] See Ref. [7].
[f] See Ref. [14].

particular, both the mono-(**20**, $EC_{50} = 17.2$ μM) and trimethoxylated derivatives (**5**, $EC_{50} = 3$ μM) on C-rings displayed a potency on μM range, while the potency of the disubstituted derivatives (**1a**,**b**) is submicromolar.

On this basis we decide to replace the C-ring with hetero-aryl frameworks in order to assess the effects of this type of substitution on the P-gp inhibition activity. In Table 2 the activity of arylmethyloxyphenyl derivatives in which the C-ring has been replaced by a pyrimidine (**12**), a thiophene (**15**) or 3,4-methylenedioxybenzene (**14**) are reported. All these compounds show to be inactive towards P-gp ($EC_{50} > 100$ μM) excepting for pyrimidine derivative **12** ($EC_{50} = 32.5$ μM) that displayed lower activity with respect to the pyridine homologue previously synthesized **22** ($EC_{50} = 27.6$ μM). Also the replacement of methoxy groups on C-ring with chlorine cause a loss of P-gp inhibitory activity (compound **13**, $EC_{50} > 100$ μM).

In order to investigate the role of oxygen on the spacer linking the B-ring to the C-ring the amine derivative (**16**) has been synthesized. We were encouraged in investigating this aspect because previous SAR studies demonstrated that the presence of NH group determined an increase of P-gp activity (**23**: $EC_{50} = 0.48$ μM vs. **20**: $EC_{50} = 17.2$ μM) [8].

The result disagreed with our previous studies because **16** showed lower P-gp activity than the corresponding compound **21** bearing oxygen in the spacer. ($EC_{50} = 11.5$ μM vs. 0.85 μM, respectively).

The replacement of the spacer linking B-ring with C-ring by an anilide group to confer both conformational restriction and to evaluate the role of basicity, (compounds **17** and **18**) have been studied. The results showed that tested compounds were poorly active towards P-gp.

3.1. P-gp interacting mechanism investigation

In a monolayer efflux assay, the Apparent Permeability (P_{app}) in both basolateral to apical [P_{app}(B–A)] and apical to basolateral [P_{app}(A–B)] directions was determined for each compound. The BA/AB ratio is ranging from 2.7 to 4.1 for compounds **6** and **17** respectively. It is noteworthy that compounds showing BA/AB ratio up to 2 were not effluxed by P-gp, while compounds with BA/AB ratio >2 were P-gp transported and consequently considered substrates of this pump [12]. Taking that into account, all the compounds synthesised should be considered as P-gp substrates.

As regards ATP-ase activation, the compounds **5**, **6** and **11** were unable to deplete ATP from cells. The compounds that reduced the cell ATP level were considered P-gp substrates, while the compounds with unmodified ATP cell content were not effluxed by P-gp.

Combining the biological assays results, compound **6**, could be claimed “saturating transported” P-gp substrate because it was effluxed by P-gp, inhibited calcein transport, and it was unable to deplete ATP.

All the other compounds were effluxed by P-gp and they depleted ATP so they were considered P-gp substrates. Therefore, the best P-gp ligand **10** is a potent P-gp substrate.

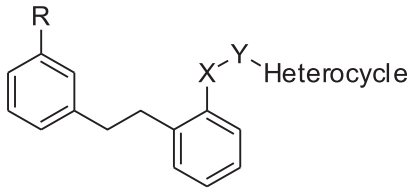
Moreover all tested compounds were found inactive towards MRP1 pump at 100 μM.

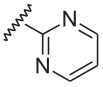
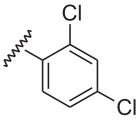
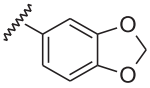
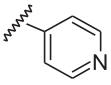
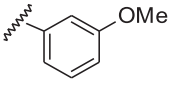
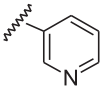
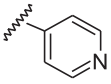
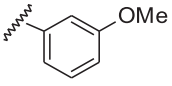
3.2. Cytotoxicity evaluation of compound 10 with MC18

In order to confirm the mechanism of the best compound **10**, the cytotoxicity of this compound in the absence and in presence of **MC18**, a P-gp inhibitor [13], has been performed in cells overexpressing P-gp (MDCK-MDR1 cell line) at 24 h and 48 h (Fig. 2). Compound **10** was inactive at 24 h while displayed moderate cytotoxicity at 48 h (40% at 1 μM). Therefore, the cytotoxicity of compound **10** at 48 h has been evaluated in the presence of 1 μM **MC18** that is inactive in these conditions (48 h and 1 μM).

The results showed that compound **10** at 1 μM increased its cytotoxicity from 40% to 63% in the presence of P-gp inhibitor **MC18**. This increase in terms of cytotoxicity is due to the additional

Table 2
Biological properties of heterocycle derivatives **12**–**18**.



Compds	R	X	Y	Heterocycle	Calcein-AM transport inhibition EC_{50} ± SEM (μM)[a]	ATP-ase activation[b]	P_{app} (B–A) P_{app} (A–B)[c]
12	OMe	O	CH_2		32.5 ± 4.0	Y	2.91
13	H	O	CH_2		>100	–	NT
14	OMe	O	CH_2		>100	Y	NT
15	OMe	O	CH_2		>100	Y	NT
16	OMe	N	CH_2		11.5 ± 1.7	Y	3.00
17	OMe	NH	CO		10.9 ± 1.60	Y	4.1
18	OMe	NH	CO		43.7 ± 5.60	Y	NT
21[d]	OMe	O	CH_2		0.85 ± 0.05	Y	2.1
22[e]	OMe	O	CH_2		27.6 ± 0.02	N	–
23[d]	OMe	N	CH_2		0.48 ± 0.002	N	2.9
verapamil					20 ± 1.0	Y	>2.0[f]
tariquidar					0.044[f]	Y (30%)	22.0[f]

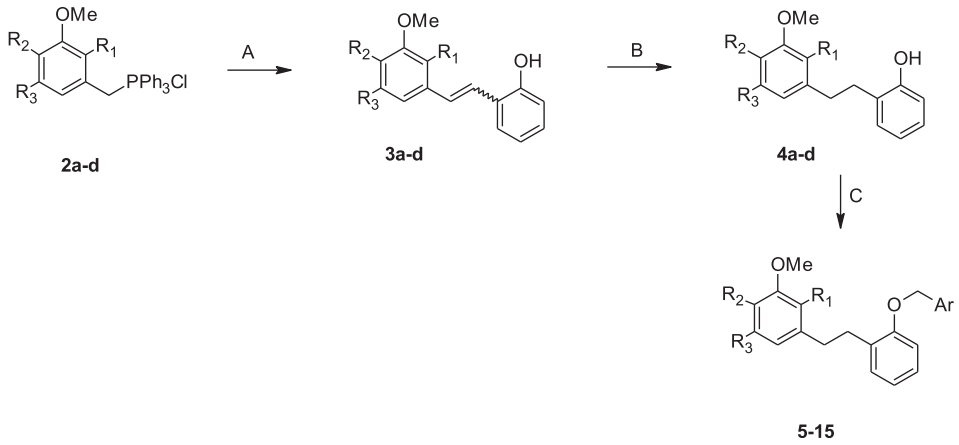
(NT) not tested; (Y) ATPase activation; (N) no ATPase activation.
[a] Data are the mean of three independent determinations, samples in triplicate.
[b] Effect measured at 100 μM. Data are the mean of three independent determinations with samples in triplicate.
[c] Data are the mean of three independent determinations (samples in triplicate).
[d] See Ref. [8].
[e] See Ref. [7].
[f] See Ref. [14].

cell accumulation of compound **10** in the presence of P-gp inhibitor (Fig. 2).

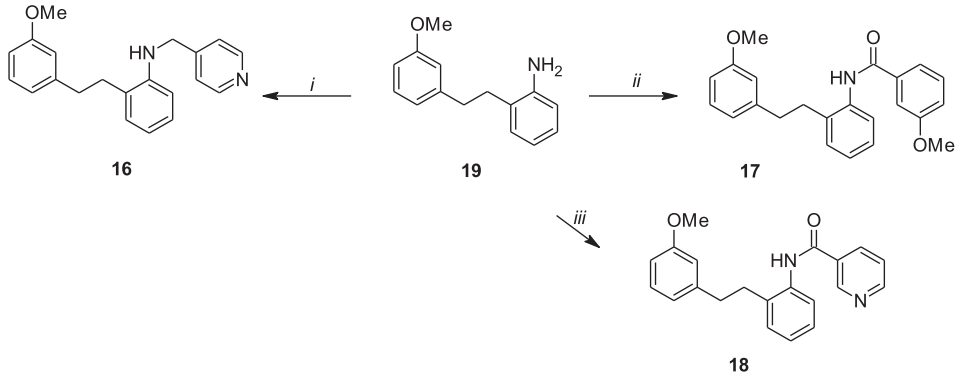
This assay confirms that **10** is a P-gp substrate and the increase of cytotoxic effect could be correlated to the cell concentration measured at 48 h.

4. Conclusion

This work demonstrated that P-gp modulation is strongly correlated to the number of methoxy groups in the A-ring whereas the methoxylation of C-ring seems to poorly affect P-gp activity.



Scheme 1. Reagents and Conditions: (A) salicylaldehyde, DBU, CH_3CN, refluxed, 12 h; (B) H_2, Pd/C 10%, rt, 24 h; (C) appropriate arylmethylchloride, KOH, DMSO, rt, 24 h.



Scheme 2. Reagents and Conditions (*i*) pyridine-4-carbaldehyde, $NaBH_4$, EtOH, refluxed, 12 h; (*ii*) 3-methoxybenzoylchloride, TEA, DCM, 12 h; (*iii*) pyridine-3-carbonylchloride, TEA, THF, refluxed, 48 h.

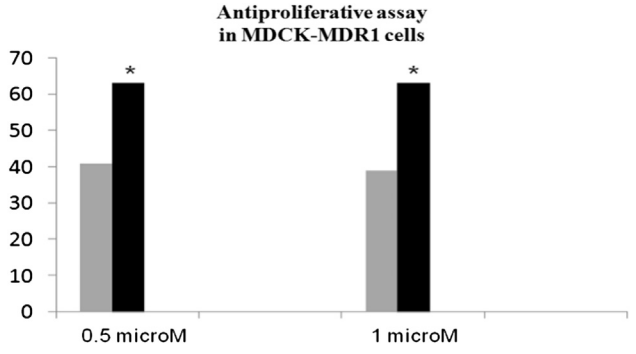


Fig. 2. Antiproliferative activity of compound **10** (0.5 μM and 1.0 μM) in absence (gray bar) and in the presence of 1 μM MC18 (black bar) in MDCK-MDR1 cells at 48 h. MC18 was not-cytotoxic at this concentration and at 48 h. The results are the mean of three independent experiments; (*) $P > 0.05$, significant.

The replacement of C-ring by a pyrimidine, a thiophene or a 3,4-methylenedioxybenzene did not lead to optimize P-gp effect. The arylmethyloxyphenyl derivative **10**, bearing small electron-donor groups (OMe) on the A-and C-rings, displayed nanomolar affinity towards P-gp ($EC_{50} = 7.1$ nM), good selectivity towards the P-gp since it was inactive towards MRP1 pump and a substrate profile.

In conclusion, the present paper report the discovery of compound **10** as potent P-gp ligand which could represent a suitable tool to study P-gp activity in *in vitro* and/or *in vivo* models. In particular, the presence and the position of methoxy groups permit to plan in the next future a specific chemical pathway to obtain radiolabelled compound(s) useful for imaging *in vitro* and *in vivo* P-gp. In particular methoxy substituent is an easy point to obtain both [^{3}H]OCH_3 and [^{18}F]-CH_2CH_2F for *in vitro* and *in vivo* PET studies, respectively.

5. Experimental section

5.1. Chemistry

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were obtained with a Varian Gemini 200 MHz spectrometer. Chemical shifts (δ) are reported in parts per million downfield from tetramethylsilane and referenced from solvent references; coupling constants *J* are reported in hertz; ^{13}C NMR spectra were fully decoupled. The following abbreviations are used: singlet (s), doublet (d), triplet (t), double-doublet (dd), and multiplet (m). Mass spectra were obtained on a Hewlett–Packard 5988 A spectrometer by using a direct injection probe and an electron beam energy of 70 eV. Analyses indicated by the symbols of the elements or functions were within ±0.4% of the theoretical values. Chromatographic separation was performed on silica gel columns by flash (Kieselgel 40, 0.040–0.063 mm; Merck) or gravity column (Kieselgel 60, 0.063–0.200 mm; Merck) chromatography. Reactions were followed by thin-layer chromatography (TLC) on Merck aluminum silica gel (60 F254) sheets that were visualized under a UV lamp. Evaporation was performed in vacuo (rotating evaporator). Sodium sulfate was always used as the drying agent. Commercially available chemicals

were purchased from Sigma–Aldrich. The UV–vis spectra of compounds and the corresponding calibration curves were recorded with a PerkinElmer LAMBDA BIO-20 spectrophotometer.

*5.1.1. General procedure for the synthesis of compounds **5**–**15***

A solution of appropriate phenol **4a**–**d** (1.0 mmol) in a small amount of DMSO (2 mL) was added to a solution of KOH (174 mg, 3.11 mmol) in DMSO (2.5 mL). The mixture was stirred at rt for 15 min, and then a solution of the appropriate arylmethylchloride (1.0 mmol) in DMSO (2 mL) was added dropwise to the solution of **4**. The suspension was stirred at room temperature for 12 h, and then it was diluted with AcOEt and washed with water and brine. The organic layer was dried and concentrated.

*5.1.1.1. 1-[(3,4,5-Trimethoxybenzyl)oxy]-2-[2-(3-methoxyphenyl) ethyl]-benzene (**5**).* The crude oil did not necessitate further purification (75% yield). ^{1}H NMR ($CDCl_3$): δ 2.87–3.07 (m, 4H, CH_2CH_2), 3.76 (s, 3H, OCH_3), 3.85 (s, 6H, OCH_3), 3.89 (s, 3H, OCH_3), 5.04 (s, 2H, OCH_2), 6.72–6.81 (m, 5H, Ar), 6.95 (d, 2H, $J = 7.9$ Hz, Ar), 7.16–7.26 (m, 3H, Ar). ^{13}C NMR ($CDCl_3$): δ 159.79, 156.73, 153.56, 144.04, 138.03, 133.15, 130.80, 130.16, 129.25, 127.32, 121.02, 120.91, 114.36, 112.08, 111.32, 104.67, 70.40, 60.94, 56.33, 55.22, 36.57, 32.79. Anal. ($C_{25}H_{28}O_5$) C, H. UV–vis (solvent: PBS) $\lambda = 230$ nm, $\varepsilon = 16{,}350$.

*5.1.1.2. 1-[(3,4-Dimethoxybenzyl)oxy]-2-[2-(3,4-dimethoxyphenyl) ethyl]-benzene (**6**).* The crude product was purified by crystallization from AcOEt, affording **5** as a white solid (40% yield). Mp: 127–129 °C. ^{1}H NMR ($CDCl_3$): δ 2.79–2.98 (m, 4H, CH_2); 3.75 (s, 3H, OCH_3); 3.85 (s, 6H, OCH3); 3.90 (s, 3H, OCH_3); 5.02 (s, 2H, CH_2); 6.60 (d, 1H, $J = 1.5$, Ar); 6.66–6.78 (m, 2H, Ar); 6.85–7.01 (m, 5H, Ar); 7.11–7.25 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$): δ 157.44, 150.01, 149.65, 148.06, 135.90, 131.40, 130.82, 127.81, 121.44, 121.02, 120.62, 113.05, 112.65, 112.37, 112.21, 111.83, 70.84, 56.77, 56.66, 56.51, 36.72, 33.81. Anal. ($C_{25}H_{28}O_5$) C, H.

*5.1.1.3. 1-[(3,4,5-Trimethoxybenzyl)oxy]-2-[2-(2,3-dimethoxyphenyl) ethyl]-benzene (**7**).* Purified with hexane/AcOEt affording **7** (50% yield) as an oil. ^{1}H NMR ($CDCl_3$): δ 2.93–2.99 (m, 4H, CH_2); 3.72 (s, 3H, OCH_3); 3.84 (s, 6H, OCH_3); 3.85 (s, 3H, OCH_3); 3.86 (s, 3H, OCH_3); 5.03 (s, 2H, CH_2); 6.70–6.78 (m, 4H, Ar); 6.87–6.99 (m, 3H, Ar); 7.14–7.23 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$): δ 156.77, 153.54, 152.87, 147.50, 137.99, 136.21, 133.20, 131.18, 130.20, 127.21, 123.68, 122.04, 120.99, 112.06, 110.63, 104.73, 70.40, 60.94, 60.59, 56.33, 55.93, 32.02, 30.55. Anal. ($C_{26}H_{30}O_6$) C, H.

*5.1.1.4. 1-[(2,3-Dimethoxybenzyl)oxy]-2-[2-(3,4,5-trimethoxyphenyl) ethyl]-benzene (**8**).* The crude solid did not necessitate further purification (59% yield). Mp: 68–70 °C. ^{1}H NMR ($CDCl_3$): δ 2.78–2.99 (m, 4H, CH_2); 3,77 (s, 6H, OCH_3); 3.81 (s, 3H, OCH_3); 3.89 (s, 6H, OCH_3); 5.15 (s, 2H, CH_2); 6.35 (s, 2H, Ar); 6.87–6.94 (m, 2H, Ar); 6.99–7.24 (m, 5H, Ar). ^{13}C NMR ($CDCl_3$): δ 156.27, 152.47, 152.05, 146.44, 137.59, 135.88, 130.78, 129.93, 129.53, 126.70, 123.43, 120.22, 120.13, 111.77, 111.37, 105.29, 64.71, 60.44, 60.26, 55.56, 55.36, 36.39, 32.55. Anal. ($C_{26}H_{30}O_6$) C, H.

*5.1.1.5. 1-[(3,4-Dimethoxybenzyl)oxy]-2-[2-(3,4,5-trimethoxyphenyl) ethyl]-benzene (**9**).* Purified with hexane/AcOEt (70:30) affording **9** (41% yield) as an oil. ^{1}H NMR ($CDCl_3$): δ 2.78–2.98 (m, 4H, CH_2); 3.73 (s, 6H, OCH_3); 3.81 (s, 3H, OCH_3); 3.85 (s, 3H, OCH_3); 3.89 (s, 3H, OCH_3); 5.01 (s, 2H, CH_2); 6.30 (s, 2H, Ar); 6.85–7.02 (m, 5H, Ar); 7.12–7.24 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$): δ 156.89, 153.14, 149.39, 149.08, 138.14, 136.61, 130.67, 130.25, 127.34, 120.89, 120.13, 112.03, 111.59, 111.35, 105.93, 70.30, 60.92, 56.20, 56.09, 36.97, 33.17. Anal. ($C_{26}H_{30}O_6$) C, H.

*5.1.1.6. 1-[(3,4,5-Triimethoxybenzyl)oxy]-2-[2-(3,4,5-trimethoxyphenyl)ethyl]-benzene (**10**).* The crude oil did not necessitate further purification (71% yield). ^{1}H NMR ($CDCl_3$): δ 2.86–2.97 (m, 4H, CH_2); 3,74 (s, 3H, OCH_3); 3.81 (s, 3H, OCH_3); 3.83 (s, 3H, OCH_3); 3.84 (s, 3H, OCH_3); 3.85 (s, 3H, OCH_3); 3.87 (s, 3H, OCH_3); 4.99 (s, 2H, CH_2); 6.31 (s, 2H, Ar); 6.69 (s, 2H, Ar); 6.88–6.95 (m, 2H, Ar); 7.13–7.24 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$): δ 156.73, 153.49, 153.11, 137.99, 136.54, 133.08, 130.58, 130.25, 127.34, 120.97, 111.97, 105.82, 104.71, 70.35, 60.88, 56.27, 56.15, 36.90, 33.00, 29.80. Anal. ($C_{26}H_{30}O_6$) C, H.

*5.1.1.7. 1-[(3,5-Dimethoxybenzyl)oxy]-2-[2-(3,4,5-trimethoxyphenyl) ethyl]-benzene (**11**).* Purified with hexane/AcOEt (70:30) (27% yield) as an oil. ^{1}H NMR ($CDCl_3$): δ 2.82–3.03 (m, 4H, CH_2); 3.79 (s, 9H, OCH_3); 3.83 (s, 6H, OCH_3); 5.04 (s, 2H, CH_2); 6.37 (s, 2H, Ar); 6.40–6.44 (m, 1H, Ar); 6.64 (d, 2H, $J = 2.2$ Hz, Ar); 6.94 (d, 2H, $J = 7.7$ Hz, Ar); 7.09–7.25 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$): δ 161.15, 156.73, 153.12, 139.96, 138.14, 136.54, 130.64, 130.27, 127.34, 120.93, 111.94, 105.89, 105.29, 99.74, 70.13, 60.88, 56.15, 55.40, 37.01, 33.17. Anal. ($C_{26}H_{30}O_6$) C, H.

*5.1.1.8. 2-({2-[2-(3-Methoxyphenyl)ethyl]phenoxy}methyl)pyrimidine (**12**).* Purified with hexane/AcOEt (50:50) (50% yield) as an oil. ^{1}H NMR ($CDCl_3$): δ 2.89–3.07 (m, 4H, CH_2CH_2); 3.76 (s, 3H, OCH_3); 5.31 (s, 2H, OCH_2); 6.70–6.92 (m, 5H, Ar); 7.10–7.28 (m, 4H, Ar); 8.78–8.80 (d, 2H, $J = 4.8$ Hz, Pyrimidine). ^{13}C NMR ($CDCl_3$): δ 219.06, 166.54, 159.57, 157.37, 156.67, 144.33, 130.95, 120.15, 129.11, 127.14, 121.11, 119.93, 114.47, 112.23, 111.14, 71.37, 55.22, 36.39, 32.86. Anal. ($C_{20}H_{20}N_2O_2$) C, H, N. UV–vis (solvent: PBS) $\lambda = 225$ nm, $\varepsilon = 22{,}560$.

*5.1.1.9. 1-[(2,4-Dichlorobenzyl)oxy]-2-(2-phenylethyl)benzene (**13**).* The crude product was purified by crystallization from MeOH affording **13** (30% yield) as a white solid. mp: 68–70 °C. ^{1}H NMR ($CDCl_3$): δ 2.86–3.05 (m, 4H, CH_2); 5.12 (s, 2H, OCH_2); 6.87–6.96 (m, 2H, Ar); 7.15–7.30 (m, 7H, Ar); 7.43–7.52 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$): δ 156.33, 142.38, 134.21, 134.04, 133.26, 130.86, 130.38, 129.60, 129.34, 128.58, 128.38, 127.41, 125.94, 121.39, 111.99, 66.98, 36.70, 32.79. Anal. ($C_{21}H_{18}Cl_2O$) C, H. UV–vis (solvent: PBS) $\lambda = 230$ nm, $\varepsilon = 16{,}350$.

*5.1.1.10. 5-({2-[2-(3-Methoxyphenyl)ethyl]phenoxy}methyl)1,3benzodioxole (**14**).* Purified with $CHCl_3$ (80% yield) as an oil. ^{1}H NMR ($CDCl_3$): δ 2.81–3.00 (m, 4H, CH_2); (s, 3H, OCH_3); 4.98 (s, 2H, OCH_2); 5.97 (s, 2H, OCH_2O); 6.70–6.94 (m, 8H, Ar); 7.12–7.22 (m, 3H, Ar). ^{13}C NMR ($CDCl_3$): δ 166.73, 159.75, 157.40, 156.84, 144.42, 131.15, 130.24, 129.14, 127.18, 121.21, 119.93, 114.61, 112.45, 111.28, 71.57, 55.31, 36.48, 32.89, 29.87. Anal. ($C_{23}H_{22}O_4$) C, H. UV–vis (solvent: PBS) $\lambda = 225$ nm, $\varepsilon = 22{,}560$.

*5.1.1.11. 2-({2-[2-(3-Methoxyphenyl)ethyl]phenoxy}methyl)thiophene (**15**).* Purified with hexane/AcOEt (80:20) (50% yield) as an oil. ^{1}H NMR ($CDCl_3$): δ 2.80–2.99 (m, 4H, CH_2CH_2); 3.75 (s, 3H, OCH_3); 5.25 (s, 2H, OCH_2); 6.71–6.80 (m, 3H, Ar); 6.87–7.04 (m, 3H, Ar); 7.12–7.34 (m, 5H, Ar). ^{13}C NMR ($CDCl_3$): δ 159.77, 156.46, 144.18, 140.12, 131.11, 130.36, 129.23, 127.25, 126.80, 126.18, 125.88, 121.26, 121.11, 114.30, 112.17, 111.57, 65.58, 55.27, 36.68, 32.95. Anal. ($C_{20}H_{20}SO_2$) C, H. UV–vis (solvent: PBS) $\lambda = 225$ nm, $\varepsilon = 22{,}560$.

*5.1.2. 2-[2-(3-Methoxyphenyl)ethyl]-N-(pyridin-4-ylmethyl)aniline (**16**)*

To a solution of **19** [8] (0.88 mmol) in ethanol (10 mL) was added the pyridine-4-carbaldehyde (0.88 mmol), and the resulting solution was stirred and refluxed for 12 h until the disappearance of aniline. The reaction mixture was then cooled at 0 °C and treated with a solution of $NaBH_4$ (33.3 mg, 0.88 mmol) in H_2O (2 mL). The

suspension was stirred at room temperature for 3 h, and then was concentrated to dryness. The crude product was purified by crystallization from iPrOH and transformation into hydrochloride salt giving **16** (104.1 mg, 0.28 mmol, 32% yield) as a white solid: Mp 88–90 °C. ^{1}H NMR ($CDCl_3$): δ 2.90–2.98 (m, 4H, CH_2); 3.72 (s, 3H, OCH_3); 4.74 (s, 2H, NCH_2); 6.09 (d, 1H, J = 7.8 Hz, Ar); 6.68–6.85 (m, 4H, Ar); 7.02 (t, 1H, J = 7.8 Hz, Ar); 7.17–7.26 (m, 2H, Ar); 7.75 (s, 2H, Py); 8.62 (s, 2H, Py). ^{13}C NMR (CD_3OD): δ 165.70, 161.05, 145.56, 144.70, 142.08, 130.86, 130.17, 128.00, 127.64, 126.51, 122.05, 119.01, 115.33, 112.55, 111.51, 55.63, 47.96, 36.64, 33.85. Anal. ($C_{21}H_{23}N_2ClO$) C, H, N.

5.1.3. 3-Methoxy-N-{2-[2-(3-methoxyphenyl)ethyl]phenyl} benzamide (*17*)

To a solution of aniline **19** [8] (0.44 mmol) and TEA (161 mg, 1.6 mmol) in DCM (7 mL) was added dropwise at 0 °C and under argon the 3-methoxybenzoyl-chloride (1.4 mmol) dissolved in DCM (3 mL). The mixture was stirred for 12 h and then the organic layer was washed with H_2O, dried and concentrated under vacuum to obtain **17** as crude product which was purified by silica gel chromatography eluting with hexane/AcOEt (30:70) to obtain **17** as an oil (99 mg, 0.27 mmol, 62% yield). ^{1}H NMR ($CDCl_3$): δ 2.92 (s, 4H, CH_2); 3.65 (s, 3H, OCH_3); 3.86 (s, 3H, OCH_3); 6.55–6.65 (m, 2H, Ar); 6.72–6.77 (m, 1H, Ar); 7.04–7.21 (m, 4H, Ar); 7.23–7.38 (m, 4H, Ar) 7.71–7.75 (m, 1H, Ar). ^{13}C NMR ($CDCl_3$): δ 165.76, 160.12, 159.95, 142.82, 136.41, 135.61, 134.20, 129.96, 129.65, 129.74, 127.09, 126.14, 125.07, 120.93, 118.86, 118.00, 114.20, 112.88, 112.26, 55.60, 55.18, 37.36, 33.81. Anal. ($C_{23}H_{23}NO_3$) C, H, N.

5.1.4. N-{2-[2-(3-Methoxyphenyl)ethyl]phenyl}nicotinamide (*18*)

Nicotinic acid (1.6 mmol) and $SOCl_2$ (2.6 g, 21.8 mmol) were stirred at 55 °C for 2 h. Then $SOCl_2$ was concentrated under argon and to the residual material in THF (7 mL) was added TEA (167 mg, 1.65 mmol) and aniline **19** (120 mg, 0.81 mmol) in THF (5 mL). The mixture was stirred and refluxed for 48 h. Then the solution was filtered and concentrated under vacuum. Residue was dissolved into DCM, washed with H_2O and the organic layer was concentrated under vacuum affording **18** as crude product. The crude product was purified by transformation into hydrochloride salt and crystallization from iPrOH gave **18** (83.0 mg, 0.22 mmol, 28% yield) as a white solid. Mp 109–111 °C. ^{1}H NMR ($CDCl_3$): δ 2.86–3.07 (m, 4H, CH_2); 3.65 (s, 3H, OCH_3); 6.61–6.73 (m, 3H, Ar); 7.10 (t, 1H, J = 8.0 Hz, Ar); 7.24 (d, 2H, Ar); 7.41–7.44 (m, 1H, Ar), 7.87–7.94 (m, 1H, Ar); 8.69 (d, 1H, J = 4.7 Hz, Ar), 8.98 (d, 1H, J = 8 Hz, Ar), 10.13 (s, 1H, Ar). ^{13}C NMR (CD_3OD): δ 162.93, 161.00, 145.99, 145.01, 144.41, 142.99, 139.09, 135.62, 135.35, 131.21, 130.26, 128.64, 128.59, 128.21, 127.79, 121.81, 115.22, 112.42, 55.57, 37.60, 34.49. Anal. ($C_{21}H_{21}N_2O_2Cl$) C, H, N.

5.1.5. General procedure for the synthesis of arylmethyl(chloro) triphenylphosphorane (*2a–d*)

A stirred solution of arylmethylhalide (31.6 mmol) in CH_3CN (20 mL) was treated with PPh_3 (8.57 g, 32.7 mmol), and the mixture was vigorously stirred, refluxed for 12 h, and then evaporated.

*5.1.5.1. 2,3-Dimethoxybenzyl(bromo)triphenylphosphorane (**2b**).* The crude product was purified by crystallization from $CHCl_3/Et_2O$, affording **2b**, (14.43 g, 30.02 mmol, 95% yield) as a white solid. ^{1}H NMR ($CDCl_3$): δ 3.40 (s, 3H, OCH_3); 3.63 (s, 3H, OCH_3) 4.97 (d, 2H, J = 14.3 Hz, CH_2P), 6.54–6.72 (m, 3H, Ar), 7.49–7.68 (m, 15H, Ph). Anal. ($C_{27}H_{26}PBrO_2$) C, H.

*5.1.5.2. 3,4-Dimethoxybenzyl(chloro)triphenylphosphorane (**2c**).* The crude product was purified by crystallization from $CHCl_3/Et_2O$, affording **2c**, (13.46 g, 30.02 mmol, 95% yield) as a white solid. ^{1}H NMR ($CDCl_3$): δ 3.47 (s, 3H, OCH_3); 3.75 (s, 3H, OCH_3) 5.31 (d, 2H, J = 13.9 Hz, CH_2P), 6.58–6.71 (m, 3H, Ar), 7.54–7.74 (m, 15H, Ph). Anal. ($C_{27}H_{26}PClO_2$) C, H.

*5.1.5.3. 3,4,5-Trimethoxybenzyl(chloro)triphenylphosphorane (**2d**).* The crude product was purified by crystallization from $CHCl_3/Et_2O$, affording **2d**, (8.31 g, 17.38 mmol, 55% yield) as a white solid. ^{1}H NMR: 3.49 (s, 6H, OCH_3); 3.75 (s, 3H, OCH_3); 5.48 (d, 2H, J = 14.1 Hz, CH_2); 6.42 (d, 2H, J = 2.7 Hz, Ar); 7.57–7.81 (m, 15H, Ph). Anal. ($C_{28}H_{28}PClO_3$) C, H.

5.1.6. General procedure for the synthesis of 2-[(E/Z)-2-(phenyl) vinyl]phenol derivatives *3a–d*

A solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (1.17 g, 7.7 mmol), salicylaldehyde (0.9 g, 7.45 mmol), and the appropriated triphenylphosphonium salt **2a–d** (7.45 mmol) in CH_3CN (12 mL) was stirred and refluxed for 12 h. Then the organic layer was concentrated under vacuum and the residual product was diluted with $CHCl_3$ and washed with water, 1 N HCl, and brine. The organic layer was dried and evaporated to afford a mixture of *cis*- and *trans*-stilbene derivatives **3a–d** which were purified by chromatography on a silica gel column.

*5.1.6.1. 2-[(E/Z)-2-(2,3-dimethoxyphenyl)vinyl]phenol (**3b**).* Purified eluting with hexane/AcOEt (70:30) affording **3b** (59% yield). ^{1}H NMR ($CDCl_3$): δ 3.86 (s, 3H, OCH_3); 3.88 (s, 3H, OCH_3); 6.78–6.86 (m, 1H, CH=CH); 6.91–7.18 (m, 4H, Ar, CH=CH); 7.29 (dd, 1H, J = 8.0, 1.5 Hz, Ar); 7.43–7.44 (m, 2H, Ar); 7.59 (dd, 1H, J = 7.7, 1.6 Hz, Ar)ppm.

*5.1.6.2. 2-[(E/Z)-2-(3,4-dimethoxyphenyl)vinyl]phenol (**3c**).* Purified with hexane/AcOEt (70:30) affording **3c** (35% yield) as an oil. ^{1}H NMR ($CDCl_3$): δ 3.90 (s, 3H, OCH_3); 3.94 (s, 3H, OCH_3); 6.79–6.90 (m, 1H, CH=CH); 6.93–7.17 (m, 6H, Ar, CH=CH); 7.21–7.29 (m, 2H, Ar); 7.51 (dd, 1H, J = 7.7, 1.5 Hz, Ar) ppm.

*5.1.6.3. 2-[(Z)-2-(3,4,5-trimethoxyphenyl)vinyl]phenol (**Z–3d**).* Purified with hexane/AcOEt (70:30) affording **3d** (17% yield) as an oil. ^{1}H NMR ($CDCl_3$): δ 3.87 (s, 3H, OCH_3); 3.91 (s, 6H, OCH_3); 6.75 (s, 2H, Ar); 6.82 (dd, 1H, J = 7.9, 1.1 Hz, Ar); 6.90–6.98 (m, 1H, Ar); 7.00 (d, 1H, J = 16.3 Hz, CH=CH); 7.10–7.19 (m, 1H, Ar); 7.28 (d, 1H, J = 16.3 Hz, CH=CH); 7.51 (dd, 1H, J = 7.7, 1.5 Hz, Ar) ppm.

*5.1.6.4. 2-[(E)-2-(3,4,5-trimethoxyphenyl)vinyl]phenol (**E–3d**).* Purified with hexane/AcOEt (70:30) affording **3d** (41% yield) as an oil. ^{1}H NMR ($CDCl_3$): δ 3.60 (s, 6H, OCH_3); 3.81 (s, 3H, OCH_3); 6.45 (s, 2H, Ar); 6.54 (d, 1H, J = 12.0 Hz, CH=CH); 6.68 (d, 1H, J = 12.0 Hz, CH=CH); 6.87–6.95 (m, 2H, Ar); 7.14–7.22 (m, 2H, Ar).

5.1.7. General procedure for the synthesis of 2-[(aryl)ethyl]phenol derivatives *4a–d*

A mixture of the appropriated *cis*- and *trans*-stilbene derivatives **3a–d** (5.75 mmol) was hydrogenated in EtOH (30 mL) in the presence of 10% Pd–C (177 mg, 1.67 mmol) for 12 h. Then the catalyst was filtered off and the solution was evaporated to dryness to obtain the crude products as an oil.

*5.1.7.1. 2-[2-(2,3-Dimethoxyphenyl)ethyl]phenol (**4b**).* The crude product (86% yield) was submitted to the subsequent reaction without any further purification. ^{1}H NMR ($CDCl_3$): δ 2.76–2.85 (m, 4H, CH_2CH_2); 3.89 (s, 3H, OCH_3); 3.92 (s, 3H, OCH_3); 6.79–6.91 (m, 4H, Ar); 6.98–7.18 (m, 3H, Ar).

*5.1.7.2. 2-[2-(3,4-Dimethoxyphenyl)ethyl]phenol (**4c**).* The crude product (75% yield) was submitted to the subsequent reaction

without any further purification. ^{1}H NMR ($CDCl_3$): δ 2.83–2.93 (m, 4H, CH_2CH_2); 3.81 (s, 3H, OCH_3); 3.86 (s, 3H, OCH_3); 6.65 (d, 1H, J = 1.6 Hz, Ar); 6.72–6.89 (m, 4H, Ar); 7.05–7.13 (m, 2H, Ar).

*5.1.7.3. 2-[2-(3,4,5-Trimethoxyphenyl)ethyl]phenol (**4d**).* The crude product (85% yield) was submitted to the subsequent reaction without any further purification. ^{1}H NMR ($CDCl_3$): δ 2.83–2.94 (m, 4H, CH_2CH_2); 3.81 (s, 6H, OCH_3); 3.83 (s, 3H, OCH_3); 6.38 (s, 2H, Ar); 6.74–6.89 (m, 2H, Ar); 7.05–7.13 (m, 2H, Ar).

5.2. Biology

5.2.1. Materials

Cell culture reagents were purchased from Celbio s.r.l. (Milano, Italy). CulturePlate 96/wells plates were purchased from PerkinElmer Life Science; Calcein-AM, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) were obtained from Sigma–Aldrich (Milan, Italy).

5.2.2. Cell cultures

MDCK-MDR1, MDCK-MRP1 cell lines are a gift of Prof. P. Borst, NKI-AVL Institute, Amsterdam, Netherland. Caco-2 cells were a gift of Dr. Aldo Cavallini and Dr. Caterina Messa from the Laboratory of Biochemistry, National Institute for Digestive Diseases, "S. de Bellis", Bari (Italy). All 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.

5.2.3. Calcein-AM experiment

These experiments were carried out as described by Feng et al. [10] with minor modifications. Each cell line (50,000 cells per well) was seeded into black CulturePlate 96/wells plate with 100 μl medium and allowed to become confluent overnight. 100 μl of test compounds were solubilized in culture medium and added to monolayers. 96/Wells plate was incubated at 37 °C for 30 min. Calcein-AM was added in 100 μl of Phosphate Buffered Saline (PBS) to yield a final concentration of 2.5 μM and plate was incubated for 30 min. Each well was washed 3 times with ice cold PBS. Saline buffer was added to each well and the plate was read to Victor 3 (PerkinElmer) at excitation and emission wavelengths of 485 nm and 535 nm, respectively. In these experimental conditions Calcein cell accumulation in the absence and in the presence of tested compounds was evaluated and fluorescence basal level was estimated by untreated cells. In treated wells the increase of fluorescence with respect to basal level was measured. EC_{50} values were determined by fitting the fluorescence increase percentage vs. log [dose].

5.2.4. ATPlite assay

The MDCK-MDR1 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 of test compounds was added. The plate was incubated for 2 h in a humidified atmosphere 5% CO_2 at 37 °C. 50 μl of mammalian cell lysis solution was added to all wells and the plate shaked for 5 min in an orbital shaker. 50 μl of substrate solution was added to all wells and the plate shaked for 5 min in an orbital shaker. The plate was dark adapted for 10 min and the luminescence was measured.

5.2.5. Permeability experiments

5.2.5.1. Preparation of Caco-2 monolayer. Caco-2 cells were seeded onto a Millicell® assay system (Millipore), where a cell monolayer is set in between a filter cell and a receiver plate, at a density of 20,000 cells/well. The culture medium was replaced every 48 h and the cells kept for 21 days in culture. The Trans Epithelial Electrical Resistance (TEER) of the monolayers was measured daily, before and after the experiment, using an epithelial voltohometer (Millicell®-ERS). Generally, TEER values greater than 1000 Ω for a 21day culture, are considered optimal.

5.2.5.2. Drug transport experiment. After 21 days of Caco-2 cell growth, the medium was removed from filter wells and from the receiver plate, which were filled with fresh HBSS buffer (Invitrogen). 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 and reference compounds, were added to the filter well at concentration of 100 μM, while fresh HBSS was added to the receiver plate. The plates were incubated at 37 °C for 120 min. Afterwards, samples were removed from the apical (filter well) and basolateral (receiver plate) side of the monolayer to measure the permeability.

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

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

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

5.2.5.3. Cell viability assay. Determination of cell growth was performed using the MTT assay at 24 h and 48 h. On day 1, 20,000 cells/well were seeded into 96-well plates in a volume of 100 μL. On day 2, the various concentrations (1 nM-1 μM) of compound **10** were added in the absence and presence of 1 μM MC18. In all the experiments, the drug-solvent (DMSO) was added in each control to evaluate a possible solvent cytotoxicity. 1 μM MC18 at 24 h and 48 h did not exert cytotoxicity. After the established incubation time (24 h and 48 h) with compound **10**, MTT (0.5 mg/mL) was added to each well, and after 3 h incubation at 37 °C, the supernatant was removed. The formazan crystals were solubilized using 100 μL of DMSO and the absorbance values at 570 nm were determined on the microplate reader Victor 3 from PerkinElmer Life Sciences.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.02.051.

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