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# 3,5-Dibenzoyl-4-(3-phenoxyphenyl)-1,4-dihydro-2,6-dimethylpyridine (DP7) as a new multidrug resistance reverting agent devoid of effects on vascular smooth muscle contractility

[1]Simona Saponara, [2]Masami Kawase, [3]Anamik Shah, [4]Noboru Motohashi, [5]Joseph Molnar, [5]Katalin Ugocsai, *,[1]Giampietro Sgaragli & [1]Fabio Fusi

[1]Istituto di Scienze Farmacologiche, Università degli Studi di Siena, via A. Moro 2, 53100 Siena, Italy; [2]Faculty of Pharmaceutical Sciences, Josai University, Saitama 350-0295, Japan; [3]Department of Chemistry, Saurashtra University, Rajkot-360 005, India; [4]Meiji Pharmaceutical University, Tokyo 204-8588, Japan and [5]Faculty of Medicine, Institute of Microbiology, Albert Szent-Györgyi Medical University Dom ter 10, Szeged H-6720, Hungary

**1** The aim of this study was to investigate the effects of 3,5-diacetyl- (DP1–DP5) and 3,5-dibenzoyl-1,4-dihydropyridines (DP6–DP11) on vascular functions *in vitro*, by comparing their mechanical and electrophysiological actions in rat aorta rings and single rat tail artery myocytes, respectively, and to quantify their multidrug resistance (MDR)-reversing activity in L5178 Y mouse T-lymphoma cells transfected with MDR1 gene.

**2** In rat aorta, the 11 compounds tested, but 3,5-dibenzoyl-4-(3-phenoxyphenyl)-1,4-dihydro-2,6-dimethylpyridine (DP7), 3,5-dibenzoyl-4-(3-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine (DP9), 3,5-dibenzoyl-4-(4-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine (DP10) and 3,5-dibenzoyl-4-phenyl-1,4-dihydro-2,6-dimethylpyridine (DP11), antagonized 60 mM $K^+$ (K60)-induced contraction in a concentration-dependent manner, with $IC_{50}$ (M) values ranging between $5.65 \times 10^{-7}$ and $2.23 \times 10^{-5}$.

**3** The 11 dihydropyridines tested, but DP7, inhibited L-type $Ca^{2+}$ current recorded in artery myocytes in a concentration-dependent manner, with $IC_{50}$ (M) values ranging between $1.12 \times 10^{-6}$ and $6.90 \times 10^{-5}$.

**4** The $K^+$-channel opener cromakalim inhibited the $Ca^{2+}$-induced contraction in K30 but not that evoked in K60. On the contrary, DP7 was ineffective in both experimental conditions.

**5** When the rings were preincubated with 1 mM $Ni^{2+}$ plus 1 $\mu$M nifedipine, the response to phenylephrine was significantly reduced by 2,5-di-*t*-butyl-1,4-benzohydroquinone (BHQ), a well-known endoplasmic reticulum $Ca^{2+}$-ATPase inhibitor. DP7 had no effects on this model system.

**6** In L5178 MDR cell line, the 11 dihydropyridines tested, but 3,5-diacetyl-4-(2-nitrophenyl)-1,4-dihydro-2,6-dimethylpyridine (DP1), 3,5-diacetyl-4-(3-phenoxyphenyl)-1,4-dihydro-2,6-dimethylpyridine (DP2) and 3,5-diacetyl-4-(3-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine (DP4), exhibited an MDR-reversing activity, with $IC_{50}$ values ranging between $3.02 \times 10^{-7}$ and $4.27 \times 10^{-5}$, DP7 being the most potent.

**7** In conclusion, DP7 may represent a lead compound for the development of potent dihydropyridine MDR chemosensitizers devoid of vascular effects.
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**Keywords:** MDR reverter; rat aorta ring; rat tail artery smooth muscle; whole-cell patch-clamp; L-type $Ca^{2+}$ channel inhibitor; dihydropyridines; rhodamine 123; mouse T-lymphoma cell

**Abbreviations:** BHQ, 2,5-di-*t*-butyl-1,4-benzohydroquinone; DP, dihydropyridine; $I_{Ca(L)}$, L-type $Ca^{2+}$ current; K60, 60 mM $K^+$; MDR, multidrug resistance; MRPs, multidrug resistance-related proteins; P-gp, P-glycoprotein 170; PSS, physiological salt solution; R123, rhodamine 123; TEA, tetraethylammonium; $V_h$, holding potential; VOCCs, voltage-operated $Ca^{2+}$ channels

## Introduction

A large number of drugs are planar lipophilic agents and thus theoretical substrates for the ATP-binding cassette of membrane transporters, including P-glycoprotein 170 (P-gp) and various multidrug resistance (MDR)-related proteins (MRPs), acting as energy-dependent extrusion pumps. The overexpression of these transporters confers a MDR phenotype on cells in various diseases, including many forms of cancer (Gottesman *et al*., 2002), rheumatoid arthritis (Llorente *et al*., 2000), inflammatory bowel disease (Farrell *et al*., 2000) and epilepsy (Siddiqui *et al*., 2003).

Development of MDR is one of the main reasons of failure in malignant tumor chemotherapy, as tumor cells, by increasing drug efflux, acquire cross-resistance to many structurally and functionally unrelated anticancer agents,

*Author for correspondence; E-mail: sgaragli@unisi.it
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which therefore never achieve effective intracellular concentrations (Krishna & Mayer, 2000).

To restore the inherent potency of antitumor agents, several compounds have been studied as inhibitors of P-gp-mediated transport, and then proposed as reverters of cellular resistance (Ford, 1996). So far, MDR reverters belong to various chemical or pharmacological classes including $Ca^{2+}$ channel blockers, calmodulin inhibitors, coronary vasodilators, indole alkaloids, quinolines, hormones, cyclosporines, surfactants and antibodies (Krishna & Mayer, 2000; Kawase & Motohashi, 2003). Among them, $Ca^{2+}$ channel blockers, such as verapamil and some dihydropyridines related to nifedipine, have been extensively investigated (Tsuruo, 1986; Hollt *et al*., 1992; Toffoli *et al*., 1995). They are endowed with inherent cardiovascular activity, however, so that the doses required to overcome MDR were found to be associated with severe and sometimes life-threatening cardiovascular toxicity such as atria-ventricular block and hypotension, thus rendering their clinical application impossible. Thus, it is desirable to have compounds that can be used at much lower doses than those shown to be cardiotoxic, or are devoid of cardiovascular effects at the doses effective for reverting MDR. As a consequence, in the last few years, much attention has been focused on the development of safer MDR inhibitors characterized by proper potency, selectivity and pharmacokinetics.

Recently, novel 3,5-diacetyl- (DP1–DP5) and 3,5-dibenzoyl-1,4-dihydropyridine (DP6–DP11) derivatives (Table 1) were synthesized and screened for their ability to reverse MDR in *in vitro* assay systems (Shah *et al*., 2000; Kawase *et al*., 2002). Some of them were proved to reduce the activity of P-gp, and therefore they were envisaged as new candidates for MDR reversion in cancer treatment. However, as dihydropyridine derivatives, these compounds are expected to be active also on the cardiovascular system.

The aim of this study, therefore, was to investigate the effects of these dihydropyridines on vascular function *in vitro,* by comparing their mechanical and electrophysiological actions, as well as to quantify their MDR reversal properties.

## Methods

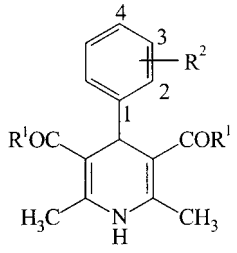
### *Aorta rings preparation*

Aorta rings deprived of endothelium were prepared from male Wistar rats (350–450 g) anesthetized with a mixture of Ketavet® (Gellini, Italy) and Xilor® (BIO 98 srl, Italy), decapitated, and bled. The thoracic aorta was immediately removed, cleaned of adhering fat and connective tissue, and cut into 2.5-mm wide rings with a razor blade slicing device. Each arterial ring was mounted over two rigid parallel stainless steel tubes, one fixed in place and the other attached to an isometric transducer (Basile, Varese, Italy) connected to a kymograph-like pen recorder (Basile, Varese, Italy), or to a flat bed recorder (Linseis, Bolzano, Italy). The preparation was immersed in a water-jacketed organ bath (37°C) containing 5 ml of a modified Krebs–Henseleit physiological salt solution (PSS; see below).

### *Equilibration period*

Rings were allowed to equilibrate for 1 h at a resting tension of 1 g. During this equilibration period, PSS was changed every 15 min and passive tension was re-adjusted to 1 g. Following this equilibration period, rings were stimulated with 60 mM $K^+$ (K60) PSS, until a sustained response was obtained ($\sim$15 min) in order to test their contractile capacity. At the end of a 30-min washout period, the absence of functional endothelium was assessed by the lack of response to 10 $\mu$M acetylcholine in rings precontracted with 0.3 $\mu$M phenylephrine. Under these conditions, maximal plateau values for active tension of $671.4 \pm 14.6$ mg ($n = 102$) were obtained. Rings were then washed and equilibrated for another 45-min

**Table 1** Structures of the dihydropyridine derivatives tested in the present study




| *Compounds* | | $R^1$ | $R^2$ |
|---|---|---|---|
| DP1 | 3,5-Diacetyl-4-(2-nitrophenyl)-1,4-dihydro-2,6-dimethylpyridine | $CH_3$ | 2-$NO_2$ |
| DP2 | 3,5-Diacetyl-4-(3-phenoxyphenyl)-1,4-dihydro-2,6-dimethylpyridine | $CH_3$ | 3-PhO |
| DP3 | 3,5-Diacetyl-4-(2-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine | $CH_3$ | 2-Cl |
| DP4 | 3,5-Diacetyl-4-(3-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine | $CH_3$ | 3-Cl |
| DP5 | 3,5-Diacetyl-4-(4-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine | $CH_3$ | 4-Cl |
| DP6 | 3,5-Dibenzoyl-4-(2-nitrophenyl)-1,4-dihydro-2,6-dimethylpyridine | Ph | 2-$NO_2$ |
| DP7 | 3,5-Dibenzoyl-4-(3-phenoxyphenyl)-1,4-dihydro-2,6-dimethylpyridine | Ph | 3-PhO |
| DP8 | 3,5-Dibenzoyl-4-(2-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine | Ph | 2-Cl |
| DP9 | 3,5-Dibenzoyl-4-(3-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine | Ph | 3-Cl |
| DP10 | 3,5-Dibenzoyl-4-(4-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine | Ph | 4-Cl |
| DP11 | 3,5-Dibenzoyl-4-phenyl-1,4-dihydro-2,6-dimethylpyridine | Ph | H |
| Nifedipine | 3,5-Dicarbomethoxy-4-(2-nitrophenyl)-1,4-dihydro-2,6-dimethylpyridine | $OCH_3$ | 2-$NO_2$ |

period before performing the various experimental tests (see below). Control preparations were treated with the vehicle only.

### *Effects of dihydropyridine (DP) derivatives and nifedipine on rat aorta rings precontracted with K60*

The relaxation produced by DP derivatives and nifedipine was assessed in rings precontracted with K60. Cumulative concentration–response curves for each drug were made. Each concentration was left in contact with the ring for long enough to allow full development of the effect. Relaxation was then evaluated as a percentage of the initial tension (taken as 100%).

### *Assessment of $K^+$ channel-opening activity*

The antispasmodic activity of DP7 was evaluated under different conditions of high $K^+$-evoked depolarization (K30 or K60) and compared to the activity of cromakalim (Gurney, 1994). Preparations were stimulated with consecutive additions of K30 or K60, intervaled by washout with PSS, until a stable response was obtained. Following a 45-min washout period, the ring was preincubated with the test drug for 10 min and then stimulated in the presence of the drug, with K30 or K60, respectively. This response was evaluated as a percentage of the response to 0.3 $\mu$M phenylephrine.

### *Assessment of endoplasmic reticulum $Ca^{2+}$-mobilizing activity*

The effect of DP7 on $Ca^{2+}$ mobilization from the endoplasmic reticulum was evaluated in aortic rings stimulated with phenylephrine, and compared to the activity of 2,5-di-*t*-butyl-1,4-benzohydroquinone (BHQ) (Fusi *et al*., 1998).

Preparations were incubated with 1 $\mu$M nifedipine. After 15 min, PSS was drained and quickly replaced with K60 containing 1 $\mu$M nifedipine. Under these conditions, K60 failed to contract the rings, demonstrating that extracellular $Ca^{2+}$ influx *via* L-type $Ca^{2+}$ channels had been blocked. After 5 min, the substances being studied or vehicle were added, for a 15-min incubation period. The rings were then contracted with 1 $\mu$M phenylephrine and the tension expressed as a percentage of the response to 0.3 $\mu$M phenylephrine in PSS. The response to phenylephrine in the presence of nifedipine was taken to represent the contribution of intracellular $Ca^{2+}$ stores to the contractile response. In some experiments, 1 mM $Ni^{2+}$ was added together with nifedipine in order to block $Ni^{2+}$-sensitive $Ca^{2+}$ channels (see Fusi *et al*., 1998).

### *Cell isolation procedure*

Smooth muscle cells, freshly isolated from the tail main artery of male rats (350–450 g) by means of collagenase treatment in the presence of bovine serum albumin and trypsin inhibitor, as previously described (Fusi *et al*., 2001), exhibited an ellipsoid form (10–15 $\mu$m in width, 35–55 $\mu$m in length). The cells were continuously superfused with external solution (see below for composition) using a peristaltic pump (LKB 2132), at a flow rate of 500 $\mu$l min$^{-1}$. Electrophysiological responses were tested at room temperature (22–24°C) only in those cells that were phase dense. Cell membrane capacitance averaged out at $48.55 \pm 1.52$ pF ($n = 50$).

### *Whole-cell patch-clamp recording*

Conventional whole-cell patch-clamp method (Hamill *et al*., 1981) was employed to voltage-clamp the smooth muscle cells. Recording electrodes were pulled from borosilicate glass capillaries (WPI, Berlin, Germany) and fire-polished to give a pipette resistance of 2–5 MΩ when filled with internal solution (see below). A low-noise, high-performance Axopatch 200B (Axon Instruments, U.S.A.) patch-clamp amplifier, driven by an IBM computer in conjunction with an A/D, D/A board (DigiData 1200 A/B series interface, Axon Instruments, U.S.A.), was used to generate and apply voltage pulses to the clamped cells and record the corresponding membrane currents. Current signals, after compensation for whole-cell capacitance, series resistance and liquid junction potential, were low-pass filtered at 1 kHz and digitized at 3 kHz prior to being stored on the computer hard disk. Long-lasting nifedipine-blockable inward currents passing through L-type $Ca^{2+}$ channels [$I_{Ca(L)}$], in 5 mM $Ca^{2+}$-containing external solution (see below), were elicited with 250-ms depolarizing voltage-clamp pulses to test potentials of 10 mV from a holding potential ($V_h$) of −50 mV. Data were collected once the current amplitude had been stabilized (usually 8–10 min after the whole-cell configuration had been obtained). $I_{Ca(L)}$ did not run down during the following 30–40 min under these conditions.

$K^+$ currents were blocked with 30 mM tetraethylammonium (TEA) in the external solution and $Cs^+$ in the internal solution.

Current values were corrected for leakage using 1 $\mu$M nifedipine, which was assumed to completely block $I_{Ca(L)}$.

### *Rhodamine 123 (R123) uptake assay*

The L5178 Y mouse T-lymphoma parent cell line was transfected with a recombinant MDR1/A retroviral vector (pHa MDR1/A), as previously described by Pastan *et al*. (1988). MDR1-expressing cell lines were selected by culturing the infected cells with 60 ng ml$^{-1}$ colchicine to maintain the expression of the MDR phenotype (Weaver *et al*., 1993). The L5178 MDR cell line and the L5178 Y parent cell line were grown in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, 2 mM L-glutamine and 20 $\mu$g ml$^{-1}$ penicillin, 20 $\mu$g ml$^{-1}$ streptomycin and 8300 U l$^{-1}$ nystatine.

Cells ($2 \times 10^6$ ml$^{-1}$) were resuspended in serum-free McCoy's 5A medium, and 0.5 ml aliquot of the cell suspension was distributed into each Eppendorf centrifuge tube. Compounds to be tested were added at different concentrations and samples were incubated for 10 min at room temperature. Then, R123 indicator was added to the samples at a final concentration of 5.2 $\mu$M and cells were incubated for 20 min at 37°C, washed twice and resuspended in 0.5 ml phosphate-buffered saline. The uptake of R123 by cells was quantified as fluorescence by flow cytometry, using a FACScan instrument (Beckton Dickinson, Oxford, U.K.) at 488 nm and 300 mW. In order to measure P-gp inhibition in cells treated with DP derivatives, the fluorescence intensity of treated cells/fluorescence intensity of untreated cells ratio was transformed into its reciprocal and multiplied by 100. The percent residual of

fluorescence intensity, thus obtained, was subtracted from 100, to give the percent P-gp inhibition value.

### *Solutions and chemicals*

PSS contained (in mM) 118 NaCl, 4.75 KCl, 2.5 $CaCl_2$, 1.19 $MgCl_2$, 1.19 $KH_2PO_4$, 25 $NaHCO_3$ and 11.5 glucose, bubbled with a 95% $O_2$–5% $CO_2$ gas mixture to give a pH of 7.4. PSS containing KCl at a concentration of more than 4.75 mM was prepared by replacing NaCl with equimolar KCl.

$Ca^{2+}$-free external solution contained (in mM): 110 NaCl; 5.6 KCl; 10 HEPES; 20 taurine; 20 glucose; 1.2 $MgCl_2$ and 5 Na-pyruvate (pH 7.4).

Internal solution (pCa 8.4) consisted of (in mM): 100 CsCl, 10 HEPES, 11 EGTA, 2 $MgCl_2$, 1 $CaCl_2$, 5 Na-pyruvate, 5 succinic acid, 5 oxalacetic acid, 3 $Na_2ATP$ and 5 phosphocreatine; pH was adjusted to 7.4 with CsOH.

The osmolarity of external solution was adjusted to 335 mosm, and that of the internal solution to 310 mosm (Stansfeld & Mathie, 1993), by means of an osmometer (Osmostat OM 6020, Menarini Diagnostics, Italy).

The chemicals used were: collagenase (type XI), TEA, bovine serum albumin, trypsin inhibitor, verapamil, nifedipine, BHQ, cromakalim, colchicine, horse serum, L-glutamine, penicillin, streptomycin, nystatine, McCoy's 5A medium, phosphate-buffered saline and R123 (Sigma Chimica, Italy).

DP1–DP5 and DP6–DP11 were synthesized by Shah *et al*. (2000) and Kawase *et al*. (2002), respectively.

Nifedipine dissolved directly in ethanol, and cromakalim, BHQ and DP derivatives, dissolved in DMSO, were diluted in PSS, external solution or McCoy's 5A medium, prior to use. The resulting concentrations of DMSO and ethanol (below 1 or 0.55% for aorta rings, 0.3 or 0.1% for tail myocytes and 0.5 or 1% for L5178 MDR cell line, respectively) did not affect the responses (data not shown).

### *Statistical analysis*

Acquisition and analysis of data were accomplished using pClamp 8.2.0.232 software (Axon Instruments, U.S.A.) and GraphPad Prism version 3.03 (GraphPad Software, U.S.A.). Data are reported as mean ± s.e.m.; *n* represents the number of aorta rings or tail myocytes, isolated from at least three animals.

Statistical analyses and significance, as measured by ANOVA, followed by Dunnett's post test, were obtained using GraphPad InStat version 3.05 (GraphPad Software, U.S.A.). In all comparisons, $P<0.05$ was considered significant.

## Results

### *Effects of DP derivatives and nifedipine on contraction promoted by extracellular $Ca^{2+}$ influx at K60*

In this set of experiments, muscle tension developed at K60 was taken as an index of $Ca^{2+}$ influx from the extracellular space promoted by membrane depolarization. The effect of DP derivatives is summarized in Figure 1b. DP4 was the most potent myorelaxing agent (Figure 1a, b), although characterized by an $IC_{50}$ two orders of magnitude higher than that of nifedipine (Table 2). DP6, DP8, DP1 and DP2 exhibited $IC_{50}$ values one order of magnitude higher than that of DP4,

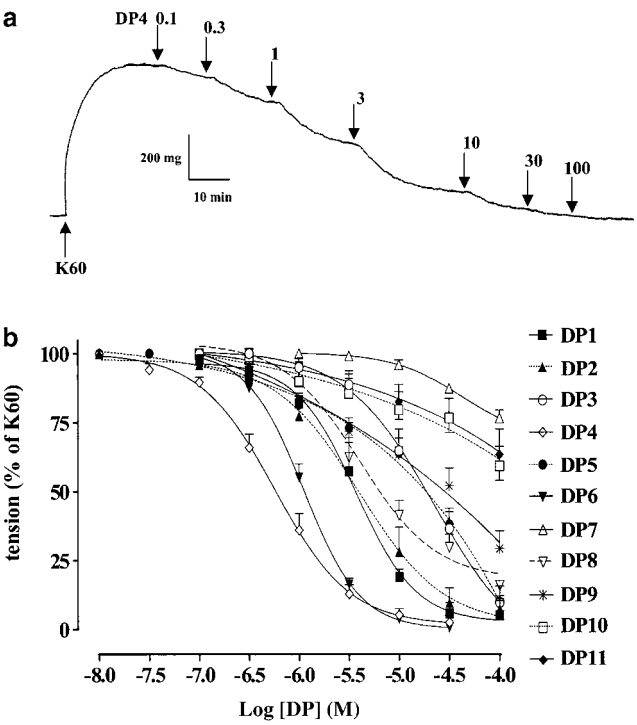



**Figure 1** Effect of DP derivatives on $Ca^{2+}$-induced contraction in rat aorta rings depolarized with K60. (a) Trace (representative of at least four experiments) of relaxation developed in response to cumulative concentrations ($\mu$M) of DP4 added on the plateau of K60-elicited contraction. (b) Concentration–response curves for DP derivatives. Data points are mean ± s.e.m. ($n=3$–6).

**Table 2** Inhibition of $Ca^{2+}$-induced contractions by dihydropyridine derivatives in rat aorta rings depolarised with K60 (figures are means ± s.e.m. ($n=3$–6))

| *Compounds* | $IC_{50}$ (M) |
|---|---|
| DP1 | $3.69\pm0.03\times10^{-6}$ |
| DP2 | $3.97\pm0.04\times10^{-6}$ |
| DP3 | $2.23\pm0.03\times10^{-5}$ |
| DP4 | $5.65\pm0.02\times10^{-7}$ |
| DP5 | $1.39\pm0.02\times10^{-5}$ |
| DP6 | $1.11\pm0.01\times10^{-6}$ |
| DP7[a] | $>3\times10^{-5}$ |
| DP8 | $4.04\pm0.08\times10^{-6}$ |
| DP9[a] | $>3\times10^{-5}$ |
| DP10[a] | $>3\times10^{-5}$ |
| DP11[a] | $>3\times10^{-5}$ |
| Nifedipine | $1.72\pm0.03\times10^{-9}$ |

[a]Figures reported refer to the highest concentration tested, at which the effect observed did not exceed 50%.

although DP8 at the maximum concentration tested caused a spamolysis that did not exceed 70.4%. DP5 and DP3 $IC_{50}$ values were about two orders of magnitude higher than that of DP4. The compounds endowed with the lowest spasmolytic activity (DP9, DP10, DP11 and DP7 up to 30 $\mu$M) relaxed K60-induced contraction by less than 50%.

### *Effects of DP derivatives and nifedipine on $I_{Ca(L)}$*

All the novel dihydropyridine derivatives, but DP7, inhibited $I_{Ca(L)}$ in a concentration-dependent manner (Figure 2b). As shown in Table 3, DP6 was the most potent $I_{Ca(L)}$ inhibitor

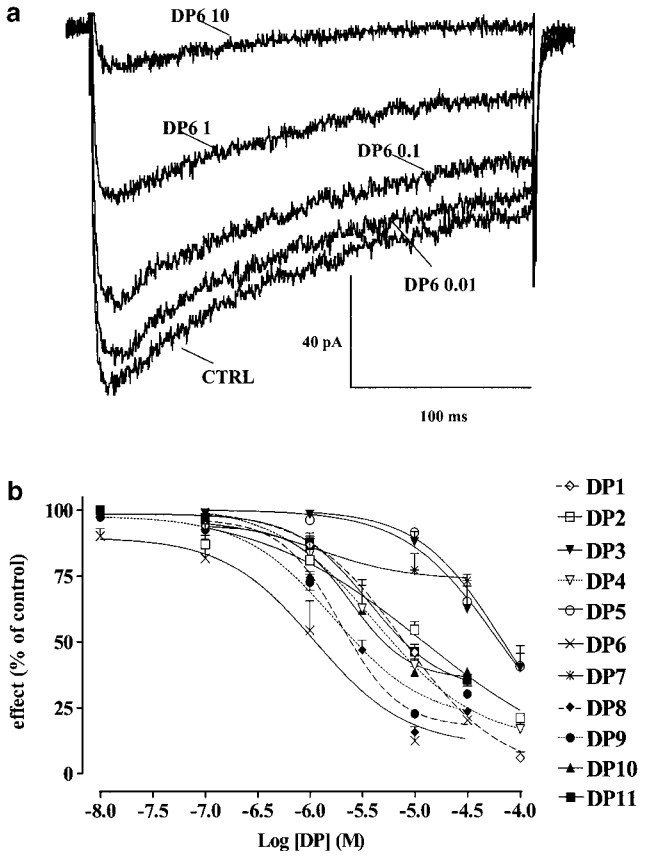



**Figure 2** Effect of DP derivatives on $I_{Ca(L)}$ recorded in rat tail myocytes. (a) Original recordings of conventional whole-cell $I_{Ca(L)}$ elicited with 250-ms clamp pulses to 10 mV from a $V_h$ of −50 mV, measured in the absence (CTRL) or presence of DP6 (0.01–10 $\mu$M). (b) Concentration–response curves for DP derivatives. Data points are mean ± s.e.m. ($n = 3$–6).

**Table 3** Inhibition of $I_{Ca(L)}$ by dihydropyridine derivatives in rat tail artery myocytes (figures are means ± s.e.m. ($n = 3$–6))

| *Compounds* | *$IC_{50}$* (M) |
|---|---|
| DP1 | $7.93 \pm 0.08 \times 10^{-6}$ |
| DP2 | $1.21 \pm 0.03 \times 10^{-5}$ |
| DP3 | $6.35 \pm 0.05 \times 10^{-5}$ |
| DP4 | $4.34 \pm 0.02 \times 10^{-6}$ |
| DP5 | $6.90 \pm 0.07 \times 10^{-5}$ |
| DP6 | $1.12 \pm 0.04 \times 10^{-6}$ |
| DP7[a] | $> 3 \times 10^{-5}$ |
| DP8 | $1.95 \pm 0.06 \times 10^{-6}$ |
| DP9 | $1.71 \pm 0.08 \times 10^{-6}$ |
| DP10 | $2.48 \pm 0.05 \times 10^{-6}$ |
| DP11 | $3.71 \pm 0.03 \times 10^{-6}$ |
| Nifedipine | $4.48 \pm 0.07 \times 10^{-9}$ |

[a]Figure reported refers to the highest concentration tested, at which the effect observed did not exceed 20%.

(Figure 2a), although its $IC_{50}$ value was three orders of magnitude higher than that of nifedipine.

DP1, DP4, DP8, DP9, DP10 and DP11 also exhibited remarkable $Ca^{2+}$ antagonist properties and showed $IC_{50}$ values ranging between 1.95 and $7.93 \times 10^{-6}$, while DP2, DP3 and DP5 inhibited $I_{Ca(L)}$ with $IC_{50}$ values one order of magnitude higher than that of DP6.

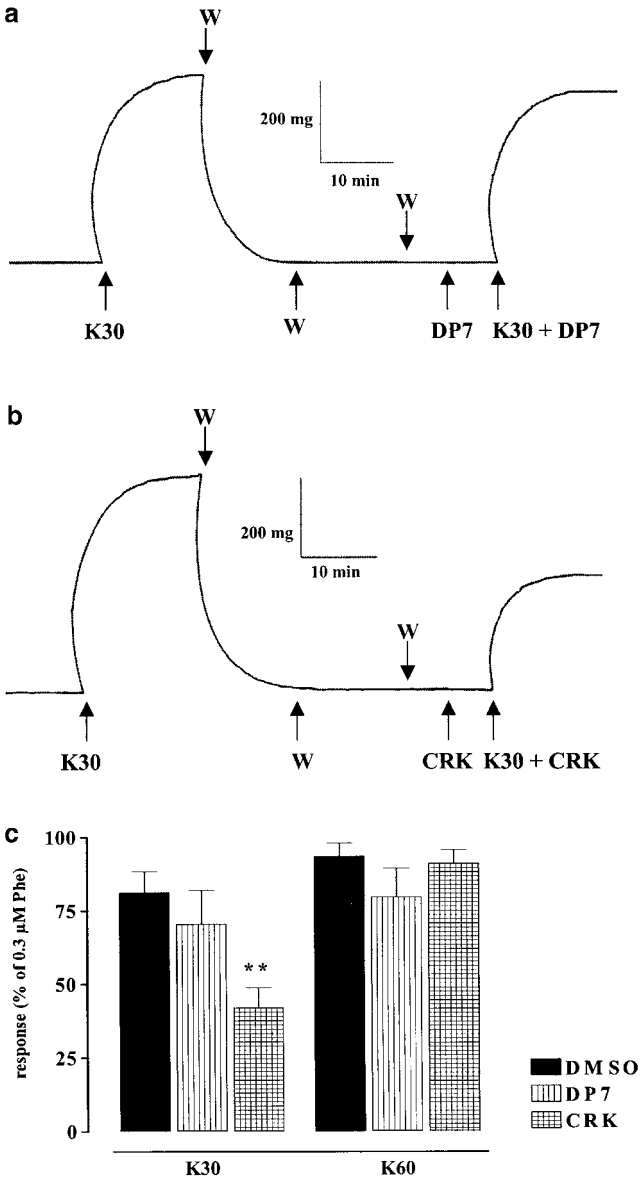



**Figure 3** Effect of DP7 and cromakalim (CRK) on $Ca^{2+}$-induced contractions in rat aorta rings depolarized with either K30 or K60. Rings were bathed with K30 for 15 min and then washed in PSS, until a stable response was obtained. DP7 (30 $\mu$M) (a) or CRK (500 nM) (b) were added 5 min before K30. Traces are representative of at least four experiments. (c) Rings were stimulated with either K30 or K60 in the presence of DMSO (vehicle), 30 $\mu$M DP7 or 500 nM CRK. Columns represent mean ± s.e.m. ($n = 4$–8). $^{**}P < 0.01$ (Dunnett's post-test).

### *Effects of DP7 and cromakalim on contractions promoted by extracellular $Ca^{2+}$ influx at either K30 or K60*

$K^+$ channel-opening activity was assessed by stimulating aorta rings with K30 (Figure 3a, b) or K60 in the presence of the tested compounds. K30 and K60 evoked contractile responses averaging 81.1 ± 7.2% ($n = 6$) and 93.5 ± 4.5% ($n = 8$), respectively, of the maximum attainable contraction (Figure 3c).

In rings preincubated with 30 $\mu$M DP7, both K30- and K60-induced contractions were not significantly different from control. In contrast, the relaxant effect of cromakalim, a $K^+$ channel opener, could be observed at K30 but not at K60.

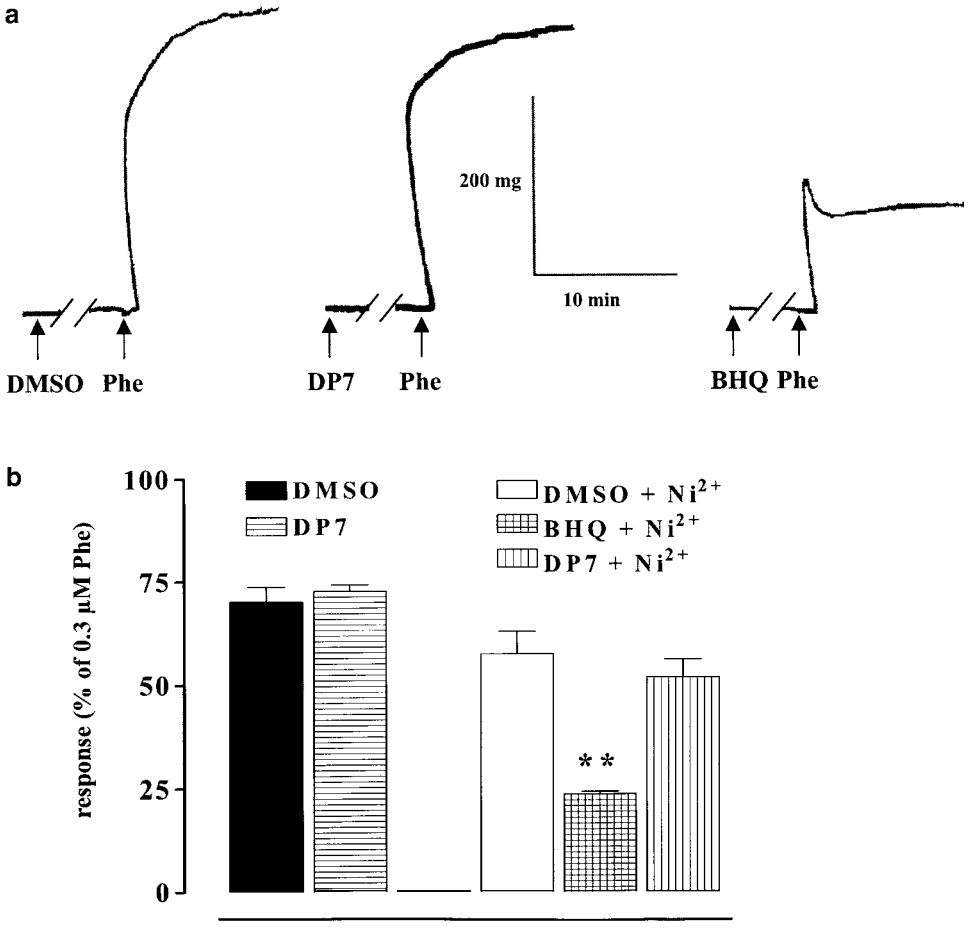



**Figure 4** Effect of DP7 and BHQ on $Ca^{2+}$ mobilization from intracellular stores. All traces show 1 $\mu$M phenylephrine (Phe)-induced contraction of rat aorta rings incubated in K60 containing 1 $\mu$M nifedipine and 1 mM $Ni^{2+}$. This response was assumed to represent the contribution of intracellular $Ca^{2+}$ sources to the contractile response. (a) Phe-induced response after 10 min incubation with DMSO (vehicle), with 30 $\mu$M DP7, or with 10 $\mu$M BHQ. Traces are representative of at least four experiments. (b) Effect of DP7 and BHQ on Phe-induced contraction in K60 containing nifedipine or nifedipine plus $Ni^{2+}$. Columns represent mean $\pm$ s.e.m. ($n=4$). $^{**}P<0.01$ (Dunnett's post-test).

### *Effects of DP7 and BHQ on contraction promoted by $Ca^{2+}$ mobilization from the endoplasmic reticulum*

The amount of $Ca^{2+}$ stored intracellularly was estimated from the amplitude of the phenylephrine-induced contraction in the presence of K60 and nifedipine or nifedipine plus $Ni^{2+}$. Figure 4a shows the typical traces of 1 $\mu$M phenylephrine-induced contraction in rings depolarized with K60 in the presence of nifedipine plus $Ni^{2+}$ with or without 30 $\mu$M DP7 or 10 $\mu$M BHQ. In the presence of nifedipine or nifedipine plus $Ni^{2+}$, phenylephrine evoked tonic responses (70.1 $\pm$ 3.7 and 57.5 $\pm$ 5.4%, respectively, of the maximum attainable contractions, $n=4$), which were not affected by the presence of DP7 (Figure 4b). On the contrary, when tissues were preincubated with BHQ, a quite selective endoplasmic reticulum $Ca^{2+}$-ATPase inhibitor (Moore *et al*., 1987), the response to phenylephrine was significantly reduced and characterized by an initially phasic component, followed by a tonic contraction.

### *Effects of DP derivatives and nifedipine on P-gp-mediated R123 efflux*

All the novel dihydropyridine derivatives, but DP1, DP2 and DP4, inhibited P-gp-mediated R123 efflux in a concentration-dependent manner (Table 4). DP7 was the most potent P-gp inhibitor with an $IC_{50}$ value two orders of magnitude lower than that of nifedipine. DP9 and DP10 showed $IC_{50}$ values comparable to that of DP7. DP6, DP8 and DP11 also exhibited a remarkable MDR-reversing activity, and showed $IC_{50}$ values ranging between 2.74 and $7.35 \times 10^{-6}$, while DP3 and DP5 were as active as nifedipine.

DP1, DP2 and DP4, at the maximum concentration tested (30 $\mu$M for DP1 and DP2, and 165 $\mu$M for DP4), inhibited P-gp activity by only 30%.

## Discussion

Endeavouring to find MDR reverters is a crucial task for anticancer chemotherapeutic intervention. In the past few years, extensive studies have been performed with the aim of developing effective chemosensitizers to overcome MDR of human cancer cells. Potent P-gp inhibitors have been tested in clinical trials so far, including $Ca^{2+}$ channel blockers such as verapamil and dihydropyridines (Bates *et al*., 1994; Bellamy, 1996). However, clinical application of these agents has not been extensively pursued to date, owing to their unwanted cardiovascular side effects. This has promoted the search for

**Table 4** Inhibition of Pgp-mediated R123 efflux by dihydropyridine derivatives in L5178 MDR cell line (figures are means ± s.e.m. from triplicate experiments)

| *Compounds* | $IC_{50}$ (M) |
|---|---|
| DP1[a] | $>3\times10^{-5}$ |
| DP2[a] | $>3\times10^{-5}$ |
| DP3 | $4.27\pm0.19\times10^{-5}$ |
| DP4[a] | $>16.5\times10^{-5}$ |
| DP5 | $2.15\pm0.06\times10^{-5}$ |
| DP6 | $2.74\pm0.07\times10^{-6}$ |
| DP7 | $3.02\pm0.07\times10^{-7}$ |
| DP8 | $5.92\pm0.11\times10^{-6}$ |
| DP9 | $6.90\pm0.05\times10^{-7}$ |
| DP10 | $7.07\pm0.19\times10^{-7}$ |
| DP11 | $7.35\pm0.10\times10^{-6}$ |
| Nifedipine | $2.01\pm0.04\times10^{-5}$ |

[a]Figures reported refer to the highest concentration tested, at which the effect observed did not exceed 30%.

congeners of this first-generation-MDR reverters with reduced cardiovascular toxicity.

A series of 3,5-diacetyl- (DP1–DP5) and 3,5-dibenzoyl-1,4-dihydropyridine (DP6–DP11) derivatives (Table 1), recently synthesized and preliminarily assessed for their ability to reverse MDR in *in vitro* assay systems, have revealed a noteworthy P-gp inhibitory activity in L5178 Y mouse T-lymphoma cells transfected with MDR1 gene (Shah *et al*., 2000; Kawase *et al*., 2002). In order to provide further information on these new candidates for MDR cancer cell treatment, their effects on vascular functions *in vitro* have been investigated and compared to their MDR-reversing potency. All compounds tested, but DP9, DP10, DP11 and DP7, antagonized $K^+$-induced contraction in a concentration-dependent manner similar to the well-known $Ca^{2+}$-channel-blocking drug nifedipine. $K^+$-induced contraction is the result of an increased $Ca^{2+}$ influx through voltage-operated $Ca^{2+}$ channels (VOCCs). Therefore, its inhibition by the DP derivatives might be interpreted as a consequence of the blockade of these channels. The electrophysiological data presented here, in fact, have directly confirmed this hypothesis, since all dihydropyridines tested, but DP7, inhibited, although to a different extent, $I_{Ca(L)}$ recorded in rat tail myocytes.

Comparison of the $Ca^{2+}$ antagonist activity between DP compounds and nifedipine has shown that the latter was about 1000-fold more potent in both model systems. This could be due to the replacement of the 3,5-dicarbomethoxy groups of nifedipine by either 3,5-diacetyl (DP1–DP5) or 3,5-dibenzoyl (DP6–DP11) groups. This is in agreement with what previously reported by Loev *et al*. (1974) and Janis & Triggle (1983), who showed that replacement of 3,5-dicarbomethoxy groups by other electron-withdrawing substituents markedly reduces the $Ca^{2+}$-blocking activity of the resulting compounds.

DP1, DP3, DP5, DP6 and DP8 showed an approximately equivalent activity in both model systems. In contrast, DP9, DP10 and DP11 were more effective in inhibiting $I_{Ca(L)}$ in single smooth muscle cell than in relaxing aorta rings, suggesting that a different diffusion of the compounds across intact tissues and isolated myocytes might account for this difference. However, this feature alone cannot account for their lower effectiveness towards $K^+$-induced contraction, since DP9, DP10 and DP11 are equally lipophilic to DP6 and DP8 (Shah *et al*., 2000; Kawase *et al*., 2002). It is likely that the position of the substituent in the 4-phenyl ring may be critical: para (DP10) and meta (DP9 and DP11) substitution invariably decreased the spasmolytic activity, whereas ortho (DP6, DP8) substitution increased it. Moreover, slight differences in L-type $Ca^{2+}$ channels expressed in the two tissues examined might explain the incomplete matching of data from the two experimental settings. Finally, DP2 and DP4 caused a much higher spasmolysis than what could be expected from their $Ca^{2+}$ channel-blocking activity, thus advocating their interaction with targets, beyond VOCCs, crucial for the maintenance of vascular smooth muscle tone.

Noteworthy, among the seven derivatives (DP1, DP2, DP3, DP4, DP5, DP6 and DP8) exhibiting both spasmolytic and $Ca^{2+}$ channel-blocking activity, a linear and significant correlation was found ($r^2=0.65$, $P<0.05$), thus indicating that homologies between the two vascular preparations employed in this study are more relevant than differences.

Safak *et al*. (1990) have already tested the $Ca^{2+}$ antagonist activity of DP1 and DP3 in $K^+$-depolarized guinea-pig *Taenia coli* strips. In this preparation, DP1 and DP3 showed $IC_{50}$ values one order of magnitude lower than those reported here. The higher concentration of extracellular $Ca^{2+}$ used in the present work (2.5 mM *versus* 1 mM in *Taenia coli* strips) might account for this difference, since the extent of inhibition of muscle contraction by $Ca^{2+}$ channel-blocking agents is known to be inversely correlated to the extracellular $Ca^{2+}$ concentration (see Karaki, 1987).

Since DP7 was almost ineffective as a vascular $Ca^{2+}$ channel blocker, its effects on other functions that regulate vascular smooth muscle tone were assessed.

The antispasmodic activity of $K^+$ channel openers, such as cromakalim (Norman *et al*., 1994), is reduced when extracellular $K^+$ concentration is raised; invariably, when the $K^+$ gradient across the membrane drops, the effect of $K^+$ channel openers disappears (Gurney, 1994; this paper). In contrast to what observed with cromakalim, however, DP7 did not show any antispasmodic activity even when the external $K^+$ concentration was kept at a value (30 mM) which allows $K^+$ channel activation to be displayed, nor did it affect muscle contraction caused by intracellular stored $Ca^{2+}$ mobilization.

In conclusion, DP7, at a concentration two orders of magnitude higher than $IC_{50}$ as a P-gp inhibitor, was neither an antispasmodic nor a spasmolytic agent and inhibited $I_{Ca(L)}$ by a mere 20%, thus outlining the clear divergence of its P-gp inhibition and the vascular activity. The absence of vascular activity *per se*, however, is not sufficient to guarantee for DP7 safety, and further experiments on other physiological systems are needed before it can be addressed to the clinical investigation. Nevertheless, since reversion of MDR is a field of research of overwhelming importance, DP7 may represent a lead compound for the design of novel, safe and potent MDR chemosensitizers needed for the chemotherapy of cancer and other diseases.

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