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A potent and selective P-gp modulator for altering Multidrug Resistance due to pump overexpression.

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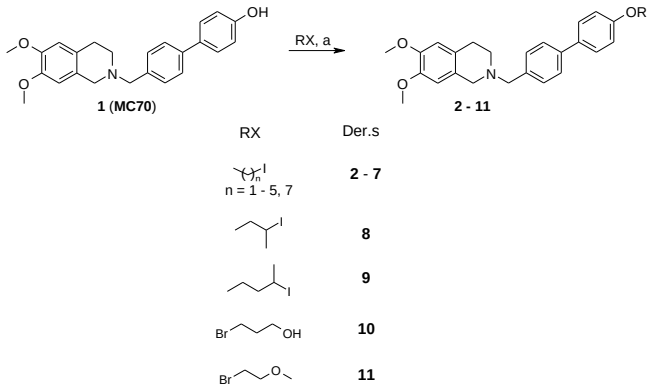
Abstract: P-gp is a membrane protein responsible for the active transport of several endogenous and exogenous substances. It constitutes a defense mechanism and, at the same time, it severely compromises the success rate of antitumor chemotherapy. In the present study a small library of alkyl/oxyalkyl derivatives of **MC70**, a well-known P-gp inhibitor, was synthesized through straightforward functionalization of the phenolic group present in its structure. All compounds were characterized for their effect on P-gp, proving capable of blocking the P-gp-mediated Calcein-AM efflux with micromolar potency, following their ability to act as high-affinity substrates of this transporter. Excitingly, compound **4** exhibited low nanomolar potency (5.2 nM) and had a peculiar activity profile, acting both as a positive allosteric modulator and as a substrate of the transporter. A new and more efficient synthesis of **MC70** is also described.

The major limit on the full success of cancer chemotherapy is the progressive unresponsiveness of the tumor towards anticancer drugs differing in structure and action mechanism. This phenomenon is known as Multi Drug Resistance (MDR). A number of mechanisms underlie MDR; the most important is the overexpression of integral membrane proteins known as ATP binding cassette (ABC) transporters which pump drugs out of the cell thus reducing their efficacy. These transporters use hydrolysis of ATP as their source of energy for the translocation of their substrates. ABC-B1, better known as P-glycoprotein (P-gp), ABC-C1-6 (MRP1-6 multidrug resistance associated proteins), and Breast Cancer Resistant Protein (BCRP) are the most significant transporters.[1-3] A strategy that has been suggested for reversing MDR is to co-administer the antitumor agent with a product able to inhibit drug efflux by the pump.[4]

Most of these products have been studied for their effect on P-gp, and according to their action mechanism can be classified into three groups: substrates, inhibitors and modulators. The substrates are actively-transported molecules and high concentrations of these products saturate the binding sites of the protein, thus blocking it. The inhibitors block the translocation activity of the pump by inhibiting the ATP binding to the protein's two nucleotide binding domains (NBD), and consequently inhibiting its hydrolysis. The modulators reduce the availability of substrate binding to its site through a negative allosteric interaction.[5] Biphenyl derivatives bearing a tetrahydroisoquinoline moiety have emerged as an interesting class of products displaying P-gp inhibiting activity, some of them also show selectivity towards BCRP transporter.[6] The most active member of this series is compound **1** (**MC70**), which bears a hydroxyl group at the 4' position of the biphenyl scaffold.[6,7] This product is unable to activate ATP-ase, thus it must be considered a P-gp inhibitor. It acts as a potent inhibitor (EC_{50} = 0.69 μM) and is unable to interact effectively with the BCRP pump (19% of inhibition at 100 μM). Antiproliferative activity of Doxorubicin (5 μM) on the MCF-7/Adr cell line after pre-treatment with **1**, at 2 and 20 μM increased from 28 % to 42 % and to 98 % respectively. In order to shed light on the structure-activity relationships of this lead compound, the results are reported of a study investigating the capacity of a series of simple alkyl and oxyalkyl derivatives (**2**-**11**) of **MC70** to block P-gp. An improved synthetic route to **MC70** is also proposed.

The target compounds were synthesized through alkylation of phenolic derivative **1** (**MC70**) with the appropriate commercially-available iodo- (der.s **2**-**9**) or bromo- (der.s **10**, **11**) reagents, in refluxing CH_3CN, with K_2CO_3 as base (Scheme 1). The derivatives were obtained in moderate to good yields (see supporting information).



Scheme 1. Reagents and conditions. a) K_2CO_3, CH_3CN, reflux.

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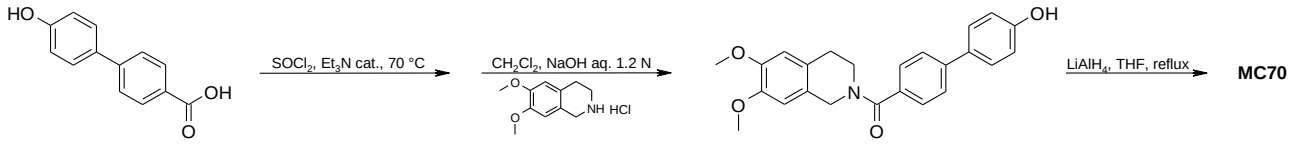
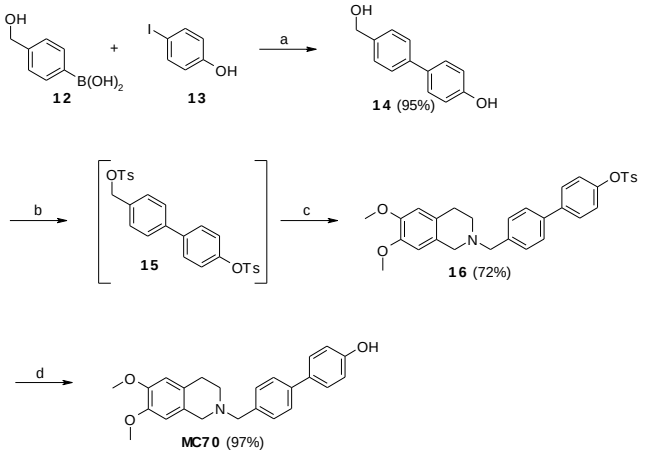


Scheme 2. Reported synthesis for **MC70**.[6]

A procedure to synthesize the starting material **MC70** has already been reported.[6] Although the synthesis is straightforward (Scheme 2), the two steps lead to a low overall yield (16%) and the reaction mixtures are difficult to treat and purify.In view of these drawbacks a new synthetic route is described here (Scheme 3); it is based on the well-known Suzuki-Miyaura coupling reaction.[8] In particular, the biphenyl intermediate **14** was obtained starting from the commercially-available 4-hydroxymethylphenylboronic acid (**12**) and 4-iodophenol (**13**), through a recently reported procedure for the protecting group-free coupling of halophenols;[9] the catalyst used was the inexpensive palladium on activated carbon and the reaction was conducted using water as solvent; compound **14** was isolated in high yield after work-up without the need of further purification. In the subsequent step, the activation of benzylic alcohol was achieved with *p*-toluenesulfonyl chloride in basic medium. In this reaction, the experimental conditions were not controlled, and an excess of *p*-toluenesulfonyl chloride was used, leading to the formation of the doubly sulfonylated compound **15**. This intermediate was immediately reacted with 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in mild conditions; as expected this reaction afforded the benzylic amine **16** as sole reaction product, in 72% yield over the two steps. Hydrolysis of the *p*-toluenesulfonate group was easily achieved by heating the compound **16** in a 1/1 mixture of CH_3CN and 1N aqueous sodium hydroxide, giving **MC70** in a 66% overall yield.



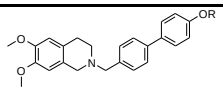
Scheme 3. Reagents and conditions. a) K_2CO_3,Pd/C cat., H_2O, 80 °C, 45 min. b) *p*-toluensulfonylchloride, Et_3N, DMAP cat., CH_2Cl_2, room temp., 4 h. c) 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride, DBU, CH_3CN 65 °C, 2 h. d) 1N NaOH/CH_3CN 1/1, 80 °C, 6 h.

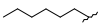
In order to evaluate the potency of each synthesized ligand towards P-gp and the P-gp interacting mechanism, all derivatives were tested by three combined assays: 1) apparent permeability determination in Caco-2 cells monolayer;[10] 2) inhibition of the transport of Calcein-AM, a profluorescent P-gp substrate, in cells overexpressing P-gp (MDCK-MDR1 cells);[10] 3) determination of the consumption of ATP in MDCK-MDR1 cells.[10] By the calcein-AM assay, the potency of the interaction towards P-gp can be defined. The activity profile (as substrate or inhibitor) of all ligands can be determined by combining the three above assays. *Substrates* inhibit transport of Calcein-AM, as transported, induce a decrease in ATP cell level and show a P_{app} value > 2; *inhibitors* inhibit Calcein-AM transport, but, not being transported, do not induce a decrease in ATP level and show a P_{app} value < 2. This experimental classification can be used to easily distinguish between an inhibitor and a substrate. A particular kind of substrates exists, which are named also *modulator*.[11] Cyclosporin A (CSA) is a substrate/modulator belonging to the category IIB3,[11] since it is transported, it competes with a P-gp subtsrate but it is unable to activate ATPase. Modulators and inhibitors exert the same final biological effect, restoring cell sensitivity to chemotherapeutic agents and are usually co-administered with a radiotracers to study the pharmakodynamic profile in vivo of a new PET radiotracer candidate.
The selectivity of all ligands was established by testing their effect towards MRP1 (calcein-AM assay on MDCK cells overexpressing MRP1).

As depicted in Table 1, all compounds displayed a substrate behavior (P_{app} >2 in all cases) and had potencies comparable to that of the lead. Compounds **10** and **11**, which bear oxyalkyl chains, were the most active agents of the series. Compound **4** represents an exception to this trend, showing a particularly high activity (5.2 nM).

In order to shed light on the reasons for the very high activity of **4**, its behavior was studied in the presence of 30 μM CSA, a well-known product able to bind the modulator site of P-gp.[12] We select this dose (30 μM) since we preliminarily probed all the concentrations of modulator (from 0.5 to 30 μM) and we found that CSA exerted the highest effect at 30 μM. In this experiment, our compound **4** was used at concentrations ranging from 0.1 nM to 100 nM. Under these conditions, the compound can only bind the substrate site, since the modulator site is occupied by CSA. Figure 1, in which the results of the experiments are reported, shows that the activity on P-gp strongly decreases when CSA is present, thus suggesting that the binding to the modulator site positively modulates the P-gp substrate site. Figure 1, which gives the results of the experiments, shows that the activity on P-gp decreases markedly when CSA is present, suggesting that binding to the modulator site positively modulates the P-gp substrate site.

Table 1. Biochemical characterization of compounds **2**-**11**.



No	R	EC_{50} P-gp[a]	EC_{50} MRP1[a]	P_{app}[c]
2		0.63 ± 0.12 μM	na[b]	3.96
3		1.20 ± 0.20 μM	na[b]	> 20
4		0.0052 ± 0.001 μM	na[b]	> 20
5		0.96 ± 0.22 μM	na[b]	> 20
6		na[b]	na[b]	> 20
7		na[b]	na[b]	15
8		0.82 ± 0.16 μM	na[b]	3.3
9		1.25 ± 0.25μM	na[b]	3.5
10		0.42 ± 0.08 μM	na[b]	9.8
11		0.32 ± 0.06 μM	na[b]	4.1
1 (**MC70**)	-H	0.69 μM[d]	9.30 μM[d]	1.3[e]

[a] Data ± SEM are the mean of three independent determinations (samples in triplicate). [b] Not active 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) each with SEM < 10%. [d] See reference 7. [e] See reference 6.

Similar experiments carried out with the higher and lower homologues **5** and **3**, showed that their activity was not influenced by the presence of CSA (data not shown). Thus, the dual nature of substrate/modulator P-gp ligand might legitimately be responsible for the high activity of **4**.

In conclusion, the present study proposed a new and more efficient synthetic route for the preparation of **MC70**, a well-known P-gp inhibitor. A small library of alkyl/oxyalkyl derivatives of **MC70** were synthesized, and studied to elucidate their pharmacological profiles in terms of potency, selectivity, and mechanism of interaction with P-gp. Most compounds proved to be selective inhibitors of P-gp mediated Calcein-AM efflux, through a substrate mechanism, and were endowed with potency in the low or sub-micromolar range, comparable to that of the lead.

Excitingly, compound **4** showed a dual P-gp interacting activity *i.e.* modulator/substrate. This compound indeed seems to modulate P-gp substrate site as for CSA, and comparing to the immunomodulator, it is the first molecule devoid of any other pharmacological activities exerting this effect. Therefore, compound **4** discloses new perspectives in reverting MDR by the co-administration of chemotherapeutic drugs and P-gp modulator.

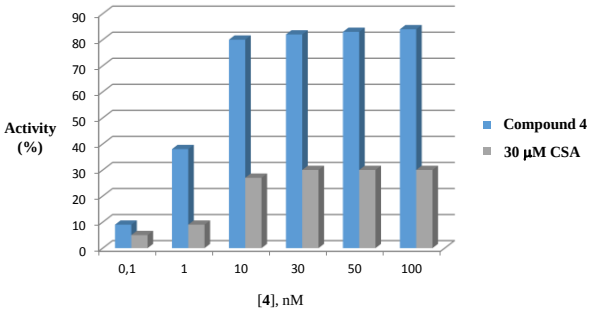


Figure 1. Representative P-gp activity evaluation of compound **4** at several concentrations (0.1-100 nM) in the absence (blue bars) and in the presence of 30 μM CSA (grey bars). The results are the mean of three independent experiments (samples for each concentration in duplicate). Statistical evaluation gave $P = 0.001$ ($P < 0.005$, significant).

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Keywords: P-glycoprotein • Multidrug resistance • Anti tumor agents • Proteins • C-C coupling.

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