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Iodination Increases the Activity of Verapamil Derivatives in Reversing PGP Multidrug Resistance

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Abstract. *Iodinated derivatives of verapamil were synthesized and tested as P-glycoprotein (Pgp)-mediated multidrug resistance (MDR) reversal agents. The ability of these compounds to revert MDR was evaluated on daunorubicin-resistant K562 cells, by measuring the intracellular accumulation of rhodamine 123, a fluorescent probe of Pgp transport activity. One of the investigated compounds (**16c**) was found to be a more potent MDR reversal agent than verapamil and cyclosporin A, used as reference molecules. Further in vitro studies showed that compound **16c** restored daunorubicin activity and, when used alone, did not induce cell death, cell cycle perturbation and modification of calcium channel activity in comparison with verapamil.*

Drug resistance is one of the major obstacles to the successful treatment of cancer by chemotherapy and one way for tumoral cells to survive to antitumor drug administration is through a multidrug resistance MDR phenotype which can be the result of a variety of mechanisms (1, 2). However, the most implicated mechanism is the overexpression of drug-transmembrane transporter proteins (3, 4). The best known such proteins are P-glycoprotein (Pgp) (5, 6), encoded by *MDR1* gene (7, 8), and, to a lesser extent, multidrug resistance-associated protein MRP-1 (9, 10) encoded by *MRP1* gene (3, 11), and breast cancer resistance protein BCRP (12, 13). These belong to the ATP binding cassette (ABC) superfamily of transporters and use ATP hydrolysis to extrude various drugs from cancer cells (14).

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One approach to overcome such resistance is to develop modulators that would inhibit Pgp or other ABC transporters involved in MDR. In the past few years, a wide range of chemical compounds has been identified as being able to revert MDR (15), including verapamil. Verapamil has been used for decades in the treatment of cardiovascular diseases according to its calcium-channel blocking properties and was shown for the first time to be able to inhibit Pgp activity in resistant cells in the 1980s (16). As a result, verapamil is one of the most studied modulators, both *in vitro* and *in vivo*, and has been used as well as cyclosporin A to identify more potent and selective drugs. Several chemical modifications have been attempted on the verapamil backbone to reach derivatives with low calcium channel blocking activity and high MDR-reverting action (17-23). Reversal studies of structure–activity relationships carried out on these verapamil derivatives concluded that the aromatic moiety of verapamil bearing the quaternary carbon substituted with isopropyl and cyano groups (the left part of verapamil) is essential for MDR modulation (18, 21). Other modifications were also made on aromatic moieties with halogens. It seems that the substitution of aromatic rings with fluorine reduces the MDR activity (19), whereas that with chlorine (18) and bromine (21) induce no significant increase or only slight increase respectively. Curiously, as yet, the effect of iodination on the reversal of Pgp-mediated MDR of verapamil derivatives has never been described in the literature. We are interested in the design of new reversing agents based on verapamil (24). Introduction of iodine atoms may potentially increase the affinity for Pgp as was described for iodinated derivatives of chalcones (25).

This article describes the *in vitro* evaluation of cytotoxicity and MDR reversal potential of non-iodinated and iodinated verapamil derivatives.

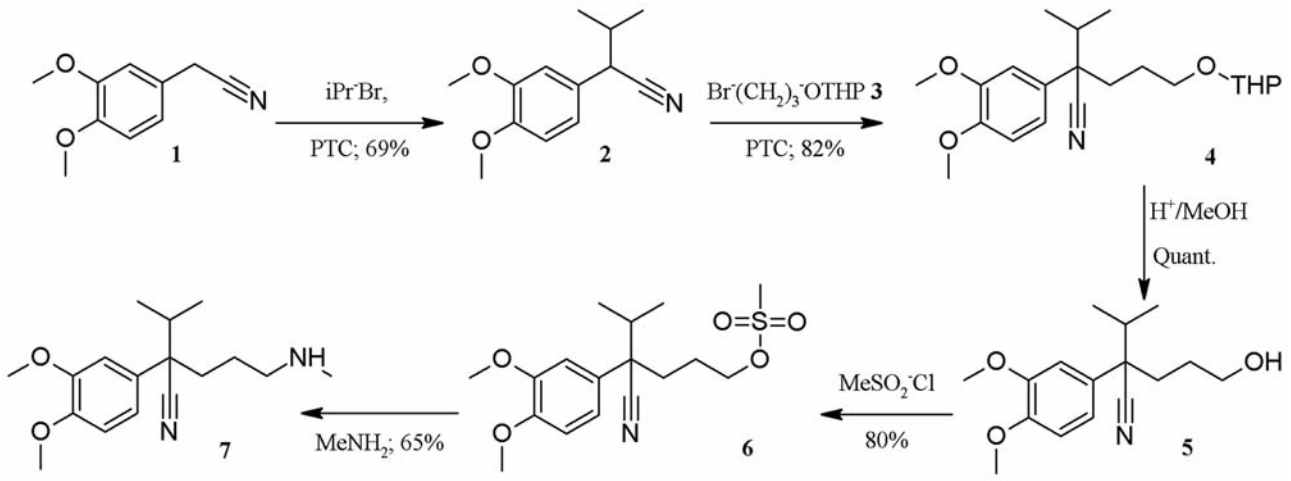


Figure 1. *Synthesis of the left part of verapamil.*

Materials and Methods

Chemistry. The synthesis of the left part of verapamil starts from homoveratronitrile **1** (scheme shown in Figure 1). According to the literature, this can be alkylated with 2-bromopropane first, and then with a bromopropyl tetrahydropyranyl (THP) derivative **3** using NaH (26) or BuLi (21). However, we found it more convenient to proceed by phase-transfer catalysis (PTC) (27, 28): it gave a good yield of the tetrahydropyranyl ether **4** without the manipulation of NaH and BuLi which are water sensitive and dangerous reactants. In turn, the tetrahydropyranyl group (THP) of **4** was removed and gave the alcohol **5** quantitatively. Subsequent mesylation with Me-SO_2-Cl in pyridine afforded **6** in good yield. The conversion of **6** to **7** was performed with methylamine in moderate yield (65%) (Figure 1).

The target molecules were prepared following the general route depicted in the scheme shown in Figure 2. Starting from 2-(4-methoxyphenyl)-ethanol **8**, mesylation followed by amination with **7** afforded compound **16a** (non-iodinated) in a satisfactory yield (51%, two steps).

For the synthesis of iodinated derivatives, it was not possible to perform the iodination satisfactorily on **16a** because both aromatic rings had very similar reactivity towards iodinating agents. The iodine atom was consequently introduced earlier in the synthesis before the condensation of the right part, on the 2-(4-methoxyphenyl)-ethanol. We found that chloramine-T, and iodine in ammonia were inefficient to perform iodination (29). It was necessary to generate the electrophilic I^+ with iodine and sodium periodate in acetic acid to achieve the iodination step. But in such conditions, acetylation of the alcohol function was noted (compounds **9** and **10**) and it was necessary to cleave this ester with potassium carbonate in methanol to afford quantitatively the desired iodinated alcohols **11** and **12**, which were then easily mesylated. The target compounds **16b-c** were finally prepared in moderate yield. All the analytical data (^1H, ^{13}C, IR, mass spectrometry and elemental analysis) are in agreement with the structure of the compounds.

Cells and culture conditions. The human myeloid leukemic K562 cell line and its adriamycin-resistant subline K562/Adr, which expresses high levels of Pgp, were generously provided by J.P. Marie (Hôtel-Dieu Hospital, Paris). K562 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 IU/ml penicillin and 100 ng/ml streptomycin (Invitrogen, Cergy Pontoise, France) at 37˚C in a humidified atmosphere with 5% CO_2. Parental and resistant K562 cultures are named in the text as K562S and K562R, respectively. The chemoresistance of K562R cells was maintained by continuous exposure to 10^{-6} M adriamycin (ADR). Resistant cells were grown in ADR-free medium for three weeks before each experiment in order to avoid interference from ADR fluorescence. Culture in ADR-free medium did not alter the resistance phenotype, as measured by rhodamine 123 expulsion.

Adult cardiomyocytes were isolated from male Wistar rats as described previously (30). About 1 to 1.5×10^6 calcium-tolerant cardiomyocytes in 15 ml of 199 culture medium (Sigma-Aldrich) added to insulin 10^{-9} M, sucrose 55 mM, Hepes 20 mM, $NaHCO_3$ 10 mM, penicillin 100 IU/ml and streptomycin 0.1 mg/ml) were placed into one Petri dish and incubated at 37˚C in an atmosphere containing 5%CO_2 and 95% air.

Rhodamine-123 accumulation test. Modulators were diluted in DMSO (10 mM) then in RPMI-1640 medium to obtain a final concentration ranging from 10^{-5} to 10^{-6} M. The cells grown in flasks up to 90% confluence were washed with serum-free RPMI and resuspended in this medium to obtain a cell density of 10^6 cells/ml. The rhodamine-123 test was performed by incubating 1 ml of cell suspension at 10^6 cells/ml with different concentrations of compound at 37˚C for 15 min, followed by adding 10^{-6} M rhodamine-123, with cyclosporin A was used as a reference modulator. After 30 min incubation, accumulation was stopped by adding 3 ml of ice-cold PBS and analyzed by flow cytometry. The results are expressed by the mean intensity of rhodamine-123 fluorescence (arbitrary unit, A.U.).

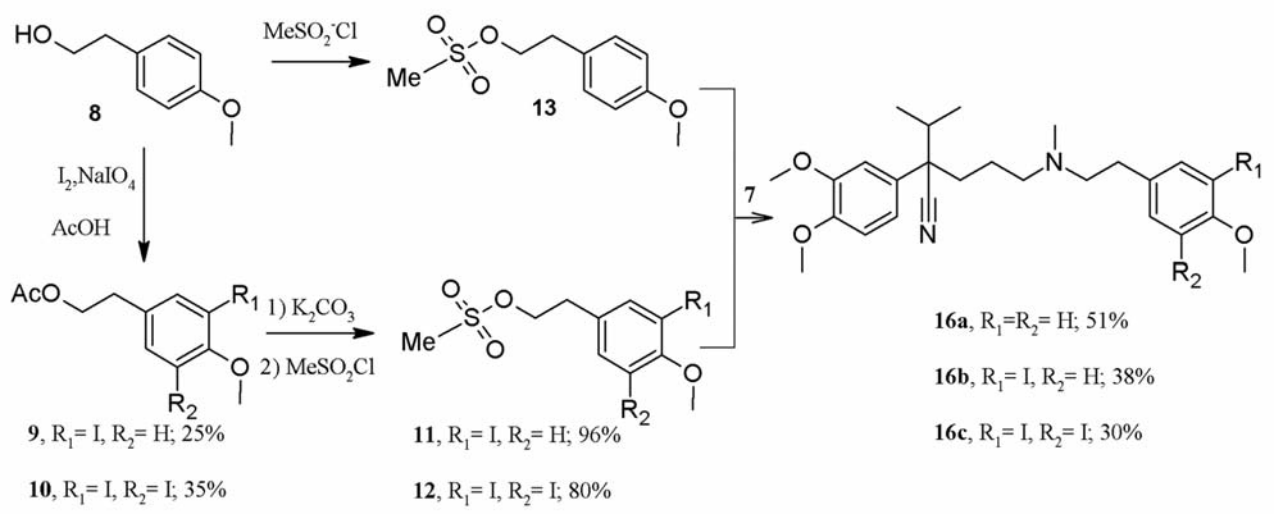


Figure 2. *Synthesis of the target molecules.*

DNR cytotoxicity studies. The effect of modulators on cellular cytotoxicity was evaluated by flow cytometry using the exclusion test of TOTO-3 (a fluorochrome not taken up by living cells) to avoid an overlap of daunorubicin spectrum, Cells were incubated with 10^{-6}M daunorubicin and various concentrations (10^{-5} to 10^{-10} M) of cyclosporine A, verapamil, or the compound to be tested. After 24, 48, or 72 h incubation at 37˚C, cells were washed twice with PBS buffer. Cell death was assessed by the uptake of 5 nM TOTO-3.

DNA content analysis. The cell cycle was evaluated with the Cycle Test™ kit (BD-Biosciences). Briefly, cells were incubated with trypsin in a spermine tetrahydrochloride detergent buffer for 10 min at room temperature. Trypsin inhibitor and ribonuclease A were added for 10 min without washing and finally, propidium iodide (PI) was added and the whole was incubated for 10 min, then cells were immediately analyzed by flow cytometry.

Flow cytometric analysis. Cell fluorescence was measured with a FACScalibur flow cytometer (BD Biosciences) equipped with a 15 mW 488 nm argon laser and a laser diode at 635 nm. Measured parameters were always expressed in A.U. Rhodamine-123 green fluorescence was collected through a 530±30 nm filter. TOTO-3 red fluorescence was collected through a 660±16 nm filter and PI orange fluorescence with a 585±44 nm filter. Data were acquired and analyzed using Cell Quest Pro software (BD Biosciences). All experiments were repeated three times.

Perforated patch-clamp. Ca^{2+} currents were recorded in the whole-cell configuration of patch-clamp technique and analyzed using PClamp (Molecular Device, Sunnyvale, CA, USA). Pipettes were pulled from Kimax 51 glass. Currents were obtained with an Axopatch 200B amplifier. All traces were corrected for leak and capacitance currents, filtered at 2 kHz, and digitized at 20 kHz msec. The pipette solution was designed to eliminate all K^+ currents and consisted of the following components (mM): Cs-glutamate (130), D-glucose (5), HEPES (10), $MgCl_2$ (2.5), TEA-Cl (20), Mg_2ATP (4), EGTA-Cs (10) pH 7.2 (adjusted with 1 N CsOH). The bath solution contained (mM): NaCl (116.4), KCl (5.36), $CaCl_2$ (1.8), $MgSO_4$ (0.4), NaH_2PO_4 (0.8), Glucose (5), HEPES (10), pH 7.2 (adjusted with 1 N NaOH). The cells were isolated in a 1 ml chamber and perfused at a rate of 4-8 ml/min. All experiments were carried out at room temperature (~25˚C).

Results

MDR reversal activity. We evaluated rhodamine-123 accumulation in K562R cells using flow cytometry in order to distinguish interesting derivatives. All derivatives were compared to verapamil. Cyclosporin A, one of the MDR modulators used clinically, which acts on Pgp transport activity, was also used as a reference. The reversing properties were tested at two concentrations (1 and 10 μM) on K562R cells. The results are expressed as the mean rhodamine-123 fluorescence intensity as shown in Figure 3. At 10^{-5} and 10^{-6} M, compound **16b** showed the same reversal activity as verapamil but the highest activity was observed for derivative **16c**.

At the same concentrations, **16c** was still strongly and even more active than cyclosporin A and verapamil. Being the most active compound **16c** was selected for further studies.

Cytotoxicity activity. The ability of compound **16c** to potentiate daunorubicin cytotoxicity on K562R cells was evaluated by flow cytometry using the exclusion test of TOTO-3. The potency of **16c** was compared with cyclosporin A and verapamil. The dose response curves which show the percentage of viable cells (corresponding to cells with a low level of TOTO-3 fluorescence) are reported in Figure 4A.

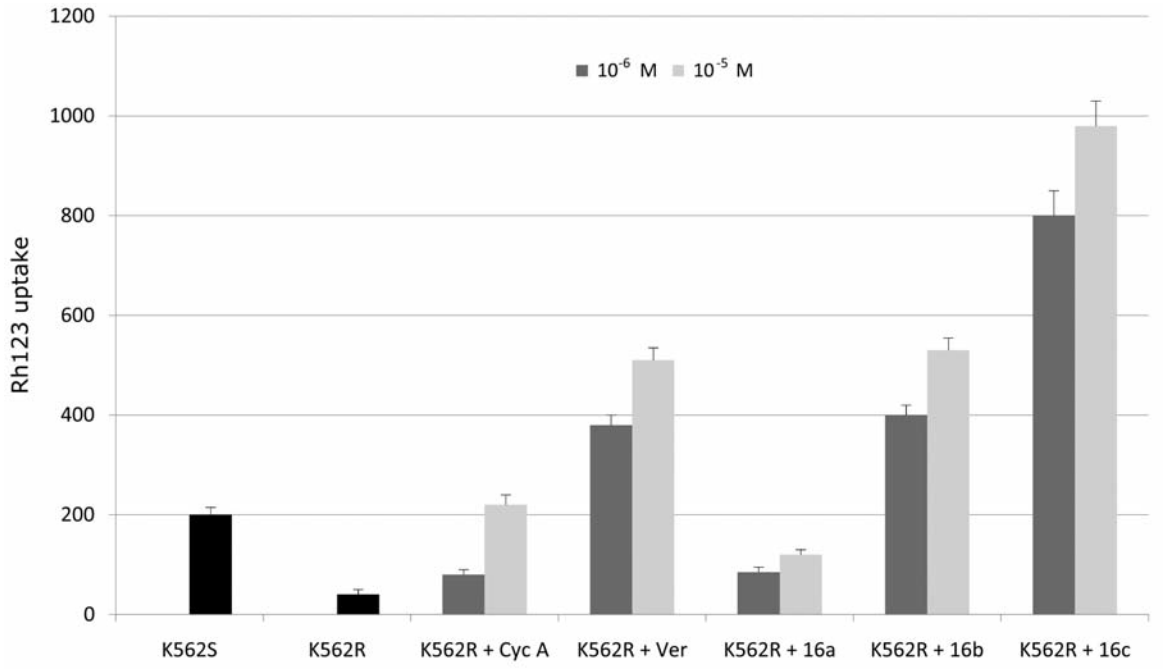


Figure 3. *Effect of verapamil derivatives (**16a-c**) on the accumulation of Rhodamine-123 in K562R. Cyclosporin A (Cyc A) and verapamil (Ver) were used as reference inhibitors of Pgp transport activity. The results are expressed as the mean intensity of rhodamine-123 (Rh123) fluorescence (arbitrary units). Each experiment was repeated three times.*

At different concentrations, compound **16c** had the same activity as verapamil. When cells were treated with 10^{-5} and 10^{-6} M daunorubicin, derivative **16c** reduced K562R viability to 45±4% and 35±3%, respectively.

Effect on cell cycle. The effect of compound **16c** on the cell cycle distribution of the different phases was investigated using flow cytometry after DNA labeling with PI. K562R cell distribution in $G_{0/1}$, S, and G_2+M phases was analyzed after treatment with 10^{-5} to 10^{-7} M concentrations of verapamil and **16c**, for 48 h. There was no significant differences between control and cells treated at the different concentrations of verapamil and **16c** since the cell cycle distribution was 57±4%, 32±4%, 11±2% for $G_{0/1}$, S and G_2+M respectively (Figure 4B).

Cardiovascular activity. Verapamil is a potent antagonist of L-type calcium channels. It was thus important to estimate the potency of its derivatives on L-type calcium channels. The ability of the **16c** derivative to inhibit L-type calcium channels was evaluated in cardiomyocytes. Freshly dissociated cardiac myocytes were patch-clamped in the whole cell configuration and depolarized from a holding potential of –60 mV to a test potential of +20 mV, at a frequency of 0.1 Hz. Figure 5 shows representative L-type current inhibition experiments with 100 nM **16c** and verapamil. The percentage of L-type current inhibition obtained with 100 nM **16c** (n=7) and verapamil (n=6) were not statistically different (p>0.1).

This protocol activates an L-type inward calcium current, due to the opening of Ca V1.2 calcium channels (inset Figure 5). The **16c** derivative or verapamil was introduced by perfusion in the bath medium at identical concentrations. In the presence of both drugs, we recorded a progressive inhibition of the amplitude of the calcium currents, with identical kinetics. At a concentration of 10^{-7} M, the amplitude of the current decreased by 75%. Different concentrations (5.10^{-8} and 10^{-6} M) were also tested and no difference was noticed in kinetics or potency (data not shown). From these results, we can conclude that the modifications introduced in the verapamil molecule to obtain the **16c** derivative do not increase the cardiac toxicity.

Discussion

The structures investigated in this study were iodinated verapamil analogs, and their MDR reversal activity was evaluated by measuring rhodamine-123 accumulation in resistant cells. On the basis of the *in vitro* results, the following remarks can be made. Non-iodinated compound **16a** showed moderate reversal and was less effective than cyclosporin A and verapamil. The mono-iodinated compound **16b** restored

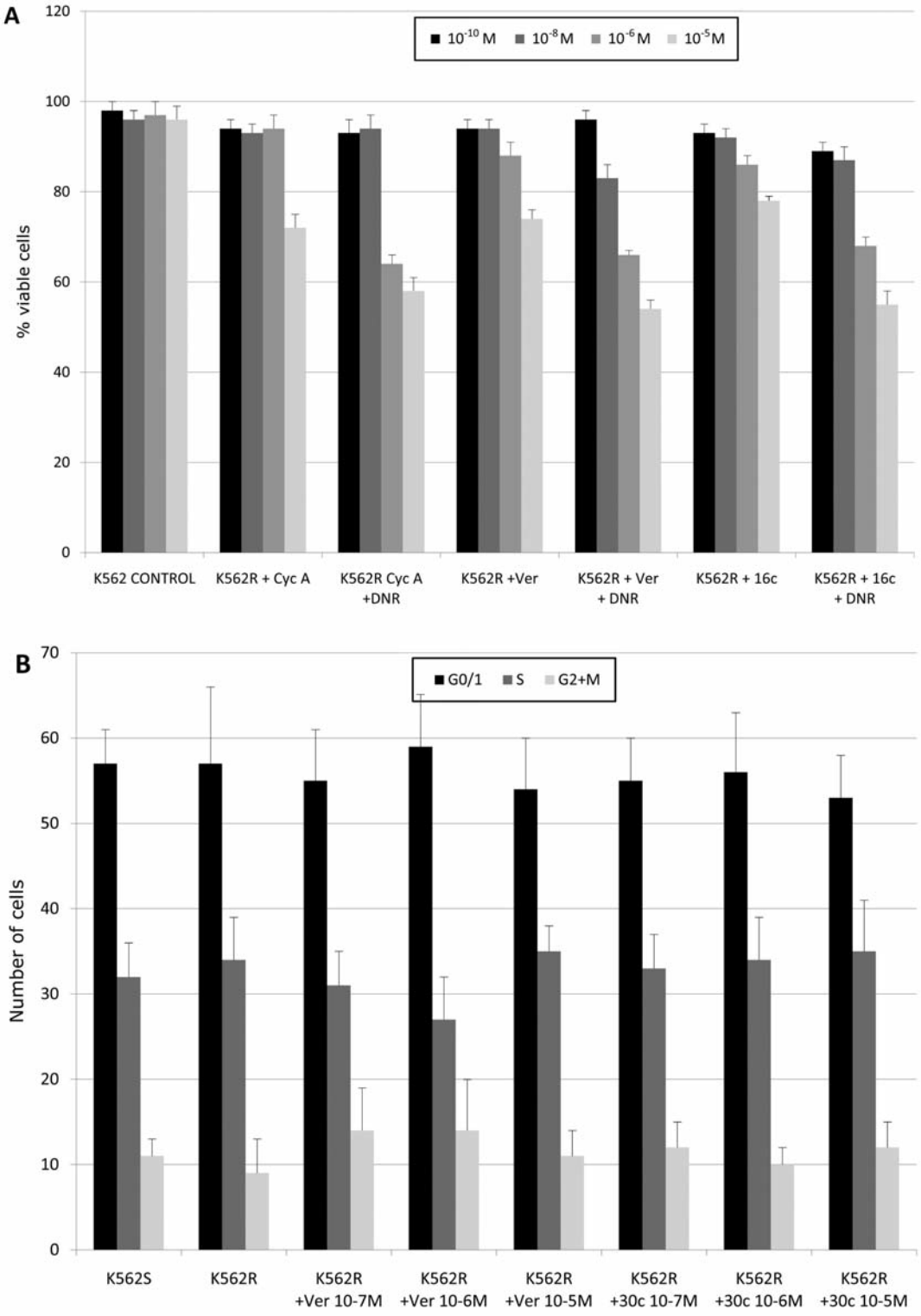


Figure 4. *A: Effect of compound* ***16c*** *on cytotoxicity of daunorubicin in K562R cells. Viability of K562R, controls and treatment with* ***16c*** *at four concentrations (10^{-5}, 10^{-6}, 10^{-8} and 10^{-10} M) after 72 h with or without 1 μM of daunorubicin was measured by flow cytometry using TOTO-3 dye. Cyclosporin A and verapamil were used as reference. Each experiment was repeated three times. B: Effect of compound* ***16c*** *on cell cycle distribution after treatment with daunorubicin in K562R cells. Cyclosporin A and verapamil were used as reference compounds. DNA content of K562R, controls and cells treated with* ***16c*** *at three concentrations (10^{-5}, 10^{-6}, 10^{-7} M) during 48 h was measured by flow cytometry using PI staining.*

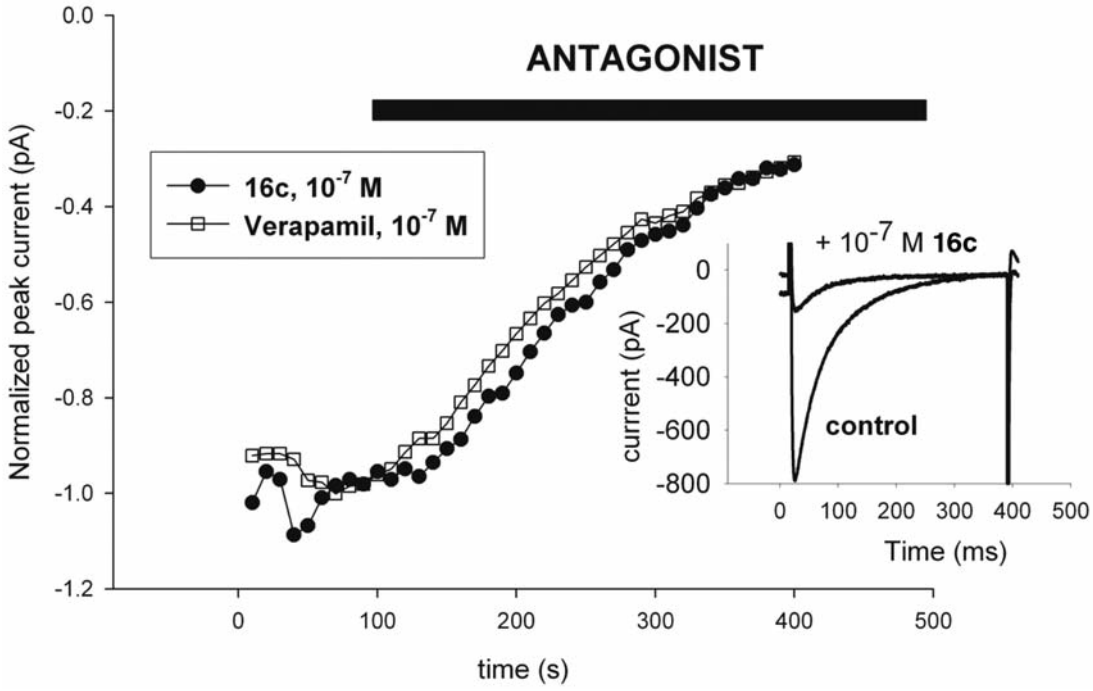


Figure 5. *Verapamil and **16c** derivative have very similar potency on L-type calcium current. Representative time courses of L-type calcium current inhibition by verapamil and **16c** derivative are shown. Cardiomyocytes were depolarized at a frequency of 0.1 Hz from a holding potential of –60 mV to a test potential of +20 mV. In the absence of antagonist, the normalized amplitude of the peak current recorded was stable. When the antagonists were introduced into the bath at a concentration of 10^{-7}M, the amplitude of the peak current was inhibited. Inset: Representative current traces showing the current before and after the application of **16c**.*

activity to that of the level of verapamil. The di-iodinated compound **16c** presented the highest reversal (practically two-fold more potent than verapamil). Hence, there is a relationship between the extent of reversal and the number of iodine atoms in the *ortho* position of the methoxy group. This result stresses the importance of the iodine atom used as a substituant of verapamil, since it had particularly beneficial effect in MDR reversal. This effect is usually correlated to the lipophilic nature of iodine, which increases the liposolubility of compounds. The better activity of compounds substituted with iodine rather than the other halogens (F, Cl, Br) (18, 19, 21) is also certainly correlated to its capability to establish strong nitrogen-halogen bonds which may effectively drive the intermolecular recognition between iodinated verapamil and the nitrogen atoms of Pgp. Indeed, it is now well established that halogen atoms are able to function as general, effective and reliable sites for directing molecular recognition processes. The strength of the interactions decreases in the order I>Br>Cl (31). This could explain our results and also those obtained for iodinated chalcones (25).

Patch-clamp experiments showed that compound **16c** presented almost the same calcium channel blocking activity as verapamil. Some verapamil derivatives described in literature are good modulators of Pgp *in vitro* and present low cardiovascular activity, such as those recently reported by Biscardi *et al*. (23). Although compound **16c** shows the same cardiovascular activity as verapamil, its reversal of MDR potency is greater.

Conclusion

Iodinated verapamil derivatives were synthesized and evaluated as Pgp reversal agents. The *in vitro* data demonstrated that compound **16c** was twice as potent as verapamil and that the iodine atom plays a particular role in this effect. Indeed, compound **16c** restored rhodamine-123 accumulation in K562R cells expressing Pgp and restored daunorubicin activity. Compound **16c** did not modify K562 cell cycle distribution and showed similar cardiomyocyte toxicity to verapamil. Other iodinated drugs are currently studied in our laboratory in order to get better understanding of the role of the iodine atom in medicinal chemistry.

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