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Iodination of verapamil for a stronger induction of death, through GSH efflux, of cancer cells overexpressing MRP1

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ABSTRACT

The multidrug resistance protein 1 (MRP1), involved in multidrug resistance (MDR) of cancer cells, was found to be modulated by verapamil, through stimulation of GSH transport, leading to apoptosis of MRP1-overexpressing cells. In this study, various iodinated derivatives of verapamil were synthesized, including iodination on the B ring, known to be involved in verapamil cardiotoxicity, and assayed for the stimulation of GSH efflux by MRP1. The iodination, for nearly all compounds, led to a higher stimulation of GSH efflux. However, determination of concomitant cytotoxicity is also important for selecting the best compound, which was found to be 10-fold more potent than verapamil. This will then allow us to design original anti-cancer compounds which could specifically kill the resistant cancer cells.

1. Introduction

The development of multidrug resistance (MDR) is a major cause of chemotherapy failure. This MDR phenotype is mostly related to an increased expression of ATP-binding cassette (ABC) transporters in cancer cell membranes, like P-glycoprotein (P-gp), multidrug resistance protein 1 (MRP1) and/or breast cancer resistance protein (BCRP).[1] These transporters actively expel anti-cancer drugs out of the cell by using the ATP hydrolysis energy, but the efflux mechanism remains unclear. MRP1 transports many anti-cancer drugs and organic anions: a broad range of substrates are usually conjugated either to glutathione (GSH), such as the proinflammatory cysteinyl leukotriene C_4 (LTC_4), or to glucuronate or sulfate.[2] Some cytotoxic drugs like vincristine and vinblastine are co-transported with GSH, and MRP1 can transport the GSH alone. A lot of efforts has been made to identify compounds able to reverse this MDR phenotype. Most of the modulators used for P-gp inhibition failed on MRP1, since P-gp transports cationic/neutral hydrophobic molecules, whereas MRP1 substrates are anionic and rather hydrophilic. Fortunately, a great number of modulators have been discovered for inhibiting MRP1. MK571, a leukotriene antagonist, is known as a reference MRP1 inhibitor.[3] Argosterol and analogs, which are sterols from marine sponge, also inhibit MRP1.[4] Another class of modulators, the flavonoids, including genistein, was shown to inhibit daunorubicin efflux from MRP1-overexpressing cells, while bioflavonoids were reported to stimulate GSH efflux. Another interesting MRP1 modulator is verapamil, which is known as a calcium channel blocker, used in therapy for cardiovascular diseases. This molecule was first described as a reference P-gp inhibitor.[5–8] No reversal in drug sensitivity of MRP1-overexpressing cells was observed with verapamil,[9,10] but it was shown to stimulate GSH efflux by MRP1.[11] Verapamil alone poorly inhibited Leukotriene C_4 transport by MRP1, but this inhibition was markedly increased in the presence of GSH.[11–13] We have shown that verapamil acts as a modulator of MRP1 since it specifically kills MRP1-overexpressing cells, by triggering apoptosis through MRP1-mediated glutathione extrusion.[14] A similar phenomenon was described later on.[15,16] Thus, verapamil activity might be used to elaborate a novel anti-cancer chemotherapeutic strategy in order to target and eliminate MRP1-overexpressing resistant cells within a tumor, taking advantage that it is not transported.[11] This would constitute an original strategy to selectively eradicate resistant cancer cells by comparison to the classical approach using efflux inhibitors. However, one major difficulty in the clinical use of verapamil is its cardiovascular activity. Thus, the design of new verapamil analogs was carried out, we modified the verapamil structure in order to increase its effectiveness on MRP1. The useful amounts of active compound could thus be decreased. Some works[17,18] have shown that modifications on the B moiety of

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verapamil (Fig. 1), particularly the phenyl ring, lead to a lower cardiovascular activity. Iodine atoms were introduced with the aim to potentially increase the affinity for MRP1, as this was observed for iodinated derivatives of chalcones in the case of P-gp.[19]

We then evaluated the ability of new iodinated verapamil derivatives to stimulate MRP1-mediated GSH efflux and to decrease the proliferation of MRP1-overexpressing cells. Our results show that iodination greatly enhances verapamil effectiveness, while length modification of the verapamil linker seems also to be critical. More potent and safer verapamil analogs could then be designed, and further develop in the frame of a new therapeutic strategy aimed at eliminating resistant cancer cells.

2. Results and discussion

Verapamil was shown to specifically kill *mrp1*-transfected BHK-21 cells by triggering apoptosis though MRP1-mediated glutathione extrusion.[14] This constitutes a promising approach since verapamil could act as a therapeutic drug able to kill MRP1-overexpresing resistant cancer cells. One of the major difficulties in using verapamil is related to its cardiovascular activity by blocking calcium channels. Thus, as soon as verapamil was discovered to modulate P-glycoprotein activity, a lot of efforts were spent to synthesize new verapamil analogs with low cardiovascular activity and high affinity for the ABC transporter.[17,18,23–25] We modified verapamil structure (Fig. 1) according to a previous study on the effects of iodinated chalcones derivatives on P-gp,[19] which showed that substitution by various halogen atoms gradually enhanced the binding affinity: I > Br > Cl > F. Furthermore, modifications of the B moiety of verapamil might decrease its cardiovascular activity.[17] In this study, synthesis of modified verapamil derivatives, particularly by iodination of the B ring, has been performed. We have evaluated their activity on both glutathione efflux and cell proliferation, using a cell line transfected by *mrp1*.

2.1. Chemistry

Synthesis of the phenol derivatives was achieved according to Scheme 1. Starting from commercial 4-hydroxyalkylphenols **3–5**, the key step of this synthesis is the selective protection of the phenol group with ethylchloroformate at −78 °C to prepare compounds **6–8**. This allowed a smooth and quantitative O-mesylation of the alcohol function to afford **9–11**. Those protected phenolic B moieties were finally condensed with the amine **2**[20] in methanol with potassium iodide and bicarbonate as a proton scavenger. Surprisingly, in such conditions the protecting group of the phenol was cleanly removed affording the desired compounds **12a–14a**. Iodination was then attempted with chloramine-T and NaI.[21] This method proved to be unsatisfactory because great difficulties were encountered to obtain a pure material. Therefore, we decided to perform this reaction simply with iodine and ammonia in acetonitrile as described by Flanagan et al. (method D1).[22] The target monoiodinated 12b–**14b** and diiodinated **12c–14c** compounds were then isolated as pure forms. The demethylated analogs **15a–c** were obtained easily in a similar manner as shown in Scheme 2 starting from the commercially available tyramine and the mesylate **1** and subsequent iodination (method D1).

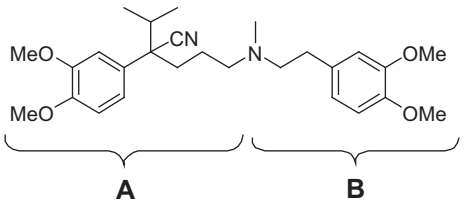


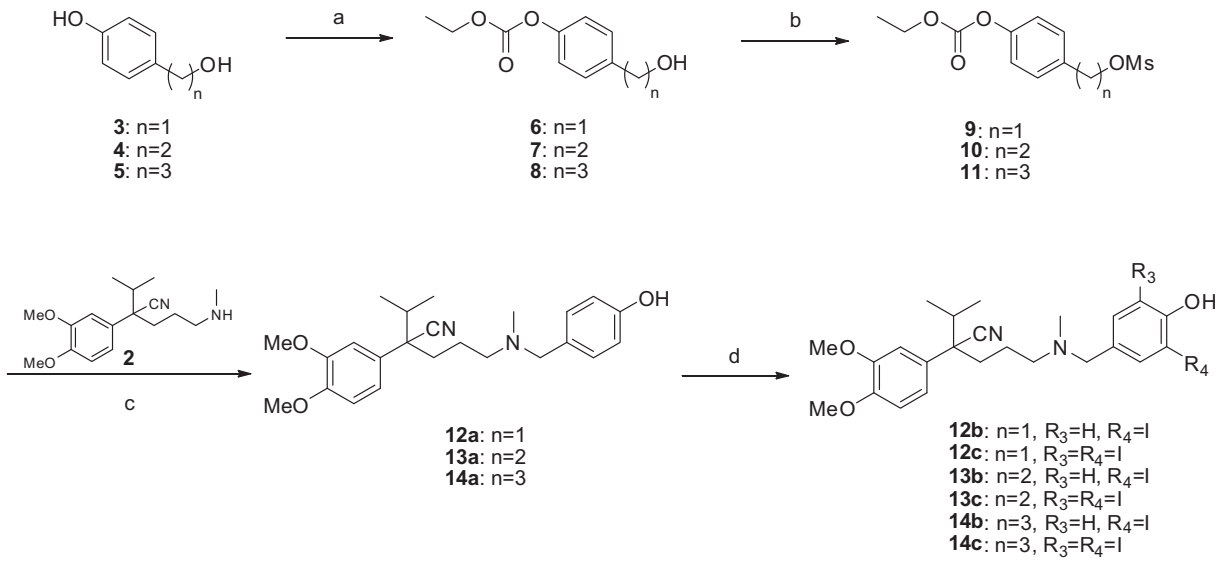
Figure 1. Chemical structure of verapamil.

The synthetic way to the methoxy compounds follows the same general route, which is depicted in Scheme 3. It was shorter since the direct mesylation of the starting compound 4-methoxyphenylethanol **16** could be performed. Alkylation of the amine **2** with the mesylate **21** afforded the desired compound **24a** in a satisfactory yield (51%). The iodinated analogs **24b–c** could not be directly obtained from **24a**, because both rings in A and B showed very similar reactivity towards iodinating agents. Therefore, the iodine atom was introduced earlier in the synthesis before the condensation of the B moiety, on the 4-methoxyphenylethanol **16**. We found that chloramine-T or iodine in ammonia was inefficient to perform iodination; it was necessary to generate the electrophilic I^+ with iodine and sodium periodate in acetic acid to achieve the iodination step (method D2). However, in such conditions, acetylation of the alcohol function was noted (compounds **17** and **18**) and it was necessary to cleave the ester bond with potassium carbonate in methanol to afford the desired iodinated alcohols **19** and **20**, which were easily mesylated. The target compounds **24b–c** were finally prepared in moderate yield.

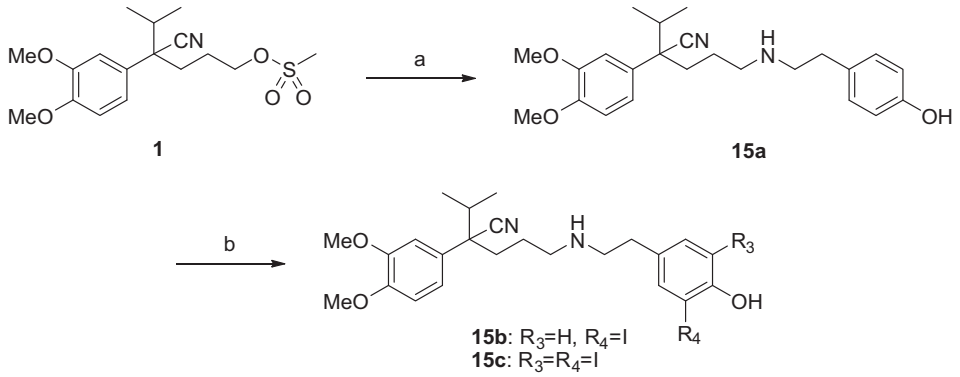
2.2. Structure–activity relationships

The results of glutathione efflux shown in Figure 2 are expressed as changes in the time for which 50% of the intracellular glutathione is lost. Indeed, verapamil was previously described to activate MRP1,[11] leading to a strong and rapid GSH efflux, with 50% of intracellular GSH lost in less than 30 min.[14] Thus, the effectiveness of the verapamil analogs was studied by measuring GSH efflux kinetics, allowing the determination of the time for which 50% of intracellular glutathione is lost. As expected, verapamil, here used at 5 μM, induces a lost of 50% cellular GSH in about 20 min (Fig. 2), whereas no GSH efflux is detected in control BHK-21 cells under the same conditions (not shown here). To investigate the role of B ring substituents, the methoxy group at position R_3 of verapamil has been suppressed, leading to the **24a–c** family of compounds (Table 1). This modification does not change effectiveness, when compared to verapamil, since glutathione efflux is similar in the presence of derivative **24a**. The additional substitution of the methoxy group at position R_2 by a hydroxyl group (Table 1: compounds **13a–c**) was also without effect, since the efflux in presence of derivative **13a** was the same as that obtained with verapamil (Fig. 2). In contrast, the methyl group on the central nitrogen atom at position R_1 is quite important, since we observed a GSH efflux twofold lower in the presence of derivative **15a** (where this methyl group is absent) by comparison with verapamil (Table 1). The length of verapamil linker also appears critical, since compounds **14a** (containing a longer linker than verapamil: $n = 3$) and **12a** (containing a shorter linker: $n = 1$) than verapamil ($n = 2$) (Table 1) failed to strongly activate MRP1, as 50% extrusion of intracellular GSH required up to 50–60 min.

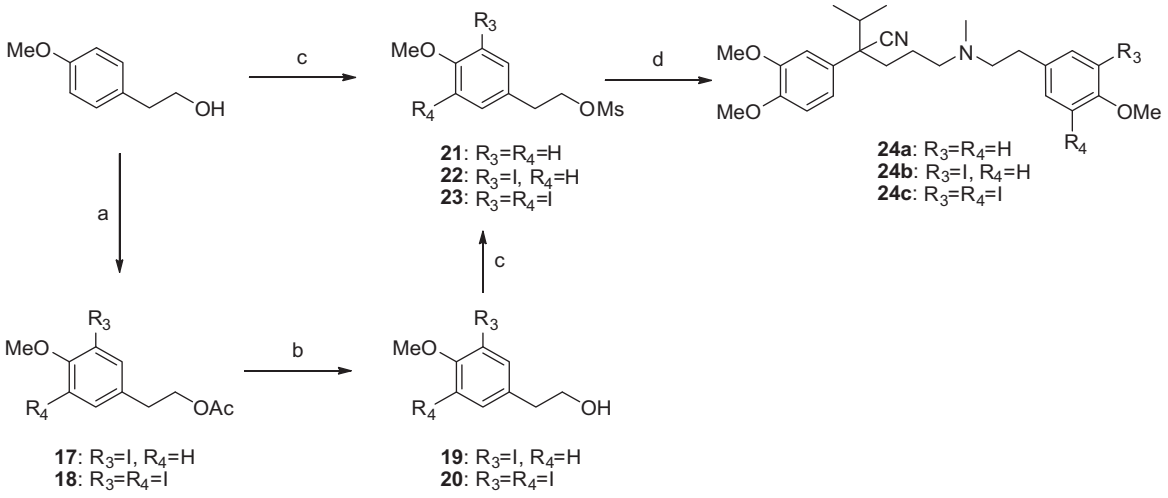
The cytotoxicity of these compounds was checked, as shown in Table 2. We must distinguish non-specific cytotoxicity, as monitored on control BHK-21 cells, from that produced on BHK-MRP1 which results from both non-specific and specific cytotoxicity through MRP1. As a reference, verapamil exhibits an IC_{50} of 10.6 ± 0.5 μM for BHK-MRP1 cells, and up to 100 μM for control cells (Table 2 and Fig. 3), as previously shown.[14] The specific cytotoxicity for BHK-MRP1 of **24a** and **13a** derivatives is not higher than that observed with verapamil (Table 2). Those of **15a** and **14a** derivatives are four times and two times lower, respectively, than that of verapamil. While compound **12a** was slightly more cytotoxic for BHK-MRP1, it was much more cytotoxic for control BHK-21 cells. Specific cytotoxicity of these various compounds showed a close relationship with GSH efflux results. The more



Scheme 1. Preparation of **12a–c**, **13a–c**, and **14a–c** (Table 1). Reagents and conditions: (a) EtOCOCl, NEt_3/CH_3CN, −80 °C; (b) $MeSO_2Cl$, NEt_3/CH_2Cl_2, 0 °C, 2h; (c) $NaHCO_3$/KI, MeOH, 65 °C, 3 days; (d) I_2, CH_3CN/NH_4OH.



Scheme 2. Preparation of **15a–c** (Table 1). Reagents and condition: (a) tyramine, CH_3CN, 82 °C, 20 h; (b) I_2, CH_3CN/NH_4OH.



Scheme 3. Preparation of **24a–c** (Table 1). Reagents and conditions: (a) $I_2/NaIO_4$, $AcOH/H_2SO_4/H_2O$, 60 °C, 1–2 days; (b) K_2CO_3, MeOH, rt, 3 h; (c) $MeSO_2Cl$, pyridine, 0 °C, 3 h; (d) amine **2**, CH_3CN/NEt_3, 82 °C, 6 days.

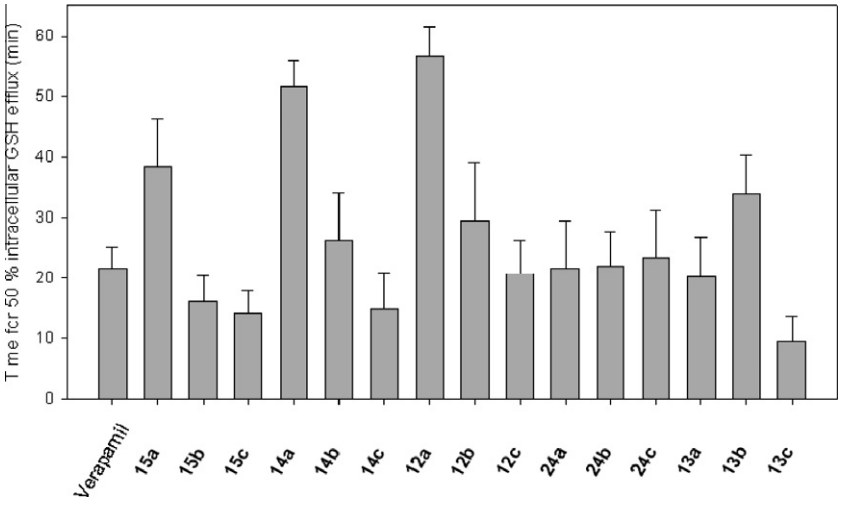
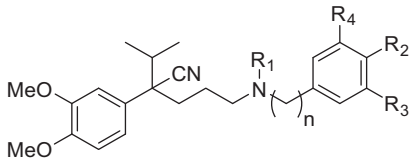


Figure 2. Effects of verapamil derivatives on the GSH content of BHK-21-MRP1 cells. MRP1-BHK-21 cells were cultured with or without 5 μM racemic verapamil or derivatives during various incubation times, from 10 to 120 min. Then total intracellular glutathione levels were determined as described in Section 4. Differences in GSH levels between cells cultured in the absence of compound (taken as 100% intracellular GSH) and cells cultured in the presence of compound (% residual intracellular GSH), corresponding to GSH efflux, were determined at each time. Data represent the values after 30 min incubation, and are the mean of triplicate determinations in a typical experiment; bars, ±SD.

Table 1
Verapamil derivatives



Compound	R_1	R_2	R_3	R_4	*n*
12a	Me	OH	H	H	1
12b	Me	OH	I	H	1
12c	Me	OH	I	I	1
13a	Me	OH	H	H	2
13b	Me	OH	I	H	2
13c	Me	OH	I	I	2
14a	Me	OH	H	H	3
14b	Me	OH	I	H	3
14c	Me	OH	I	I	3
15a	H	OH	H	H	2
15b	H	OH	I	H	2
15c	H	OH	I	I	2
24a	Me	OMe	H	H	2
24b	Me	OMe	I	H	2
24c	Me	OMe	I	I	2

R_1 is the substituent linked to the central nitrogen atom. R_2, R_3, and R_4 are the substituents of the **B** moiety. *n* is the length of the linker hydrocarbon chain between the central nitrogen atom and the benzene ring **B**.

Table 2
Cytotoxic effects of verapamil derivatives on BHK-21 and BHK-21-MRP1 cells

Compound	IC_{50} (μM)	
	BHK-21-MRP1	BHK-21
Verapamil	10.6 ± 0.5	⩾100
24a	17.2 ± 1.0	33.6 ± 7.7
24b	4.7 ± 0.6	13.0 ± 0.5
24c	1.7 ± 0.9	8.9 ± 0.3
13a	11.7 ± 0.3	55.9 ± 0.8
13b	8.6 ± 1.2	32.8 ± 0.9
13c	1.1 ± 0.9	54.7 ± 7.1
14a	20.9 ± 1.4	⩾100
14b	9.3 ± 1.4	9.4 ± 0.2
14c	6.0 ± 0.8	33.1 ± 5.1
12a	6.6 ± 0.9	28.5 ± 1.6
12b	13.0 ± 1.2	14.1 ± 0.3
12c	6.6 ± 1.2	27.4 ± 2.6
15a	38.1 ± 0.3	⩾100
15b	0.78 ± 0.7	14.2 ± 0.3
15c	1.96 ± 0.4	13.8 ± 0.5

Data are means ± SD of experiments performed in triplicate.

rapidly intracellular GSH was extruded, the more cytotoxic for BHK-MRP1 was the compound. These results confirm the assumption that the rapidity of GSH efflux indeed induces the death of cells.

These effects were highly modulated by iodination. With the **24a–c** family compounds, iodination was without effect on GSH efflux, but it strongly increased cytotoxicity for control cells. Di-iodination of compound **13a**, leading to compound **13c**, led to a higher GSH efflux and an increased cytotoxic effect on BHK-MRP1, despite a slightly increased cytotoxic effect on control BHK-21 cells. The specific cytotoxic effects produced on BHK-MRP1 by iodinated **12b–c**, **14b–c**, **15b–c** derivatives increased as GSH was extruded more rapidly. However, cytotoxic effect on control BHK-21 was also stronger. Iodination of the compounds tends to increase their toxicity. However, one of them, the diiodinated compound **13c** is very efficient, with respective IC_{50} values of 1.1 ± 0.9 and 54.7 ± 7.1 μM for BHK-MRP1 and BHK-21 control cell lines, and a glutathione efflux strongly enhanced in BHK-MRP1 cells.

3. Conclusions

Only few works have been reported on novel MRP1 verapamil analogs. Structure–activity relationship of dithiane compounds showed that the most lipophilic were the best inhibitors of LTC_4 transport.[26] We have designed here new verapamil analogs in order to enhance glutathione transport activity and to decrease proliferation of *mrp1*-transfected cells. When studies of MRP1 modulation are undertaken, it is crucial to measure in parallel GSH efflux and cytotoxicity in order to only select the MRP-effective compounds. We have modified the B ring of verapamil in order to get compounds with lower cardiovascular activity. The informations collected with these data suggest that iodination of the B ring of verapamil could be crucial for an increased activity. We previously demonstrated that verapamil directly binds to MRP1[27] and thus induces GSH transport stimulation, results which are in agreement with the fact that verapamil is not transported by MRP1.[11] We could

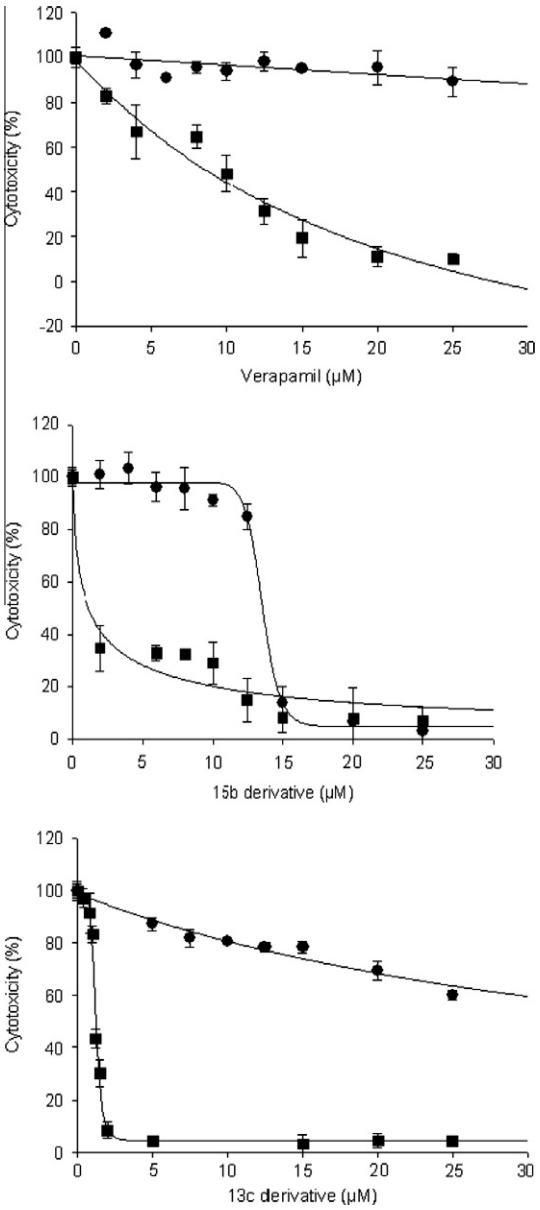


Figure 3. Cytotoxicity of verapamil derivatives on BHK-21 and BHK-21-MRP1 cells. BHK-21 control cells (circles) and MRP1-BHK-21 cells (squares) were cultured in the presence of increasing concentrations of racemic verapamil or verapamil derivative for 96 h. Cytotoxicity was measured by the MTT assay, and data were analyzed by using a sigmoidal fitting. Results are the mean of triplicate experimental points; bars, ±SD.

make the assumption that iodination of verapamil enhances its affinity for MRP1. Upon iodine addition, cell proliferation might be highly decreased and glutathione efflux strongly enhanced in BHK-MRP1. Cytotoxicity remains low in control BHK-21 cells when methoxy group in position R_2 of the B ring is suppressed, as for compound **13c**. Such an information is important for designing new verapamil analogs. Furthermore, the length modification of verapamil linker seems to be critical for MRP1, whereas it was not for P-glycoprotein since a large range in length was tolerated.[23] An important feature was brought by Perrotton et al.[27] who showed that only the *S*-isomer of verapamil is responsible of the GSH transport stimulation. From the new findings described in this paper, synthesis of the **13c** derivative of *S*-verapamil is to be considered; a very high efficiency towards MRP1 might be expected for further experiments with such a new derivative.

4. Experimental

4.1. Materials and methods

^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 (300 MHz for ^1H, 75 MHz for ^{13}C) instrument. Chemical shifts were reported as δ values (ppm) relative to Me_4Si as an internal standard. DCI mass spectra were recorded on a ThermoFinnigan Polaris Q instrument. Melting points were determined on a Büchi 300 apparatus and are uncorrected. IR spectra were recorded on a Nicolet Impact 530 spectrometer. Elemental analyses were performed by the Analytical Department of CNRS, Vernaison, France. Thin-layer chromatography (TLC) was carried out using E. Merck silica gel F-254 plates (thickness 0.20 mm). Flash chromatography was carried out using Merck or Macherey-Nagel silica gel 60 (0.04–0.063 mm). All solvents were distilled prior to use. Chemical and reagents were obtained either from Aldrich or ACROS companies and used without modification. Racemic verapamil, reduced glutathione (GSH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5.5′-dithiobis(2-nitrobenzoic acid) (DTNB), β-nicotinamide adenine dinucleotide phosphate reduced (β-NADPH), penicillin–streptomycin, and fetal bovine serum were obtained from Sigma (Saint Quentin Fallavier, France), Triton X-100 was from Merck (Darmstadt, Germany), methotrexate from Rhône-Poulenc (Montrouge, France). Glutathione Reductase (EC 1.6.4.2) was from Roche Applied Sciences (Meylan, France).

4.2. Chemistry

4.2.1. General procedure for the protection of phenol with ethylchloroformate (method A)

A solution of the appropriate phenol in CH_3CN (40 mL) and NEt_3 (1 equiv) was cooled at −80 °C, under argon. A solution of ethylchloroformate (1 equiv) in CH_3CN (10 mL) was added and the stirred reaction mixture was allowed to warm to room temperature. After adding water (80 mL), the aqueous solution was extracted with ether (3× 70 mL). The combined organic layers were washed with brine (70 mL) and dried over anhydrous Na_2SO_4. Removal of the solvent under reduced pressure gave an oily residue which was purified by silica gel column chromatography (CH_2Cl_2/AcOEt = 1:1) to afford protected phenol.

4.2.2. General procedure for mesylation of alcohols in dichloromethane and triethylamine (method B1)

Mesyl chloride (1.3 equiv) was added to an ice-bath cooled solution of the appropriate alcohol in CH_2Cl_2 (30 mL) and NEt_3 (1 equiv). After stirring at 0 °C for 2 h, the reaction mixture was allowed to warm at room temperature, then washed successively with 0.2 M $NaHCO_3$ solution (2× 30 mL), water (30 mL), brine (30 mL) and dried over anhydrous Na_2SO_4. Removal of the solvent in vacuo gave the mesylated alcohol, which was used as such in the next step.

4.2.3. General procedure for mesylation of alcohols in pyridine (method B2)

Mesyl chloride was carefully added to an ice-bath cooled solution of the appropriate alcohol in pyridine (10–15 mL), (1.4 equiv). The mixture was stirred at 0 °C for 3 h and at room temperature overnight. After adding 0.1 M sulfuric acid solution (50 mL), the aqueous phase was extracted with ether (3× 50 mL). Combined organic phases were washed successively with saturated $CuSO_4$ solution (3× 50 mL), water (2× 50 mL), brine (50 mL), then dried with anhydrous Na_2SO_4 and concentrated in vacuo, yielding mesylated alcohol.

4.2.4. General procedure for alkylation of amine using $NaHCO_3$/KI in MeOH (method C1)

The appropriate mesylate (1.2 equiv), sodium hydrogenocarbonate $NaHCO_3$ (1.8 equiv), and potassium iodide KI (0.5 equiv) were added to a solution of amine **7** in methanol (20 mL). The reaction mixture was then refluxed under argon for 3 days. After adding water (60 mL), the resulting aqueous solution was successively acidified to pH 1 by adding concentrated HCl, washed with ether (3× 60 mL), basified to pH 10 by adding 22% ammonia and finally extracted with ether (3× 60 mL). These last combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The oily residue was then purified by silica gel column chromatography to afford alkylated amine.

4.2.5. General procedure for alkylation of amine using CH_3CN/NEt_3 (method C2)

A mixture of the amine **7** and of the appropriate mesylated alcohol (1 equiv) in acetonitrile (10 mL), and triethylamine (3 equiv) was refluxed, under argon, for 6 days. (Reaction followed by TLC, elution with CH_2Cl_2/*i*PrNH$_2$ = 9.6:0.4.) The reaction was then poured into water (30 mL) and extracted with ether (3× 30 mL). Combined organic phases were washed with brine (40 mL), dried over anhydrous Na_2SO_4 and concentrated in vacuo. The oily residue was purified by silica gel column chromatography (CH_2Cl_2/*i*PrNH$_2$ = 9.6:0.4) to provide alkylated amine.

4.2.6. General procedure for the iodination of phenol with I_2 in CH_3CN/NH_4OH (method D1)

A 0.05 M iodine solution in acetonitrile (1.1 equiv for monoiodination and 2.3 equiv for diiodination), and 22% ammonia (1 mL for 5 mL of iodine solution) were added to the appropriate phenol. The reaction mixture was stirred at room temperature under argon for 2 days. The solution was then poured into water (30 mL) and the aqueous solution was extracted with ether (3× 40 mL). Combined organic layers were washed successively with 0.2 M $Na_2S_2O_3$ solution (3× 40 mL), brine (50 mL) and were dried over anhydrous Na_2SO_4. Removal of the solvent under reduced pressure provided an oily residue which was purified by silica gel column chromatography to afford mono- or diiodinated phenol.

4.2.7. General procedure for the iodination with $I_2/NaIO_4$ in $AcOH/H_2SO_4/H_2O$ (method D2)

Iodine I_2 and sodium periodate were added to a solution of the appropriate compound in acetic acid (12 mL), water (2 mL), and sulfuric acid (0.2 mL). The reaction mixture was heated at 60 °C, under argon, for 1–2 days, then allowed to warm at room temperature and poured into water (50 mL). The aqueous solution was then extracted with ether (4× 50 mL). Combined organic layers were washed successively with 0.2 M $NaHCO_3$ solution (3× 50 mL), 0.2 M $Na_2S_2O_3$ solution (3× 50 mL), brine (60 mL), and were dried over anhydrous Na_2SO_4. Removal of the solvent under reduced pressure provided an oily residue which was purified by silica gel column chromatography (pentane/AcOEt = 9.6:0.4) to afford the iodinated compound.

4.2.8. Carbonic acid ethyl ester 4-hydroxymethyl-phenyl ester (6)

A solution of 4-hydroxymethylphenol **3** (4.03 g, 32 mmol) was treated according to method A to afford compound **6** (5.06 g, 79% yield) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 1.39 (t, 3H, J = 7.17 Hz), 1.6–1.7 (OH), 4.32 (q, 2H, J = 7.17 Hz), 4.68 (s, 2H), 7.17 (d, 2H, J = 8.19 Hz), 7.38 (d, 2H, J = 8.19 Hz); ^{13}C NMR ($CDCl_3$) δ 14.2, 64.7, 64.8, 121.2, 128.0, 138.6, 150.5, 153.6; IR (KBr, cm^{-1}) 3100–3600, 3030, 3012, 2987, 2938, 2914, 2873, 1763, 1600, 1510, 1371, 1257, 1210 1061, 1020; MS (m/z) 107, 179; 196 (M^+); 214 ($M + NH_4^+$, 100%).

4.2.9. Carbonic acid ethyl ester 4-(2-hydroxy-ethyl)-phenyl ester (7)

4-(2-hydroxy-ethyl)-phenol **4** (2.77 g, 20 mmol) was treated with ethylchloroformate according to method A to afford compound **7** (4.16 g) as yellow oil, in quantitative yield. ^{1}H NMR ($CDCl_3$) δ 1.38 (t, 3H, J = 7.17 Hz), 2.85 (t, 2H, J = 6.53 Hz), 3.82 (q, 2H, J = 6.53 and 6.27 Hz), 4.30 (q, 2H, J = 7.17 Hz), 7.11 (d, 2H, J = 8.58 Hz), 7.23 (d, 2H, J = 8.58 Hz); ^{13}C NMR ($CDCl_3$) δ 14.1, 38.5, 63.4, 64.8, 121.1, 129.9, 136.4, 149.7, 153.7; IR (KBr, cm^{-1}) 3100–3600, 3050, 2984, 2930, 2875, 1761, 1508, 1370, 1256, 1217, 1200, 1057, 1018; MS (m/z) 193, 228 ($M + NH_4^+$, 100%); Anal. Calcd for $C_{11}H_{14}O_4$: C, 62.85; H, 6.71; O, 30.44. Found: C, 62.71; H, 6.78; O, 30.05.

4.2.10. Carbonic acid ethyl ester 4-(3-hydroxy-propyl)-phenyl ester (8)

Compound **8** was prepared following method A from 4-(3-hydroxy-propyl)-phenol **5** (4.34 g, 28 mmol) and was obtained as a pale yellow oil (6.30 g, 98% yield). ^{1}H NMR ($CDCl_3$) δ 1.38 (t, 3H, J = 7.17 Hz), 1.5–1.7 (sl, OH), 1.86 (m, 2H), 2.70 (t, 2H, J = 7.43 Hz), 3.65 (t, 2H, J = 6.4 Hz), 4.30 (q, 2H, J = 7.17 Hz), 7.08 (d, 2H, J = 8.7 Hz), 7.19 (d, 2H, J = 8.7 Hz); ^{13}C NMR($CDCl_3$) δ 14.1, 31.3, 34.1, 62.0, 64.7, 120.8, 129.3, 139.5, 149.2, 153.8; IR (KBr, cm^{-1}) 3100–3600, 3053, 2987, 2938, 2865, 1763, 1510, 1371, 1257, 1206, 1069, 1020; MS (m/z) 107, 135, 242 ($M + NH_4^+$, 100%); Anal. Calcd for $C_{12}H_{16}O_4$: C, 64.27; H, 7.19; O, 28.54. Found: C, 64.26; H, 7.44; O, 28.25.

4.2.11. Methanesulfonic acid 4-ethoxycarbonyloxy-benzyl ester (9)

Alcohol **6** (5.05 g; 26 mmol) was treated with mesyl chloride according to method B1 to afford compound **9** (7.03 g, 100% yield) as a white solid. Mp 60–61 °C; ^{1}H NMR ($CDCl_3$) δ 1.39 (t, 3H, J = 7.17 Hz), 2.94 (s, 3H), 4.33 (q, 2H, J = 7.17 Hz), 5.23 (s, 2H), 7.23 (d, 2H, J = 8.7 Hz), 7.45 (d, 2H, J = 8.7 Hz); ^{13}C NMR ($CDCl_3$) δ 14.1, 38.4, 65.0, 70.5, 121.6, 130.1, 131.1, 151.8, 153.3; IR (KBr, cm^{-1}) 3100–3600, 3040, 2993, 2935, 1755, 1511, 1364, 1270, 1238, 1175, 1060, 985; MS (m/z) 107, 179, 196, 292 ($M + NH_4^+$, 100%); Anal. Calcd for $C_{11}H_{14}O_6S{\cdot}1/3H_2O$: C, 47.14; H, 5.27. Found: C, 47.26; H, 5.68.

4.2.12. Methanesulfonic acid 2-(4-ethoxycarbonyloxy-phenyl)-ethyl ester (10)

Alcohol **7** (3.50 g, 16 mmol) was converted into mesylate **10** (4.78 g) as described in method B1 and was obtained as a brown solid, in quantitative yield. Mp 33–34 °C; ^{1}H NMR ($CDCl_3$) δ 1.39 (t, 3H, J = 7.17 Hz), 2.86 (s, 3H), 3.06 (t, 2H, J = 6.79 Hz), 4.31 (q, 2H, J = 7.17 Hz), 4.41 (t, 2H, J = 6.79 Hz), 7.14 (d, 2H, J = 8.58 Hz), 7.25 (d, 2H, J = 8.58 Hz); ^{13}C NMR ($CDCl_3$) δ 14.2, 35.0, 37.4, 64.8, 69.9, 121.3, 130.0, 134.1, 150.2, 153.6; IR (KBr, cm^{-1}) 3100–3600, 3044, 2995, 2930, 1755, 1510, 1363, 1273, 1240, 1175, 1061, 987; MS (m/z) 306 ($M + NH_4^+$, 100%); Anal. Calcd for $C_{12}H_{16}O_6S$: C, 49.99; H, 5.59; O, 33.30. Found: C, 50.00; H, 5.70; O, 32.99.

4.2.13. Methanesulfonic acid 3-(4-ethoxycarbonyloxy-phenyl)-propyl ester (11)

A solution of alcohol **8** (6.29 g; 28 mmol) was treated according to method B1 to afford compound **11** (8.06 g, 95% yield) as a white solid. Mp 40–41 °C; ^{1}H NMR ($CDCl_3$) δ 1.38 (t, 3H, J = 7.17 Hz), 2.06 (m, 2H), 2.75 (t, 2H, J = 7.16 Hz), 2.98 (s, 3H), 4.23 (t, 2H, J = 6.14 Hz), 4.31 (q, 2H, J = 7.17 Hz), 7.11 (d, 2H, J = 8.44 Hz), 7.19 (d, 2H, J = 8.44 Hz); ^{13}C NMR ($CDCl_3$) δ 14.2, 30.6, 30.9, 37.4, 64.8, 68.9, 121.1, 129.4, 138.1, 149.6, 153.7; IR (KBr, cm^{-1}) 3045, 3004, 2947, 2938, 2865, 1755, 1518, 1346, 1257, 1216, 1167, 1004, 930; MS (m/z) 107, 135, 207, 303 ($M+H^+$), 320 ($M + NH_4^+$, 100%);

Anal. Calcd for $C_{13}H_{18}O_6S$: C, 51.64; H, 6.00; O, 31.75. Found: C, 51.54; H, 6.15; O, 31.68.

4.2.14. 2-(3,4-Dimethoxy-phenyl)-5-[(4-hydroxy-benzyl)-methyl-amino]-2-isopropyl-pentanenitrile (12a)

Amine **2** (233 mg, 0.77 mmol) was treated with compound **9** (320 mg, 1.17 mmol) according to method C1 (heating for only 20 h). Purification by column chromatography (elution CH_2Cl_2/*i*PrNH$_2$ = 4.8:0.2) provided compound **12a** (0.230 g, 76% yield) as a pale yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.79 (d, 3H, J = 6.66 Hz), 1.17 (d, 3H, J = 6.66 Hz), 1.15–1.30 (m, 1H), 1.47–1.62 (m, 1H, 1.78–1.90 (m, 1H), 2.00–2.20 (m, 2H), 2.07 (s, 3H), 2.23–2.36 (m, 2H), 3.32 (s, 2H), 3.85 (s, 3H), 3.87 (s, 3H), 6.71 (m, 2H), 6.82 (d, 1H, J = 8.45 Hz), 6.83 (d, 1H, J = 2.18 Hz), 6.91 (dd, 1H, J = 8.45 and 2.18 Hz), 7.07 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 18.6, 18.9, 23.2, 35.5, 37.9, 41.9, 53.3, 55.9, 56.0, 56.4, 61.4, 109.5, 111.1, 115.1, 118.8, 121.5, 130.3, 130.7, 148.3, 149.0, 154.9; IR (KBr, cm^{-1}) 3300–3600, 3059, 2964, 2938, 2875, 2837, 2236, 1613, 1517, 1465, 1415, 1260, 1148, 1025, 914, 732; MS (*m*/*z*) 291, 397 (M$^+$), 398 (M+H$^+$); Anal. Calcd for $C_{24}H_{32}N_2O_3 \cdot 1/2H_2O$: C, 71.08; H, 8.20; N, 6.91. Found: C, 71.30; H, 8.27; N, 7.22.

4.2.15. 2-(3,4-Dimethoxy-phenyl)-5-[(4-hydroxy-3-iodo-benzyl)-methyl-amino]-2-isopropyl-pentanenitrile (12b)

Phenol **12a** (260 mg, 0.66 mmol) was iodinated by an I_2 solution in CH_3CN (12 mL, 1 equiv) and ammonia (2 mL), following method D1. Purification by column chromatography (AcOEt/CH_2Cl_2/MeOH = 5:4:1) afforded compound **12b** (105 mg, 31% yield) as a pale yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.79 (d, 3H, J = 6.65 Hz), 1.19 (d, 3H, J = 6.65 Hz), 1.11–1.28 (m, 1H), 1.45–1.62 (m, 1H), 1.78–1.92 (m, 1H), 2.00–2.20 (m, 2H), 2.07 (s, 3H), 2.21–2.33 (m, 2H), 3.28 (s, 2H), 3.87 (s, 3H), 3.88 (s, 3H), 6.81–6.95 (m, 4H), 7.10 (dd, 1H, J = 8.45 and 1.92 Hz), 7.58 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 18.6, 18.9, 23.3, 35.5, 38.0, 41.9, 53.3, 55.9, 56.0, 56.4, 60.7, 85.5, 109.6, 111.1, 114.6, 118.7, 121.5, 130.7, 133.9, 138.4, 148.3, 149.0, 153.5; IR (KBr, cm^{-1}) 3300–3600, 3053, 2971, 2938, 2881, 2832, 2791, 2236, 1600, 1510, 1477, 1420, 1265, 1159, 1036, 751; MS (*m*/*z*) 291, 523 (M+H$^+$, 100%); Anal. Calcd for $C_{24}H_{31}IN_2O_3 \cdot 1/2H_2O$: C, 54.24; H, 6.07; N, 5.27. Found: C, 54.11; H, 6.06; N, 5.15.

4.2.16. 2-(3,4-Dimethoxy-phenyl)-5-[(4-hydroxy-3,5-diiodo-benzyl)-methyl-amino]-2-isopropyl-pentanenitrile (12c)

Phenol **12a** (206 mg, 0.52 mmol) was treated by I_2 (30 mL of 0.05 M I_2 solution in CH_3CN, 3 equiv) and ammonia (7 mL) according to method D1. Purification by column chromatography (ether) afforded compound **12c** (102 mg, 30% yield) as a white solid. Mp 88 °C; ^{1}H NMR ($CDCl_3$) δ 0.81 (d, 3H, J = 6.65 Hz), 1.21 (d, 3H, J = 6.65 Hz), 1.08–1.25 (m, 1H), 1.45–1.62 (m, 1H), 1.78–1.92 (m, 1H), 2.00–2.20 (m, 2H), 2.07 (s, 3H), 2.20–2.35 (m, 2H), 3.25 (s, 2H), 3.88 (s, 6H), 6.83–6.86 (m, 2H), 6.92 (dd, 1H, J = 8.19 and 1.92 Hz), 7.59 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 18.7, 19.0, 23.3, 35.5, 38.0, 41.9, 53.3, 55.9, 56.0, 56.4, 60.1, 82.0, 109.6, 111.2, 118.7, 121.4, 130.7, 133.9, 139.4, 148.3, 149.0, 153.5; IR (KBr, cm^{-1}) 3200–3600, 3030, 2959, 2931, 2878, 2835, 2236, 1517, 1464, 1259, 1146, 1025, 803; MS (*m*/*z*) 291 (100%), 649 (M+H$^+$); Anal. Calcd for $C_{24}H_{30}I_2N_2O_3$: C, 44.46; H, 4.66; N, 4.32. Found: C, 44.66; H, 4.79; N, 4.31.

4.2.17. 2-(3,4-Dimethoxy-phenyl)-5-{[2-(4-hydroxy-phenyl)-ethyl]-methyl-amino}-2-isopropyl-pentanenitrile (13a)

Mixture of amine **2** (590 mg, 2.03 mmol) and compound **10** (706 mg, 2.45 mmol) was treated following method C1. Purification of crude product (elution AcOEt/CH_2Cl_2/MeOH = 5:3.5:1.5) afforded compound **13a** (0.250 g, 30% yield) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.78 (d, 3H, J = 6.78 Hz), 1.17 (d, 3H, J = 6.78 Hz), 1.10–1.30 (m, 1H), 1.46–1.62 (m, 1H), 1.83 (m, 1H, J = 4.47 Hz), 2.00–2.16 (m, 2H), 2.19 (s, 3H), 2.30–2.42 (m, 2H), 2.43–2.53 (m, 2H), 2.60–2.68 (m, 2H), 3.87 (s, 6H), 6.71 (d, 2H, J = 8.32 Hz), 6.82 (d 1H, J = 8.32 Hz), 6.85 (d, 1H, J = 2.31 Hz), 6.90 (dd, 1H, J = 8.32 and 2.31 Hz), 6.98 (d, 2H, J = 8.32 Hz); ^{13}C NMR ($CDCl_3$) δ 18.6, 18.9, 23.1, 32.4, 35.6, 37.9, 41.8, 53.3, 55.9, 56.0, 56.8, 59.4, 109.6, 111.1, 115.3, 118.7, 121.4, 129.7, 130.6, 132.1, 148.3, 149.0, 154.2; IR (KBr, cm^{-1}) 3100–3500, 2963, 2930, 2873, 2840, 2236, 1591, 1518, 1469, 1412, 1257, 1151, 1028; MS (*m*/*z*) 107, 303 (100%), 411 (M+H$^+$); Anal. Calcd for $C_{25}H_{34}N_2O_3$: C, 73.14; H, 8.35; N, 6.82. Found: C, 72.97; H, 8.29; N, 7.22.

4.2.18. 2-(3,4-Dimethoxy-phenyl)-5-{[2-(4-hydroxy-3-iodo-phenyl)-ethyl]-methyl-amino}-2-isopropyl-pentanenitrile (13b)

A mixture of compound **13a** (130 mg, 0.32 mmol), iodine solution in CH_3CN (10 mL, 1.4 equiv) and ammonia (3 mL) was treated following method D1. Purification by column chromatography (CH_2Cl_2/*i*PrNH$_2$ = 9.6:0.4) provided monoiodinated phenol **13b** (37 mg, 22% yield) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.78 (d, 3H, J = 6.66 Hz), 1.17 (d, 3H, J = 6.66 Hz), 1.10–1.30 (m, 1H), 1.50–1.60 (m, 1H), 1.87 (m, 1H, J = 4.61 Hz), 2.00–2.20 (m, 2H), 2.21 (s, 3H), 2.35–2.50 (m, 2H), 2.50–2.60 (m, 2H), 2.60–2.70 (m, 2H), 3.87 (s, 3H), 3.88 (s, 3H), 4.0–5.0 (OH), 6.75–6.95 (m, 4H), 6.99 (dd, 1H, J = 8.19 and 1.92 Hz), 7.46 (d, 1H, J = 1.92 Hz); ^{13}C NMR ($CDCl_3$) δ 18.6, 18.9, 22.9, 31.7, 35.4, 37.9, 41.5, 53.3, 55.9, 56.0, 56.7, 58.9, 85.4, 109.6, 111.1, 115.0, 118.7, 121.4, 130.3, 130.5, 133.9, 138.2, 148.3, 149.0, 153.5; IR (KBr, cm^{-1}) 3030, 2971, 2930, 2881, 2865, 2236, 1600, 1518, 1469, 1436, 1265, 1142, 1028; MS (*m*/*z*) 303, 411, 537 (M+H$^+$, 100%); Anal. Calcd for $C_{25}H_{33}IN_2O_3 \cdot H_2O$: C, 54.15; H, 6.36; N, 5.05. Found: C, 54.00; H, 6.46; N, 5.13.

4.2.19. 2-(3,4-Dimethoxy-phenyl)-5-{[2-(4-hydroxy-3,5-diiodo-phenyl)-ethyl]-methyl-amino}-2-isopropyl-pentanenitrile (13c)

Compound **13c** was prepared using method D1 from phenol **13a** (90 mg, 0.22 mmol), iodine (12 mL of 0.05 M solution in CH_3CN, 2.7 equiv) and ammonia (2 mL). Purification by column chromatography (CH_2Cl_2/*i*PrNH$_2$ = 9.6:0.4) afforded diiodinated phenol **13c** (60 mg, 41% yield) as a pale orange oil. ^{1}H NMR ($CDCl_3$) δ 0.78 (d, 3H, J = 6.66 Hz), 1.17 (d, 3H, J = 6.66 Hz), 1.10–1.27 (m, 1H), 1.45–1.62 (m, 1H), 1.78–1.91 (m, 1H, J = 4.61 Hz), 2.00–2.15 (m, 2H), 2.17 (s, 3H), 2.30–2.43 (m, 2H), 2.45–2.53 (m, 2H), 2.55–2.64 (m, 2H), 3.88 (s, 3H), 3.89 (s, 3H), 6.8–6.92 (m, 3H), 7.49 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 18.7, 18.9, 23.1, 31.4, 35.4, 37.9, 41.4, 53.3, 55.9, 56.0, 58.8, 60.4, 82.3, 109.7, 111.2, 118.6, 121.4, 130.6, 136.3, 139.3, 148.3, 149.0, 152.1; IR (KBr, cm^{-1}) 3053, 2963, 2930, 2881, 2873, 2236, 1600, 1510, 1461, 1273, 1142, 1028, 742; MS (*m*/*z*) 303 (100%), 411, 537, 663 (M+H$^+$); Anal. Calcd for $C_{25}H_{32}I_2N_2O_3$: C, 45.33; H, 4.87; N, 4.23. Found: C, 45.53; H, 5.06; N, 4.03.

4.2.20. 2-(3,4-Dimethoxy-phenyl)-5-{[3-(4-hydroxy-phenyl)-propyl]-methyl-amino}-2-isopropyl-pentanenitrile (14a)

Amine **2** (2.02 g, 7 mmol) was alkylated with compound **11** (2.77 g, 9.2 mmol) according to method C1. Purification by column chromatography (AcOEt/CH_2Cl_2/MeOH = 5:3.5:1.5) provided compound **14a** (0.970 g, 33% yield) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.77 (d, 3H, J = 6.66 Hz), 1.14 (d, 3H, J = 6.66 Hz), 1.10–1.20 (m, 1H), 1.45–1.62 (m, 1H), 1.73 (m, 2H), 1.78–1.90 (m, 1H), 1.98–2.10 (m, 2H), 2.11 (s, 3H), 2.25–2.40 (m, 4H), 2.49 (m, 2H), 3.86 (s, 3H), 3.87 (s, 3H), 6.61 (d, 2H, J = 8.45 Hz), 6.70–6.86 (m, 2H, J = 8.32 and 2.11 Hz), 6.88–6.92 (dd, 1H, J = 8.32 and 2.11 Hz), 6.94 (d, 2H, J = 8.45 Hz); ^{13}C NMR ($CDCl_3$) δ 18.5, 18.9, 22.7, 28.6, 32.5, 35.6, 37.8, 41.4, 53.3, 55.8, 55.9, 56.8, 56.9, 109.6, 111.1, 115.3, 118.7, 121.4, 129.3, 130.5, 133.1, 148.3, 149.0, 154.4; IR (KBr, cm^{-1}) 3100–3600, 3010, 2963, 2938, 2873, 2840, 2800,

2236, 1591, 1518, 1469, 1420, 1265, 1159, 1036, 806, 734; MS (*m*/*z*) 425 (M+H$^+$, 100%); Anal. Calcd for $C_{26}H_{36}N_2O_3 \cdot 1/2H_2O$: C, 70.56; H, 8.65; N, 6.33. Found: C, 70.52; H, 8.55; N, 6.15.

4.2.21. 2-(3,4-Dimethoxy-phenyl)-5-{[3-(4-hydroxy-3-iodo-phenyl)-propyl]-methyl-amino}-2-isopropyl-pentanenitrile (14b)

Phenol **14a** (181 mg, 0.43 mmol) was monoiodinated by an 0.05 M I_2 solution (8 mL, 1 equiv) and ammonia (2 mL) as described in method D1. Purification of crude product (elution AcOEt/CH_2Cl_2/MeOH = 5:4:1) provided compound **14b** (60 mg, 27% yield) as a pale yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.79 (d, 3H, *J* = 6.66 Hz), 1.18 (d, 3H, *J* = 6.66 Hz), 1.17–1.2 (m, 1H), 1.48–1.62 (m, 1H), 1.67–1.80 (m, 2H), 1.85–1.98 (m, 1H), 2.00–2.20 (m, 2H), 2.15 (s, 3H), 2.28–2.40 (m, 3H), 2.48 (t, 2H, *J* = 7.55 Hz), 3.88 (s, 3H), 3.89 (s, 3H), 6.82–6.89 (m, 3H), 6.90–6.95 (dd, 1H, *J* = 8.32, 2.17 Hz), 6.98–7.03 (dd, 1H, *J* = 8.19, 2.05 Hz), 7.45 (d, 1H, *J* = 2.05 Hz); ^{13}C NMR ($CDCl_3$) δ 18.6, 18.9, 22.9, 28.3, 32.0, 35.5, 37.9, 41.4, 53.3, 55.9, 56.0, 56.6, 56.8, 85.5, 109.6, 111.2, 118.8, 121.4, 130.0, 130.5, 135.8, 137.8, 148.3, 149.1, 153.2; IR (KBr, cm^{-1}) 3200–3500, 3053, 2971, 2938, 2881, 2236, 1591, 1518, 1412, 1265, 1151, 1028, 742; MS (*m*/*z*) 551 (M+H$^+$, 100%); Anal. Calcd for $C_{26}H_{35}IN_2O_3$: C, 56.73; H, 6.41; N, 5.09. Found: C, 56.80; H, 6.63; N, 4.96.

4.2.22. 2-(3,4-Dimethoxy-phenyl)-5-{[3-(4-hydroxy-3,5-diiodo-phenyl)-propyl]-methyl-amino}-2-isopropyl-pentanenitrile (14c)

Ammonia (4 mL) and iodine solution (16 mL, 2.2 equiv) were added to phenol **14a** (165 mg, 0.39 mmol) following method D1. Purification by column chromatography (AcOEt/CH_2Cl_2/MeOH = 5:3.5:1.5) afforded compound **14c** (133 mg, 51% yield) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.80 (d, 3H, *J* = 6.66 Hz), 1.19 (d, 3H, *J* = 6.66 Hz), 1.20–1.40 (m, 1H), 1.50–1.70 (m, 3H), 1.87–2.10 (m, 1H), 2.50–2.19 (m, 2H), 2.20 (s, 3H), 2.30–2.50 (m, 6H), 6.83–6.97 (m, 3H, *J* = 8.32 and 2.18 Hz), 7.46 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 18.6, 18.9, 22.6, 28.2, 31.4, 35.5, 37.9, 41.2, 53.3, 55.9, 56.1, 56.4, 56.7, 83.7, 109.7, 111.2, 118.8, 121.4, 130.4, 137.3, 138.9, 148.4, 149.1, 152.7; IR (KBr, cm^{-1}) 3010, 2955, 2930, 2865, 2236, 1591, 1518, 1469, 1412, 1265, 1142, 1028, 808; MS (*m*/*z*) 291, 418, 551, 677 (M+H$^+$, 100%); Anal. Calcd for $C_{26}H_{34}I_2N_2O_3$: C, 46.17; H, 5.07; N, 4.14. Found: C, 46.20; H, 5.23; N, 4.10.

4.2.23. 2-(3,4-Dimethoxy-phenyl)-5-[2-(4-hydroxy-phenyl)-ethylamino]-2-isopropyl-pentane nitrile (15a)

Tyramine (265 mg, 1.9 mmol) was added to a stirred solution of mesylated alcohol **1** (330 mg, 0.93 mmol) in dry CH_3CN (10 mL). The reaction mixture was refluxed under argon for 20 h and then allowed to warm at room temperature. After adding water (20 mL), the aqueous solution was acidified to pH 1 by adding concentrated HCl and washed with ether (3× 30 mL). The resulting aqueous phase was then basified to pH 10 by adding 22% ammonia and extracted with ether (3× 30 mL). These combined organic layers were dried with anhydrous Na_2SO_4 and concentrated in vacuo to provide compound **15a** (200 mg, 55% yield) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.77 (d, 3H, *J* = 6.79 Hz), 1.13 (d, 3H, *J* = 6.79 Hz), 1.2–1.3 (m, 1H, *J* = 12.03 Hz), 1.53 (m, 1H, *J* = 12.03 Hz, 1.82 (m, 1H, *J* = 12.03 and 4.48 Hz), 2.03 (m, 1H, *J* = 6.79 Hz), 2.10 (m, 1H, *J* = 12.03 and 4.48 Hz), 2.60 (m, 2H), 2.69 (m, 2H), 2.76 (m, 2H), 3.85 (s, 6H), 3.9–4.3 (OH), 6.68 (d, 2H, *J* = 8.34 Hz), 6.80 (d, 1H, *J* = 8.34 Hz), 6.82 (d, 1H, *J* = 2.16 Hz), 6.87 (dd, 1H, *J* = 8.34 and 2.16 Hz), 6.97 (d, 2H, *J* = 8.34 Hz); ^{13}C NMR ($CDCl_3$) δ 18.5, 18.9, 25.7, 34.8, 35.5, 37.9, 49.1, 50.7, 53.3, 55.9, 56.0, 109.6, 111.2, 115.7, 118.7, 121.3, 129.7, 129.9, 130.5, 148.3, 149.0, 155.1; IR (KBr, cm^{-1}) 3500, 3030, 2963, 2930, 2881, 2832, 2236, 1591, 1518, 1469, 1412, 1257, 1151, 1028; MS (*m*/*z*) 397 (M+H$^+$, 100%); Anal. Calcd for $C_{24}H_{32}N_2O_3 \cdot 2H_2O$: C, 66.64; H, 8.39; N, 6.48. Found: C, 66.94; H, 8.02; N, 6.44.

4.2.24. 2-(3,4-Dimethoxy-phenyl)-5-[2-(4-hydroxy-3-iodo-phenyl)-ethylamino]-2-isopropyl-pentanenitrile (15b)

Compound **15a** (238 mg, 0.6 mmol) was treated by an iodine solution in CH_3CN (13 mL, 1.06 equiv) and NH_4OH (3 mL) according to method D1. Purification of crude product by column chromatography (CH_2Cl_2/*i*PrNH$_2$ = 9.5:0.5) provided monoiodinated phenol **15b** (100 mg, 32% yield) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.78 (d, 3H, *J* = 6.66 Hz), 1.18 (d, 3H, *J* = 6.66 Hz), 1.2–1.3 (m, 1H, *J* = 12.03 Hz), 1.5–1.6 (m 1H, *J* = 12.03 Hz), 1.90 (m, 1H, *J* = 12.03 and 4.48 Hz), 2.07 (m, 1H, *J* = 6.66 Hz), 2.19 (m, 1H, *J* = 12.03 and 4.48 Hz), 2.67 (m, 2H), 2.7–2.9 (m, 4H), 3.87 (s, 3H), 3.89 (s, 3H), 6.84 (m, 3H), 6.90 (dd, 1H, *J* = 2.31 and 8.32 Hz), 7.01 (dd, 1H, *J* = 2.05 and 8.32 Hz), 7.47 (d, 1H, *J* = 2.05 Hz); ^{13}C NMR ($CDCl_3$) δ 18.6, 18.9, 25.0, 33.8, 35.4, 37.9, 48.7, 50.2, 53.3, 55.9, 56.1, 85.7, 109.6, 111.2, 115.0, 118.7, 121.3, 130.3, 130.4, 132.8, 138.2, 148.4, 149.0, 153.8; IR (KBr, cm^{-1}) 2500–3500, 3030, 3000, 2963, 2930, 2881, 2832, 2236, 1591, 1518, 1469, 1412, 1257, 1151, 1028; MS (*m*/*z*). 276, 289, 397 (100%), 523 (M+H$^+$); Anal. Calcd for $C_{24}H_{31}IN_2O_3$: C, 53.34; H, 6.15; N, 5.18. Found: C, 53.41; H, 6.34; N, 5.27.

4.2.25. 2-(3,4-Dimethoxy-phenyl)-5-[2-(4-hydroxy-3,5-diiodo-phenyl)-ethylamino]-2-isopropyl-pentanenitrile (15c)

A 0.05 M I_2 solution in CH_3CN (22 mL, 2 equiv) and 22% ammonia (4 mL) were added to compound **15a** (207 mg, 0.52 mmol). The mixture was stirred at room temperature, under argon, for 16 h. The precipitate formed was filtered and washed with CH_3CN (20 mL). The solid obtained was stirred for 12 h in cyclohexane (20 mL), filtered and dried on KOH in vacuo to afford compound **15c** (147 mg, 44% yield) as a marron solid, which was used with no further purification. ^{1}H NMR (DMSO) δ 0.72 (d, 3H, *J* = 6.60 Hz), 1.14 (d, 3H, *J* = 6.60 Hz), 1.0–1.2 (m, 1H), 1.4–1.6 (m, 1H), 2.0–2.2 (m, 2H), 2.2–2.3 (m, 1H, *J* = 6.60 Hz), 2.6–2.7 (m, 2H), 2.7–2.9 (m, 2H), 2.9–3.0 (m, 2H), 3.79 (s, 3H), 3.80 (s, 3H), 6.9–7.1 (m, 3H), 7.60 (s, 2H); ^{13}C NMR (DMSO) δ 19.3, 19.7, 25.2, 32.5, 35.0, 37.5, 48.5, 50.4, 53.8, 56.4, 56.7, 88.6, 110.3, 112.4, 119.8, 122.2, 131.1, 133.2, 139.8, 149.1, 149.9, 154.8; IR (KBr, cm^{-1}) 2500–3500, 3030, 2961, 2934, 2875, 2837, 2236, 1590, 1517, 1457, 1415, 1260, 1143, 1024, 868, 811, 737, 698; MS (*m*/*z*). 289, 397, 523, 649 (M+H$^+$, 100%); Anal. Calcd for $C_{24}H_{30}I_2N_2O_3 \cdot 3/2H_2O$: C, 42.68; H, 4.93; N, 4.15. Found: C, 42.50; H, 4.43; N, 4.38.

4.2.26. Acetic acid 2-(3-iodo-4-methoxy-phenyl)-ethyl ester (17)

2-(4-Methoxyphenyl)ethan-1-ol **16** (260 mg, 1.7 mmol) was treated by I_2 (0.216 g, 2 equiv) and $NaIO_4$ (0.055 g, 0.15 equiv) following method D2, to afford compound **16** (192 mg, 35% yield) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 2.03 (s, 3H), 2.83 (t, 2H, *J* = 7.05 Hz), 3.85 (s, 3H), 4.22 (t, 2H, *J* = 7.05 Hz), 6.75 (d, 1H, *J* = 8.31 Hz), 7.50 (dd, 1H, *J* = 2.19 and 8.31 Hz), 7.63 (d, 1H, *J* = 2.19 Hz); ^{13}C NMR ($CDCl_3$) δ 20.8, 33.6, 56.3, 64.7, 85.8, 110.8, 129.8, 131.9, 139.7, 156.8, 170.8; IR (KBr, cm^{-1}) 3030, 3000, 2963, 2930, 2900, 2840, 1745, 1600, 1493, 1363, 1240, 1053, 808; MS (*m*/*z*). 338 ($M + NH_4^+$, 100%); Anal. Calcd for $C_{11}H_{13}IO_3$: C, 41.27; H, 4.09; I, 39.64. Found: C, 41.15; H, 4.17; I, 39.34.

4.2.27. Acetic acid 2-(3,5-diiodo-4-methoxy-phenyl)-ethyl ester (18)

2-(4-Methoxyphenyl)ethan-1-ol **16** (0.538 g, 3.53 mmol) was diiodinated by I_2 (0.648 g, 3 equiv) and $NaIO_4$ (0.390 g, 0.5 equiv) as described in method D2, to provide compound **18** (0.387 g, 25% yield) as a white solid. Mp 62–63 °C; ^{1}H NMR ($CDCl_3$) δ 2.05 (s, 3H), 2.81 (t, 2H, *J* = 6.93 Hz), 3.84 (s, 3H), 4.22 (t, 2H, *J* = 6.93 Hz), 7.62 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 20.9, 33.2, 60.7, 64.2, 90.4, 137.8, 140.1, 157.6, 170.9; IR (KBr, cm^{-1}) 3030, 2986, 2950, 2934, 2878, 2845, 1744, 1456, 1390, 1226, 1027, 707; MS (*m*/*z*) 464 ($M + NH_4^+$, 100%); Anal. Calcd for $C_{11}H_{12}I_2O_3$: C, 29.62; H, 2.71. Found: C, 29.64; H, 2.59.

4.2.28. 2-(3-Iodo-4-methoxy-phenyl)-ethanol (19)

K_2CO_3 (10 equiv) was added to a solution of compound **17** (325 mg, 1 mmol) in methanol (15 mL). The mixture was stirred at room temperature for 3 h and then poured into water (30 mL). The aqueous solution was extracted with ether (3× 40 mL) and combined organic layers were dried over anhydrous Na_2SO_4. Removal of the solvent under reduced pressure provided alcohol **19** (282 mg, 100% yield) as a white oil. This product was used as such for further transformations. ^{1}H NMR ($CDCl_3$) δ 1.4–1.7 (OH), 2.77 (t, 2H, J = 6.54 Hz), 3.81 (t, 2H, J = 6.54 Hz), 3.86 (s, 3H), 6.77 (d, 1H, J = 8.31 Hz), 7.17 (dd, 1H, J = 2.07 and 8.31 Hz), 7.65 (d, 1H, J = 2.07 Hz); ^{13}C NMR ($CDCl_3$) δ 37.6, 56.4, 63.5, 86.1, 110.9, 130.0, 132.7, 139.7, 156.8; IR (KBr, cm^{-1}) 3100–3600, 2938, 2850, 2810, 1600, 1567, 1493, 1461, 1270, 1257, 1183, 1053, 1020; MS (m/z) 247, 261, 278 (M)$^+$, 296 ($M + NH_4^+$, 100%); Anal. Calcd for $C_9H_{11}IO_2$: C, 38.87; H, 3.99; I, 45.63. Found: C, 39.40; H, 4.03; I, 44.26.

4.2.29. 2-(3,5-Diiodo-4-methoxy-phenyl)-ethanol (20)

K_2CO_3 (10 equiv) was added to a solution of compound **18** (300 mg, 0.6 mmol) in methanol (15 mL). The mixture was stirred at room temperature for 3 h and then poured into water (30 mL). The aqueous solution was extracted with ether (3× 40 mL) and combined organic layers were dried over anhydrous Na_2SO_4. Removal of the solvent under reduced pressure provided alcohol **20** (257 mg, 100% yield) as a white solid. This product was used as such for further transformations. Mp 62–63 °C; ^{1}H NMR ($CDCl_3$) δ 2.05 (s, 3H), 2.81 (t, 2H, J = 6.93 Hz), 3.84 (s, 3H), 4.22 (t, 2H, J = 6.93 Hz), 7.62 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 20.9, 33.2, 60.7, 64.2, 90.4, 137.8, 140.1, 157.6, 170.9; IR (KBr, cm^{-1}) 3030, 2986, 2950, 2934, 2878, 2845, 1744, 1456, 1390, 1226, 1027, 707; MS (m/z) 464 ($M + NH_4^+$, 100%); Anal. Calcd for $C_{11}H_{12}I_2O_3$: C, 29.62; H, 2.71. Found: C, 29.64; H, 2.59.

4.2.30. Methanesulfonic acid 2-(4-methoxy-phenyl)-ethyl ester (21)

A solution of 2-(4-methoxyphenyl)ethan-1-ol **16** (498 mg; 3.3 mmol) was treated according to method B2 to afford compound **21** (500 mg, 66% yield) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 2.85 (s, 3H), 2.99 (t, 2H, J = 6.91 Hz), 3.79 (s, 3H), 4.38 (t, 2H, J = 6.91 Hz), 6.85 (d, 2H, J = 8.71 Hz), 7.15 (d, 2H, J = 8.71 Hz); ^{13}C NMR ($CDCl_3$) δ 34.7, 37.3, 55.2, 70.6, 114.1, 128.2, 130.0, 158.6; IR (KBr, cm^{-1}) 3030, 3020, 3000, 2970, 2938, 2900, 2840, 1600, 1518, 1469, 1355, 1241, 1175, 1028, 955; MS (m/z) 121, 135, 248 ($M + NH_4^+$, 100%); Anal. Calcd for $C_{10}H_{14}O_4S$: C, 52.16; H, 6.13. Found: C, 52.44; H, 6.14.

4.2.31. Methanesulfonic acid 2-(3-iodo-4-methoxy-phenyl)-ethyl ester (22)

Alcohol **19** (174 mg; 0.6 mmol) was treated with mesyl chloride according to method B2 to afford compound **22** (213 mg, 96% yield) as a white oil. ^{1}H NMR ($CDCl_3$) δ 2.90 (s, 3H), 2.95 (t, 2H, J = 6.93 Hz), 3.87 (s, 3H), 4.36 (t, 2H, J = 6.93 Hz), 6.77 (d, 1H, J = 8.34 Hz), 7.18 (dd, 1H, J = 2.16 and 8.34 Hz), 7.65 (d, 1H, J = 2.16 Hz); ^{13}C NMR ($CDCl_3$) δ 34.2, 37.5, 56.4, 69.9, 86.1, 110.9, 130.1, 130.4, 139.8, 157.3; IR (KBr, cm^{-1}) 2938, 2840, 1600, 1567, 1493, 1355, 1257, 1175, 1051, 955, 816; MS (m/z) 135, 248, 261, 356 (M^+), 374 ($M + NH_4^+$, 100%); Anal. Calcd for $C_{10}H_{13}IO_4S$: C, 33.72; H, 3.68. Found: C, 34.29; H, 3.73.

4.2.32. Methanesulfonic acid 2-(3,5-diiodo-4-methoxy-phenyl)-ethyl ester (23)

Alcohol **20** (164 mg, 0.4 mmol) was transformed into mesylate **23** (157 mg) as a yellow oil, with a yield of 80%, as described in method B2. ^{1}H NMR ($CDCl_3$) δ 2.94 (t, 2H, J = 6.78 Hz), 2.96 (s, 3H), 3.85 (s, 3H), 4.36 (t, 2H, J = 6.78 Hz), 7.64 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 33.8, 37.6, 60.7, 69.1, 90.6, 136.2, 140.2, 158.1; IR (KBr, cm^{-1}) 2970, 2938, 2840, 1575, 1534, 1461, 1420, 1355, 1248, 1167, 1061, 955; MS (m/z) 387, 482 (M^+), 500 ($M + NH_4^+$, 100%); Anal. Calcd for $C_{10}H_{12}I_2O_4S$: C, 24.91; H, 2.51. Found: C, 25.43; H, 2.60.

4.2.33. 2-(3,4-Dimethoxy-phenyl)-2-isopropyl-5-{[2-(4-methoxy-phenyl)-ethyl]-methyl-amino}-pentanenitrile (24a)

Compound **24a** was prepared from mesylate **21** (175 mg, 0.76 mmol) and amine **2** (214 mg, 0.74 mmol) according to method C2, and was obtained as a yellow oil (160 mg, 51% yield). ^{1}H NMR ($CDCl_3$) δ 0.78 (d, 3H, J = 6.65 Hz), 1.05–1.21 (m, 1H), 1.17 (d, 3H, J = 6.65 Hz), 1.40–1.60 (m, 1H), 1.81 (m, 1H, J = 4.48 Hz), 2.00–2.15 (m, 2H), 2.17 (s, 3H), 2.30–2.40 (m, 2H), 2.40–2.50 (m, 2H), 2.60–2.70 (m, 2H), 3.78 (s, 3H), 3.88 (s, 6H), 6.80–6.93 (m, 5H), 7.08 (d, 2H, J = 8.7 Hz); ^{13}C NMR ($CDCl_3$) δ 18.6, 18.9, 23.4, 32.7, 35.6, 37.9, 42.0, 53.3, 55.2, 55.9, 56.0, 56.9, 59.5, 109.6, 111.1, 113.7, 118.6, 121.4, 129.5, 130.7, 132.5, 148.2, 149.0, 157.9; IR (KBr, cm^{-1}) 2963, 2840, 2791, 2236, 1616, 1518, 1461, 1412, 1257, 1151, 1036; MS (m/z) 303, 425 (M^+, 100%); Anal. Calcd for $C_{26}H_{36}N_2O_3{\cdot}H_2O$: C, 70.56; H, 8.65; N, 6.33. Found: C, 70.52; H, 8.50; N, 6.54.

4.2.34. 2-(3,4-Dimethoxy-phenyl)-5-{[2-(3-iodo-4-methoxy-phenyl)-ethyl]-methyl-amino}-2-isopropyl-pentanenitrile (24b)

Mixture of amine **2** (135 mg, 0.46 mmol) and compound **22** (165 mg, 0.046 mmol) was treated following method C2 to afford compound **24b** (97 mg, 38% yield) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.78 (d, 3H, J = 6.66 Hz), 1.05–1.21 (m, 1H), 1.17 (d, 3H, J = 6.66 Hz), 1.45–1.61 (m, 1H), 1.81 (m, 1H, J = 4.35 Hz), 2.00–2.12 (m, 2H), 2.15 (s, 3H), 2.28–2.36 (m, 2H), 2.42–2.50 (m, 2H), 2.56–2.65 (m, 2H), 3.85 (s, 3H), 3.88 (s, 3H), 3.89 (s, 3H), 6.73 (d, 1H, J = 8.32 Hz), 6.81–6.96 (m, 3H), 7.08 (dd, 1H, J = 8.32 and 2.04 Hz), 7.59 (d, 1H, J = 2.04 Hz); ^{13}C NMR ($CDCl_3$) δ 18.6, 18.9, 23.4, 32.1, 35.5, 37.9, 41.8, 53.3, 56.0, 56.4, 56.9, 59.2, 85.8, 109.6, 110.8, 110.9, 118.7, 121.5, 129.7, 130.7, 134.8, 139.5, 148.2, 149.0, 156.4; IR (KBr, cm^{-1}) 3030, 3000, 2963, 2930, 2840, 2800, 2236, 1600, 1518, 1461, 1412, 1248, 1053, 1028, 914; MS (m/z) 291, 303, 425, 551 (M+H$^+$, 100%); Anal. Calcd for $C_{26}H_{35}IN_2O_3$: C, 56.73; H, 6.41; N, 5.05. Found: C, 56.99; H, 6.47; N, 5.12.

4.2.35. 5-{[2-(3,5-Diiodo-4-methoxy-phenyl)-ethyl]-methyl-amino}-2-(3,4-dimethoxy-phenyl)-2-isopropyl-pentanenitrile (24c)

Amine **2** (133 mg, 0.46 mmol) was alkylated by compound **23** (244 mg, 0.51 mmol), as described in method C2, to provide compound **24c** as a yellow oil (93 mg, 30% yield). ^{1}H NMR ($CDCl_3$) δ 0.78 (d, 3H, J = 6.70 Hz), 1.05–1.12 (m, 1H), 1.18 (d, 3H, J = 6.70 Hz), 1.42–1.60 (m, 1H), 1.82 (m, 1H, J = 4.48 Hz), 2.00–2.10 (m, 2H), 2.12 (s, 3H), 2.20–2.40 (m, 2H, 2.40–2.51 (m, 2H), 2.52–2.63 (m, 2H), 3.83 (s, 3H), 3.88 (s, 6H), 6.86 (m, 3H), 7.59 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 18.7, 18.9, 23.4, 31.9, 35.5, 37.9, 41.6, 53.4, 55.9, 56.0, 57.0, 58.7, 60.7, 90.1, 109.7, 111.1, 118.6, 121.4, 130.7, 140.0, 140.7, 148.2, 149.0, 157.0; IR (KBr, cm^{-1}) 3060, 2955, 2930, 2840, 2791, 2236, 1591, 1518, 1469, 1412, 1257, 1151, 1025, 1070; MS (m/z) 303, 551, 677 (M+H$^+$, 100%); Anal. Calcd for $C_{26}H_{34}I_2N_2O_3$: C, 46.17; H, 5.07; N, 4.14. Found: C, 45.95; H, 5.07; N, 4.06.

4.3. Biology

4.3.1. Cell lines

BHK-21 cells stably transfected with wild type MRP1 has been described previously. Cells were grown at 37 °C in 5% CO_2 in DMEM culture medium containing 1% penicillin–streptomycin and 5%

fetal bovine serum, in the presence of 200 μM methotrexate for transfected cells.

4.3.2. Cell proliferation determined by MTT

The MTT colorimetric assay, as described previously, was used to assess the sensitivity of control cells and *MRP1*-transfected BHK-21 cells to verapamil derivatives. Briefly, growth inhibition (IC_{50}) assays were performed by plating cells at density 1.0×10^4 cells/well in 96-well plates. Cells were cultured for 17 h before addition of verapamil derivatives diluted in complete culture medium, DMSO concentration was fixed at 0.5%. The cells were then incubated for 96 h in a wet tissue-culture chamber (37 °C, 5% CO_2). Surviving cells were detected by the bromo-3(4,5-dimethyl-2-thiazoyl)2,5-diphenyltetrazolium (MTT) assay. The MTT solution was prepared to 0.5 mg/mL, and 100 μl was added to each well and incubated for 4 h at 37 °C. Thereafter, the MTT dye was aspirated and 200 μl of isopropanol was added to each well. The formazan, resulting from the reaction, was photometrically measured at 570 nm. IC_{50} values were calculated from dose–response curves obtained from triplicate experiments.

4.3.3. Total cellular glutathione determination

The total cellular glutathione content (GSH + oxidized glutathione) was measured using the enzymatic method described by Tietze, as modified by Anderson to the microtiter plate. Cells were seeded into 96-well plates at a density of 1.0×10^5 cells/well and cultured for 17 h before addition of 5 μM verapamil derivatives; DMSO concentration did not exceed 0.5%. After various incubation times, from 10 to 120 min, cells were twice washed with 200 μl cold PBS, and then lysed by 50 μl PBS supplemented with 0.1% Triton X-100. The lysate was then homogenized and 10 μl was transferred to a new plate for GSH titration, and 5 μl to another plate for protein quantification. GSH determination was performed by adding 120 μl of reaction buffer containing 221.3 μM NADPH, 462.6 μM DTNB, 11 U/mL GSH reductase, and PBS. The plate was then placed in a microplate reader, and the absorbance was read at 412 nm every 30 s for 2 min. The content of total cellular glutathione was quantified by comparison with known glutathione standards. The slope was determined for each sample, and allowed us to determine the GSH concentration. Protein titration was performed on the remaining 5 μl by the Bradford method assay. The measured total glutathione was expressed in nanomole per milligram protein, and the results obtained were issued from triplicates.

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