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New tetrahydroisoquinoline based P-glycoprotein modulators: decoration of the biphenyl core gives selective ligands.

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Abstract: P-glycoprotein (P-gp, MDR1) is a membrane transporter expressed in several districts of our body. It exerts a crucial defense role as it effluxes hundreds of potentially toxic substances. Hovewer, P-gp is one of the main causes of the failure in cancer chemotherapy, as a number of chemotherapeutic agents are P-gp substrates. Another interesting implication regards the correlation between P-gp expression impairment and the onset of several central nervous system pathologies such as Alzheimer's and Parkinson's diseases. In view of these considerations, in the present study a new series of P-gp modulators has been designed, synthesized and evaluated for their activity towards P-gp and other two sister proteins (BCRP and MRP1). The compounds, structurally correlated to the potent but non-selective P-gp inhibitor **MC70** [4'-(6,7-dimethoxy-3,4-dihydro-1*H*-isoquinolin-2-ylmethyl)biphenyl-4-ol], proved fairly selective towards P-gp, with a potency in the micromolar range. Compounds **5a**, **5d** and **12d** proved capable to restore doxorubicin toxicity in resistant cancer cells.

Keywords: P-gp, MDR, tetrahydroisoquinoline, P-gp ligands

Introduction

P-glycoprotein (P-gp, also known as MDR1 or ABCB1) is a membrane protein belonging to ATP-binding cassettes (ABC) superfamily; it is a complex molecular machinery able to recognize and efflux hundreds of structurally uncorrelated substances exploiting energy

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derived by ATP hydrolysis. It has a strategic localization in a number of organs and tissues, thus exerting a crucial defence role against toxic substances, both of endogenous and exogenous origin, and constituting an essential component of several biological barriers.[1] P-gp has raised great attention since several years ago, owing to its involvement in multidrug resistance (MDR), a phenomenon that represents one of the main causes of cancer chemotherapy failure. In some tumors MDR appears without a previous exposure to anti-cancer drugs, whilst in other cases its onset occurs during treatment, quite often as a consequence of selection of resistant over sensitive cells in heterogeneous tumor tissue.[2] P-gp is overexpressed in cancer cells and is responsible of the efflux of several chemotherapeutic agents, such as taxanes, vinca alkaloids, doxorubicin, etoposide, topotecan, methotrexate, imatinib, dasatinib, gefitinib among others.[3] The wide expression of P-gp on the surface of endothelial cells at the blood-brain barrier makes it a gatekeeper for central nervous system (CNS), as it prevents potentially harmful substances from entering, but also hampers many structurally and functionally uncorrelated drugs, compromising the success of pharmacological treatment of different CNS disorders and tumours. More recently, an interesting connection has emerged between P-gp and the onset of Alzheimer's Disease (AD), Parkinson's Disease (PD), epilepsy and other CNS diseases: an impairment in the expression level of the protein has been observed in the early stages of these neurological disorders.[4,5] Starting from this background, medicinal chemistry efforts to target P-gp have been undertaken, mainly in the aim of obtaining inhibitors which should be co-administered with chemotherapeutic agents subjected to P-gp mediated efflux, thus restoring therapy efficacy. According to this approach a number of MDR reversal agents have been reported, which are usually classified in three generations; despite promising in vitro results, most of them failed in the clinical trial phase for several reasons: in the case of first generation agents which are drugs in clinical use for different indications, the main drawback was poor selectivity of action.[6] Modulators of the second generation, supposed to be more selective, proved toxic.[7] As to third generation agents, despite their high affinity for P-gp, they did not return satisfying results due to pharmacokinetic and safety concerns.[8] Thus the development of new agents able to impede P-gp mediated drug efflux is still an unmet need and, as MDR remains a prominent issue in cancer chemotherapy, it is still a field of interest in medicinal chemistry. In view of this, an alternative approach is the search of broad spectrum inhibitors targeting different transporters, which found a very recent exemplification in the study of Stefan et al. reporting on the design of 9-deazapurine-based inhibitors able to counteract drug efflux mediated by P-gp, multidrug resistance-associated protein 1 (MRP1, ABCC1) and breast caner resistance protein (BCRP, ABCG2).[9]

Besides the design of P-gp modulators to be flanked to chemotherapy agents, recently different approaches to counteract MDR have been undertaken by several groups. An important strategy is obviously the development of anti-cancer drugs that are not substrates of P-gp, both through design of new molecules or modification of already known drugs.[10] Some successful examples have been obtained, among others, with taxanes, with epothilones (a class of natural compounds structurally correlated to taxaxnes) and with vinca alkaloids.[11] In the setting of this strategy, given the lack of structural insights into the mechanism of interaction of anti-cancer drugs with P-gp, it is difficult to envisage

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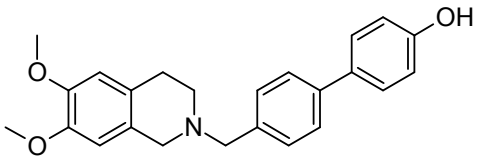
the nature and the position of the structural modifications required to evade P-gp efflux and to hold desireable cytotoxicity.

Another strategy that has been undertaken to overcome P-gp mediated efflux consists in enhancing drug uptake or modifying the mechanism of cellular uptake through non-covalent or covalent conjugation of chemotherapy agents with targeting systems such as nano-particles, liposomes, micelles, polymeric conjugates, antibodies among others. Particular attention has been paid to liposome formulation and a number of such systems is currently under clinical evaluation.[12] A word of caution must be spent regarding this approach, as the intracellular release of the drugs from these formulations may still result in P-gp efflux. Regarding covalent derivatives, strategies that gave interesting results consist in the modification of the drug through the introduction of guanidinium-rich tags, with consequent higher water solubility and modifyied recognition by P-gp[13,14], and of more complex cationic oligopeptide-based moieties, as in the case of Taxol-octaarginine conjugates.[15]

Besides the above reported approaches, based on mainly medicinal chemistry tools, in the recent past some less explored strategies have been attempted to counteract MDR, aiming at the downregulation of ABCB1 gene expression or translation, such as antisense oligonucleotides and small interfering RNA.[16]

In addition to the MDR issue, the potential application in the early diagnosis through imaging techniques such as Positron Emission Tomography (PET) of several neurological disorders has been proposed for P-gp ligands, in view of the involvement of the transporter in the onset of these CNS pathologies: in particular radiolabeled substrates are useful to measure in vivo function of the transporter at the blood brain barrier (BBB).[17]

Following the heavy effects of P-gp mediated MDR on cancer chemotherapy, despite the obstacles encountered so far, the development of new P-gp modulators still appears as a valuable and possibly straightforward route to counteract MDR. In this ongoing effort and also in view of the application of P-gp ligands in early diagnosis of some CNS degenerative diseases, in the present study the authors have carried out several modifications on **MC70** (Figure 1), a previously studied P-gp inhibitor.[18,19] **MC70** shows a good P-gp inhibiting potency (EC_{50} = 0.69 μM), on the other hand displaying a non-selective profile towards P-gp.



MC70
EC_{50} = 0.69 μM (P-gp)
EC_{50} = 9.30 μM (MRP1)

Figure 1. Structure and biological activity profile of **MC70**.

The authors have previously explored structure activity relationships of **MC70** derivatives: in particular they focused on functionalization of phenolic group with alkyl and oxyalkyl chains,[20] and with more complex moieties containing variously substituted furazan (1,2,5-

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oxadiazole) rings.[21] In the present investigation the structural modifications involve the biphenyl core: the aim of the study was to evaluate, in terms of inhibition potency and selectivity on P-gp, the effect of substituents able to modify electronic properties and endowed with hydrogen bond donor or acceptor properties. Thus at the four positions of the biphenyl moiety different substituents were inserted: fluorine, a small strongly electronegative atom, able to accept hydrogen bond, nitro group, a prototypical electron-withdrawing group with strong resonance effect and hydrogen bond acceptor properties, methoxy group, an electron-donating substituent with resonance effect and hydrogen bond acceptor properties, and amino group, electron donating with hydrogen bond donor and acceptor properties.

The compounds have been synthesized through an inexpensive and straightforward route; compared to **MC70**, potency was slightly decreased, EC_{50} values being in the micromolar range for most compounds, but all the ligands display high selectivity towards P-gp, being essentially inactive against MRP1 and BCRP. Three of them have been further evaluated in co-administration with doxorubicin and proved able to restore intracellular concentration of the anthracycline to different extent, without any effects on P-gp expression. A sensible insight of the binding pose of a selected compound has been gained through a molecular docking study.

Results and discussion

Synthesis of target compounds

The target compounds were synthesized according to the methods reported in Scheme 1 and 2. In brief, the key step is a Suzuki coupling reaction,[22] carried out with palladium on activated charcoal in a ligand free fashion in aqueous environment, starting from the 4-bromobenzylalcohols **1a-f** (Scheme 1), or the 4-bromophenols **8a-e** (Scheme 2). The substrates were coupled with 4-hydroxyphenylboronic acid or 4-hydroxymethylphenylboronic acid respectively. The coupling products were then reacted with an excess of *p*-toluensulfonyl chloride in presence of triethylamine, to give the di-tosylate derivatives **3a-f** (Scheme 1) or **10a-e** (Scheme 2). The latter was used without purification to obtain benzylation of 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline. Finally the removal of the phenyl tosylate group was achieved through hydrolysis with NaOH in THF/CH_3OH mixture.

The two benzylic alcohols **1c** and **1d** were synthesized starting from the corresponding benzoic acids through reduction with BH_3-THF complex (Scheme 1).

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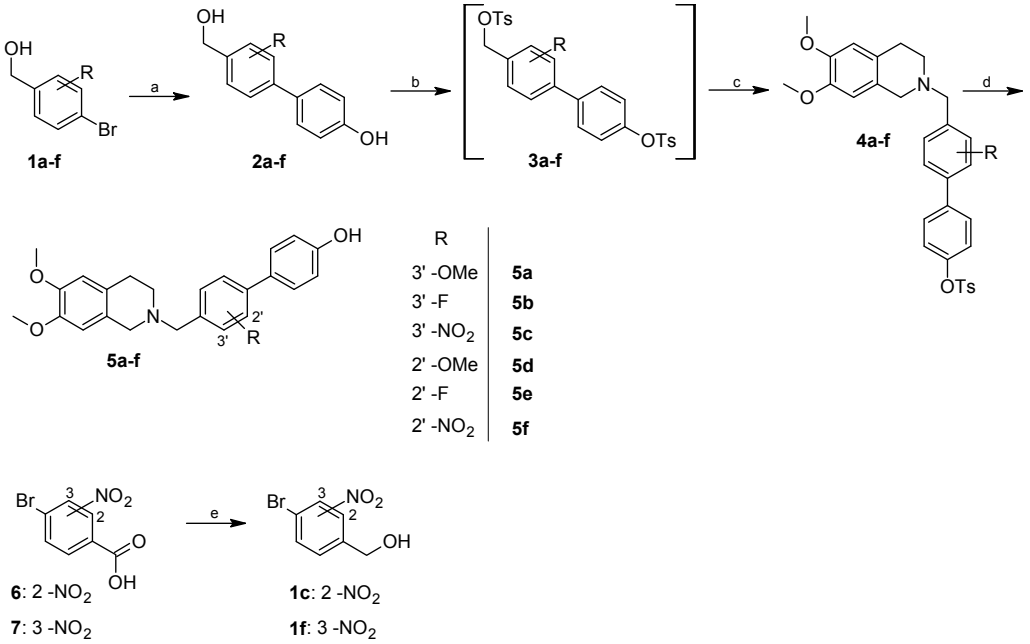

Scheme 1. Synthesis of target compounds **5a-f**. *Reagents and conditions:* a) 4-hydroxybenzeneboronic acid, KOH, Pd/C cat., H_2O, 130 °C, 3 hours; b) p-toluenesulfonyl chloride, Et_3N, DMAP cat., CH_2Cl_2, room temperature, 6 hours; c) 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride, DBU, CH_3CN, 70 °C, 18 hours; d) NaOH, THF/CH_3OH 2/1, 65 °C, 1 hour; e) BH_3–THF complex, THF, N_2, 18 hours, room temperature.

As for compound **16**, the procedure reported above for coupling reaction starting from 1-bromo-4-methoxy-2-nitrobenzene **13** did not afford the desired intermediate, probably due to the poor solubility of **13**. For this reason the Suzuki coupling reaction was carried out in a more “traditional” manner (Scheme 2) using palladium tetrakis triphenylphosphine as catalyst in 1,4-dioxane/water mixture; the intermediate **14** was subdued to hydrolysis of methoxy group with BBr_3, obtaining the concomitant bromination of benzylic alcohol. The bromo derivative **15** was then reacted with 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline, yielding compound **16**.
Finally the two amino substituted compounds **17a** and **17b** were obtained through catalytic hydrogenation of the nitro derivatives **12c** and **16** (Scheme 2).

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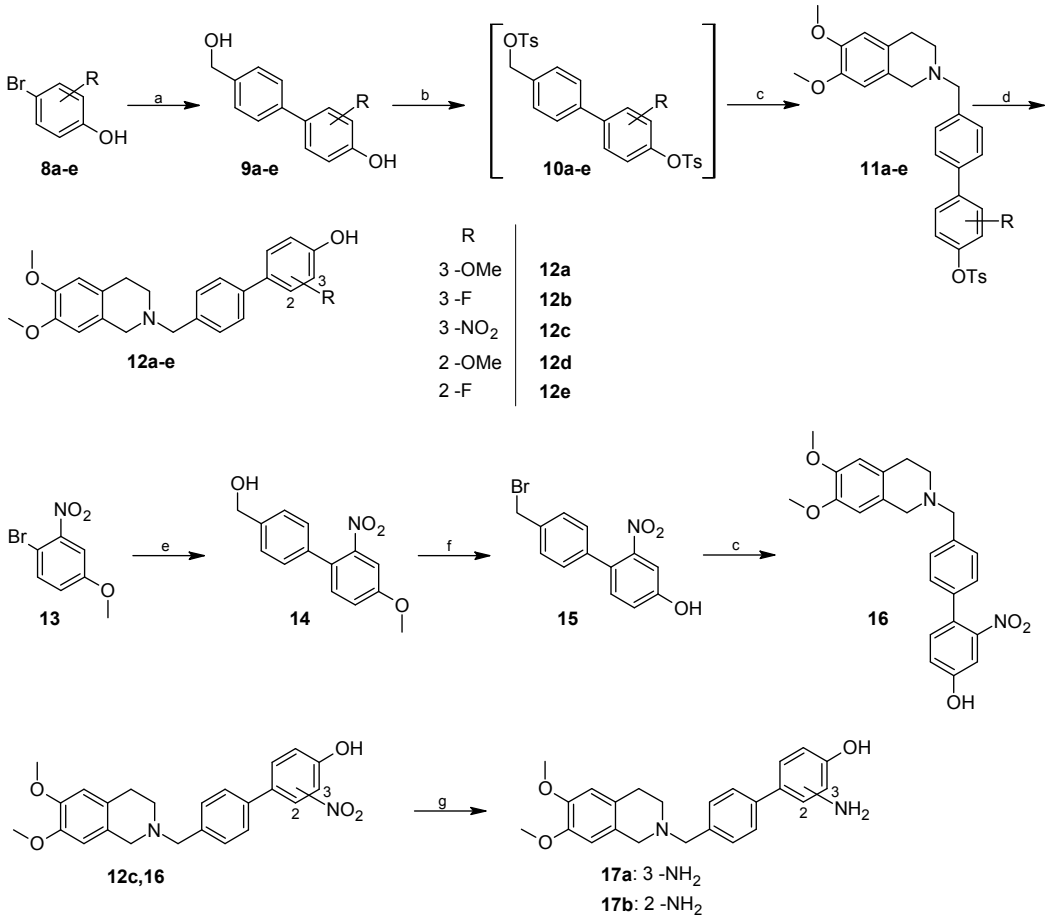

Scheme 2. Synthesis of target compounds **12a**-**e**, **16**, **17a**,**b**. *Reagents and conditions:* a) 4-hydroxymethylbenzeneboronic acid, KOH, Pd/C cat., H_2O, 130 °C, 3 hours; b) p-toluenesulfonyl chloride, Et_3N, DMAP cat., CH_2Cl_2, room temperature, 6h; c) 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride, DBU, CH_3CN, 70 °C, 18 hours; d) NaOH, THF/CH_3OH 2/1, 65 °C, 1 hour; e) 4-hydroxymethylbenzeneboronic acid, K_2CO_3, $Pd[(C_6H_5)_3P]_4$, 1,4-dioxane/water 2/1, N_2, 90 °C, 18 hours; f) BBr_3, CH_2Cl_2, 18 hours, room temperature; g) H_2, Pd/C cat., CH_3OH, room temperature, 3 hours.

Biological evaluation

All the new synthesized compounds have been tested to establish their P-gp interacting mechanism as substrate or inhibitor by three assays: 1) the inhibition of the transport of a fluorescent or a pro-fluorescent substrate of the transporter;[23] 2) the determination of the apparent permeability value (P_{app});[23] 3) the detection of the ATP cell level depletion;[23] the results are reported in Table 1.

Table 1. Biological characterization of target compounds.

Compound	Structure	MDR1 EC_{50} (μM) [a]	MRP1 EC_{50} (μM) [a]	BCRP EC_{50} (μM) [a]	ATP consumption	P_{app} [b]

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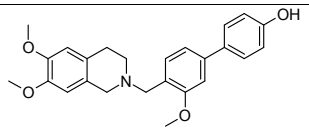
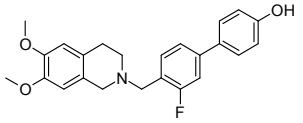
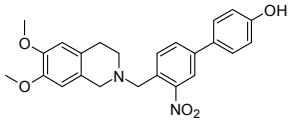
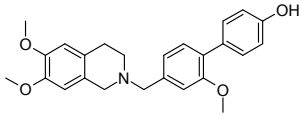
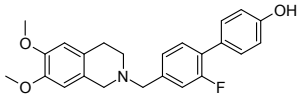
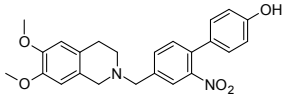
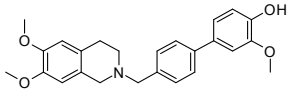
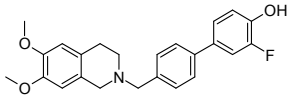
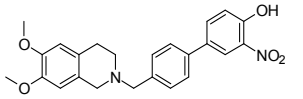
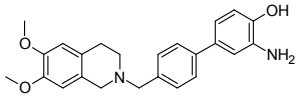
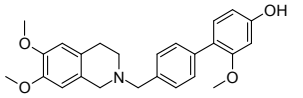
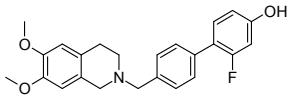
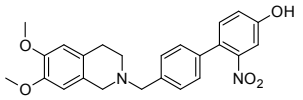
5a		1.60 ± 0.27	na[c]	na	NO[c]	4.5
5b		4.73 ± 0.90	na	na	NO	4.1
5c		8.4 ± 1.58	na	na	NO	5.8
5d		1.51 ± 0.30	38 ± 7.6	na	NO	4.4
5e		6.2 ± 1.24	na	na	NO	3.7
5f		15.2 ± 3.00	na	na	NO	2.9
12a		2.0 ± 0.40	na	na	NO	3.9
12b		2.8 ± 0.56	na	na	NO	4.9
12c		12.7 ± 2.50	28.9	na	NO	6.3
17a		6.9 ± 1.32	na	na	NO	7.5
12d		1.86 ± 0.37	62	na	NO	3.5
12e		2.17 ± 0.43	na	na	NO	4.5
16		2.54 ± 0.48	na	na	NO	11.2

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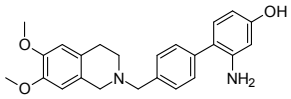
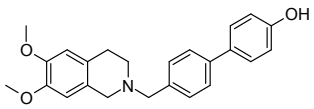
17b		5.4 ± 1.00	na	na	NO	4.5
MC70		0.69	9.30	73	NO	1.3

[a] The value is the mean of three independent experiments, sample in duplicate. [b] Apparent permeability determined as BA/AB ratio (the value is from two independent experiments). [c] Not active at 100 μM. [d] No significative ATP consumption at the activity doses.

The first assay is performed in a cell line overexpressing P-gp (MDCK-MDR1 cells) and it detects the ability of the ligands to compete with the efflux of the pro-fluorescent P-gp substrate Calcein-AM towards P-gp; it is the measure of the potency of each compound towards the target. The second assay evaluates a ratio basolateral-apical vs apical-basolateral (BA/AB) representative of two contributions, the passive diffusion (BA) and active transport (AB) in a system mimicking BBB, such as Caco2 cells; if this ratio is < 2, the compound can be considered a P-gp inhibitor, otherwise (P_{app}> 2) it behaves as a substrate. The third assay measures the consumption of ATP in MDCK-MDR1 cells. Generally, a substrate, since transported by the pump, induces an ATP consumption while an inhibitor, inhibiting the binding of ATP on its site on P-gp, is not transported and thus it does not induce a decrease in ATP cell level. The selectivity of all compounds towards the sister proteins BCRP and MRP1 has been also tested by the measure of the ability of the compounds to interfere with the transport BCRP- or MRP1-mediated of the fluorescent BCRP substrate Hoechst33342 or the pro-fluorescent MRP1 substrate Calcein-AM.

As depicted in Table 1, all of the compounds were found less active than the lead compound **MC70** (EC_{50} = 0.69 μM) displaying EC_{50} values ranging from 1.51 to 15.2 μM, and among them **5a** (EC_{50} = 1.60 μM), **5d** (EC_{50} = 1.51 μM), and **12d** (EC_{50} = 1.86 μM), displayed the best activity values. However, all compounds were more selective towards P-gp, proving inactive towards BCRP and MRP1. Only compound **5d** and **12c** showed a moderate MRP1 activity (EC_{50} = 38 μM and 28.9 μM, respectively). All the ligands, having a P_{app} values > 2 and not inducing an ATP cell depletion, behaved as substrates of the Category IIB3.[18]

As a whole only slight differences can be observed along the series; nevertheless some general considerations are worthy of note: the presence of methoxy group, electron-donating and hydrogen bond acceptor, seems to give a slightly higher activity in the series, no matter the position on the biphenyl core (EC_{50} ranging from 1.51 μM for **5d**, to 2 μM for **12a**). On the contrary the presence of a strong electron-withdrawing substituent, such as nitro group, determines a loss of activity which is more pronounced for compounds where the substituent influences to a greater extent the acidity of phenol group (position 2' and 3 of the biphenyl core) (EC_{50}= 15.2 μM for **5f** and EC_{50} = 12.7 μM for **12c**). Fluorine atom seems not to greatly influence the activity, with a slightly more detrimental effect when present on the benzylic ring (position 2' and 3') (EC_{50}= 6,2 μM for **5e**). Finally, the

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presence of amino substituent, able to donate hydrogen bond, bears a significant loss of activity (EC_{50}= 6,9 μM for **17a**).

Molecular docking study

A structure based study was then carried to furnish fresh insights into the binding mode of the new compounds and to support explication to the EC_{50} data. To fulfill this topic our previously published P-gp receptor model[21] served as valuable tool to explain ligand pose of the most active compound (**5d**). In our past study we have postulated that the "inward-outward facing" of the P-gp scaffold should facilitate the "pulling out" of substrates as soon as they pass the cell bilayer, while active inhibitors would hamper the P-gp flipping depending on molecular shape and pharmacophoric features of the molecules; in line with this view, molecular docking further supports this evidence.

As it might be perceived from Figure 2 reporting the binding mode of **5d**, the ligand scaffold properly fits the binding site space delimited by the two six helices-transmembrane domains (TMDs), and deeply locks into the crevice comprising the intracellular moiety of the same domains, with a kind of "reversed wedge" pose that might be responsible for the hampering of P-gp flipping upon ligand binding.

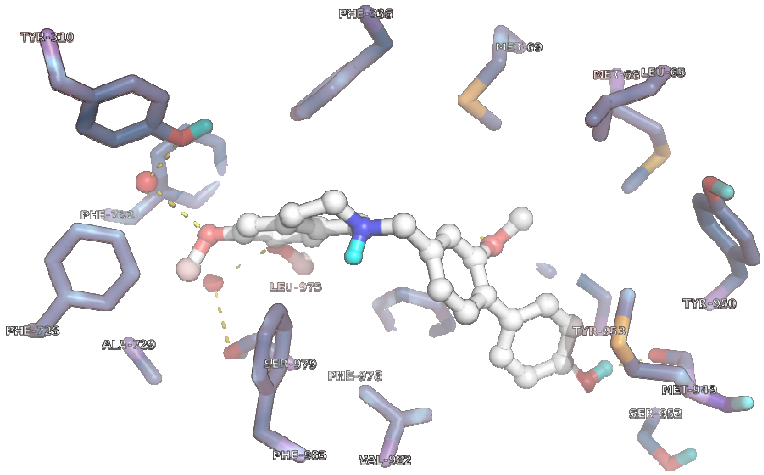

Figure 2. Binding mode of **5d** into the MDR1 binding site. Water molecules are represented as red spheres, and the extracellular and intracellular sides are at top and bottom of the scene respectively. The free energy of binding calculated with hydration force field of AutoDock is -8.62 kcal/mol, while the contact surface area measures 440 Å^2.

Interestingly the tetrahydroisoquinoline ring is placed close to one of the TMDs, and it occupies a mainly hydrophobic receptor slot, surrounded by Tyr310, Phe336, Phe728 and Phe983 generating extensive favourable contacts and π–π stacking, as well as a stable

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binding through two water molecules enlacing hydrogen bonding bridges with Tyr310 and Ser979. The rest of the aromatic moiety points to the opposite TMD with the phenolic group making polar contacts with the backbone of Met949 and the methoxy group interacting with Tyr953 throughout a water molecule coordinating a hydrogen bond, and as long as this is concerned, the same methoxy substituent should oblige, to some extent, the biphenyl motif in a non-planar conformation. Indeed, this steric hindrance, in combination with an hydrogen bond formation, might clarify, at least within this series of derivatives, the better EC_{50} value of **5d**.

Co-administration of doxorubicin with 5a, 5d and 12d

Compounds **5a**, **5d**, and **12d**, displaying the best P-gp activity profile, have been evaluated in co-administration with doxorubicin, in order to study their efficacy as doxorubicin-rehabilitating agents in the treatment of resistant tumours (Figure 3).

Preliminary data demonstrated that the three compounds were not cytotoxic at 48h and 72h (data not shown) and doxorubicin at 10 μM is not able to induce cell death as effluxed by P-gp in the resistant tumours cell model overexpressing P-gp (MDCK-MDR1 cells). When **MC70**, **5a**, **5d**, and **12d** were co-administred with doxorubicin, they restore its antineoplastic cytotoxicity. In fact, while the lead compound **MC70** and **5a**, **5d** displayed a moderate ability to restore doxorubicin effect (20% of cytotoxicity increase for **MC70** and 15% for **5a**, **5d**), **12d** was able to produce an increase of 60% of doxorubicin citotoxicity, demonstrating its ability to almost completely rehabilitate the access of the antineoplastic drug in tumour cells. Considering the structural correlation and the similarity of EC_{50} of the three tested compounds, it is difficult to envisage the reason for the efficient restoring of doxorubicin toxicity elicited by **12d**; the hypothesis that can be made is a possible reinforcement of activity at higher concentration than EC_{50}: this can be due to the peculiarity of the binding region of P-gp, which is large and comprises multiple and possibly overlapping binding sites. This feature has led several authors to postulate the possibility for some small molecules to bind simultaneously to different binding sites and this has also been exploited through the design of dimeric modulators with enhanced potency.[24]

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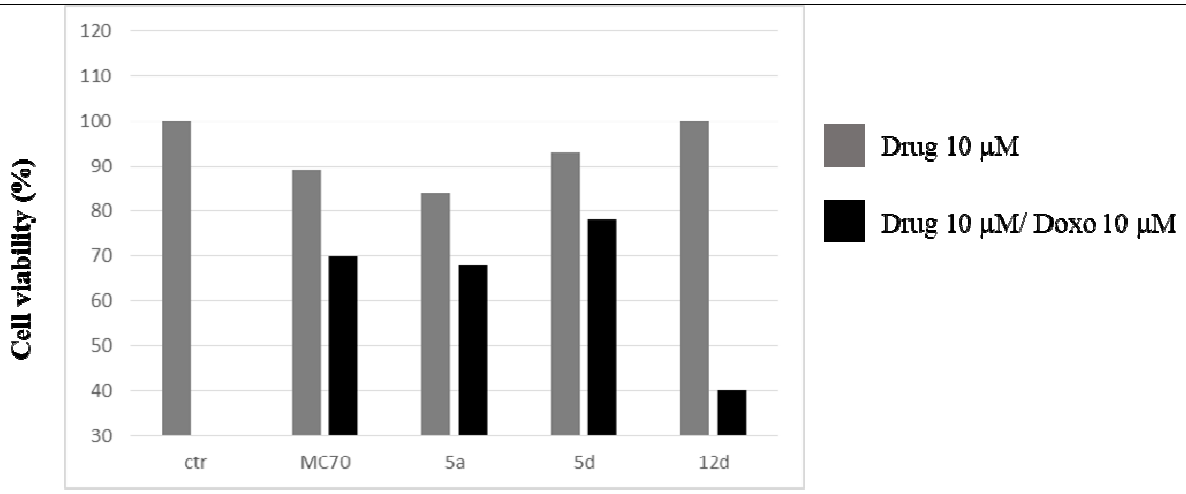

Figure 3. Co-administration of doxorubicin (10 μM) with compounds **MC70**, **5a**, **5d**, and **12d** (10 μM). Antiproliferative effect of **MC70**, **5a**, **5d**, and **12d** (10 μM) alone (gray bars) and co-administred with doxorubicin (10 μM) (black bars) at 48h in MDCK-MDR1 cell line. Ctr bar represents the administration of 10 μM doxorubicin alone.

Immunoblotting experiments on MC70, 5a, 5d and 12d

The same compounds (MC70, 5a, 5d and 12d) which were evaluated in co-administration with doxorubicin were further studied in immunoblotting experiments to verify a possible interference with P-gp expression, in the same experimental condition of cell viability tests. As it can be seen in Figure 4, no differences in P-gp expression were observed in treated cells compared to the control, thus supporting the hypothesis of a direct effect of the compounds on the transporter.

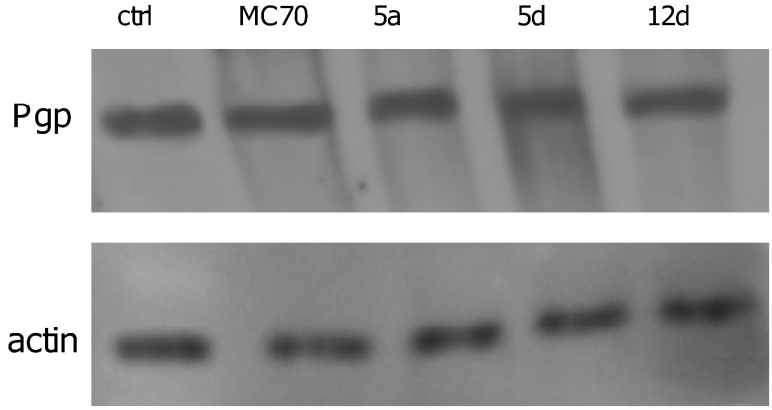

Figure 4. Immunoblotting experiment with compounds **MC70**, **5a**, **5d**, and **12d** (10 μM). Proteins were extracted from MDCK-MDR1 cells after 48 hours of incubation. Actin levels were used as protein loading control. CTRL bands refer to untreated cells. The figure is representative of 1 out of 3 experiments with similar results.

Conclusion

A new series of P-gp modulators has been developed through the “decoration” of the biphenyl moiety of **MC70** with substituents endowed with different electronic and hydrogen

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bond donor/acceptor properties. The compounds were synthesized exploiting a straightforward and inexpensive route. They displayed slightly lower potency than the parent compound but proved highly selective, thus representing suitable candidates to be used in imaging techniques for measurement of P-gp function at BBB.

A molecular docking simulation carried out for the most potent compound (**5d**) highlighted an additional anchoring for the biphenyl moiety through the methoxy substituent, which engages a hydrogen bond mediated by a water molecule. Besides the biochemical characterization aimed to clarify the mechanism of interaction of the ligands with P-gp, the most potent compounds (**5a**, **5d**, and **12d**) and **MC70** were evaluated in a co-administration assay with doxorubicin, and proved efficient to restore drug toxicity against doxorubicin resistant MDCK-MDR1 cells. A possible effect on P-gp expression was ruled out by performing immunoblotting experiment on the same selected compounds.

The present study thus represents an additional investigation of a previously started structure activity relationship study on tetrahydroisoquinoline derivatives, and furnishes additional information about the mode of binding of this class of compounds which can be useful for future development of potent P-gp modulators.

Along the series, compound **12d** deserves particular consideration: it represents a valuable candidate for further development of P-gp mediated efflux reversal agents as it displayed an interesting efficacy (60%) in restoring doxorubicin cytotoxicity in resistant tumour cells. Moreover this data probably reflects a peculiar behavior of the compound, which may interact with different binding sites simultaneously; this hypothesis deserves further in-depth analyses and, if confirmed, opens the way to the exploitation of a “dimeric analogue” approach to design P-gp modulator based on biphenyl-tetrahydroisoquinoline substructure.

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Conflict of interest

The authors declare no conflict of interest.

Contributions

S. Guglielmo and N.Colabufo desigend the study and wrote the manuscript.
S. Guglielmo planned the synthetic strategy.
M. Contino planned and performed the biological assays to define the P-gp interacting mechanism and participated in the editing of the manuscript.
M.G. Perrone performed the permeability assays.
R.Giampietro performed the co-administration assay.
A. Carrieri and D. Zaccaria performed the molecular docking study.
K. Chegaev and V. Borio carried out the synthesis of compounds.
B. Rolando carried out the structural characterization and purity assessment.
C. Riganti and K. Zabielska-Koczywąs carried out the immunoblotting experiments.

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R. Fruttero carried out literature research and wrote the manuscript.

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A new series of P-gp modulators is reported. All compounds show selectivity with EC_{50} in the low micromolar range. One of them displays good efficacy in restoring doxorubicin citotoxicity in resistant cancer cells.

12a