Differential effects of the optical isomers of KR30031 on cardiotoxicity and on multidrug resistance reversal activity

Byung Ho Lee[a], Chong Ock Lee[a], Myung-Ja Kwon[a], Kyu Yang Yi[a], Sung-eun Yoo[a] and Sang-Un Choi[a]

The present study was performed to compare the cardiovascular adverse effects of verapamil, KR30031 and their optical isomers, and also to measure their ability to overcome multidrug resistance (MDR). The *R*-isomer of KR30031 (*R*-KR30031) was equipotent with the *S*-isomer of KR30031 (*S*-KR30031) and 25-fold less potent than the *R*-isomer of verapamil (*R*-verapamil) in relaxing the aorta isolated from rat (EC_{50}: 11.8, 10.2 and 0.46 μM, respectively). The effect of *R*-KR30031 in decreasing left ventricular pressure of heart isolated from rat was 2- and 267-fold smaller than those of *S*-KR30031 and *R*-verapamil, respectively (EC_{50}: 23.9, 9.4 and 0.089 mM, respectively). The hypotensive effect of *R*-KR30031 in rat was about 2- and 23-fold smaller than those of *S*-KR30031 and *R*-verapamil, respectively (ED_{20}: 1.15, 0.60 and 0.05 mg/kg, respectively). On the other hand, *R*-KR30031 elicited potency similar to those of *S*-KR30031 and *R*-verapamil in enhancing the paclitaxel-induced cytotoxicity to HCT15/CL02 and MES-SA/DX5 cells that reveal high levels of P-glycoprotein expression (IC_{50}: 3.11, 3.04 and 2.58 μM, respectively). In addition, the intrinsic cytotoxicity of *R*-KR30031 in HCT15/CL02 and MES-SA/DX5 cells was observed only at the very high concentration of 100 μM. All these results suggest that *R*- and racemic KR30031 are active modulators of MDR with potentially minimal cardiovascular adverse activity. *Anti-Cancer Drugs* 14:175–181

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Introduction

The development of multidrug resistance (MDR) during the use of chemotherapy is a major factor limiting the success of anticancer agents. MDR is frequently associated with the overexpression of a membrane protein, P-glycoprotein (P-gp), encoded in human cells by the *mdr*1 gene, which is thought to act as an energy-dependent drug efflux pump that results in decreased drug accumulation and diminished cytotoxicity to cancer cells [1,2]. Recently, a number of pharmacological studies in experimental tumor models have sought to circumvent P-gp and have aimed at applying their findings to treatment strategies for non-responsive patients [3]. A wide range of compounds, including calcium channel blockers [4,5] and calmodulin inhibitors [5], have been shown to modulate P-gp and have been used to restore the sensitivity of resistant cells.

Since 1981, verapamil has been known to substantially reverse MDR *in vitro* [4] and also to elicit beneficial effects in patients with drug-resistant cancer [6,7]. However, the clinical application of this approach has been limited by the profound hypotension, negative inotropic effects and atrioventricular conduction delays associated with plasma levels of this drug, which in nearly all instances have been substantially lower than those targeted for reversal of resistance [8,9]. At present, the drug preparation of verapamil available for clinical usage is a racemic mixture of equal amounts of *R*- (*R*-verapamil) and *S*-isomers (*S*-verapamil) of verapamil. In a number of experiments, however, it has been demonstrated that *R*-verapamil was 3- to 10-fold less potent than *S*-verapamil with regard to the calcium channel blocking effect, such as the hypotensive, chronotropic and dromotropic effects [10–12], while *R*- and *S*-verapamil were equally effective in reversing drug resistance *in vitro* [13,14]. Accordingly, the decreased cardiovascular toxicity of the *R*-verapamil makes this drug an attractive alternative to the standard racemic verapamil preparations [15]. In previous studies, we showed that KR30031 enhanced the cytotoxicity of anticancer drugs via modulation of P-gp with reduced cardiovascular adverse effects compared with verapamil [16]. Thus, the present study was performed to evaluate the effects of optical isomers of KR30031 on the possible cardiovascular adverse effects in rats and the MDR-reversal activities in human cancer cell lines.

Materials and methods

Animals

Male Sprague-Dawley (SD) rats (400–450 g) were purchased from BioGenomics (Seoul, Korea). The animals were conditioned for 2 weeks at $22.5 \pm 1^{\circ}C$ with

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a constant humidity of 55 ± 5%, a cycle of 12-h light/dark, and free access to food and tap water. This study conformed to the *Guide for the Care and Use of Laboratory Animals*, published by the US National Institute of Health.

Cancer cells

HCT15/CL02 cells were established from the human colorectal HCT15 cancer cells by continuous and stepwise exposure to doxorubicin in the KRICT [17]. The doxorubicin-resistant subline MES-SA/DX5 established from human uterine carcinoma MES-SA cells were purchased from ATCC (Rockville, MD). The cells were cultured with RPMI1640 medium supplemented with 5% FBS as previously reported [18].

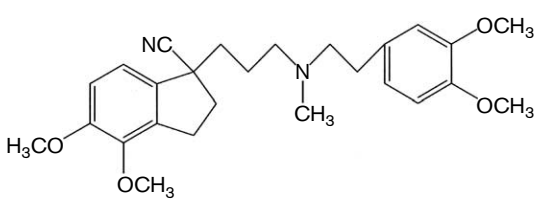
Drugs and chemicals

Verapamil and KR30031, and their respective optical isomers (Fig. 1) were synthesized at the Korea Research Institute of Chemical Technology (KRICT, Taejon, Korea). Reagents for the physiological solutions used in the isolated aorta and Langendorff experiments were purchased from Junsei (Tokyo, Japan). Sodium pentobarbital was purchased from Hanlim (Seoul, Korea). Paclitaxel and reagents for cell cultures [gentamycin, amphotericin, 1,2-cyclohexanediaminetetraacetic acid (CDTA) and sodium bicarbonate] and cytotoxicity tests [trichloroacetic acid (TCA) and sulforhodamine B (SRB)] were also purchased from Sigma (St Louis, MO). RPMI 1640 cell growth medium, trypsin, fetal bovine serum and Hank's balanced salt solution (HBSS) were obtained from Gibco (Grand Island, NY). Verapamil and KR30031, and their respective optical isomers were dissolved in ethylalcohol, and serially diluted with distilled water and 0.9% saline for *in vitro* and *in vivo* experiments, respectively. All drugs and reagents were prepared just prior to use.

Vasorelaxant effects on isolated rat aortas

Thoracic aorta was isolated from male SD rats and each aorta was cut into 2–3 mm wide rings with extreme care to preserve the endothelium [18]. The aortic preparations were suspended between wire hooks in an organ bath containing 20 ml of Krebs' bicarbonate buffer (mM: NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $NaHCO_3$, 25; $MgSO_4$, 1.2; KH_2PO_4, 1.2; and glucose, 11.0) bubbled with a gas mixture (95% O_2/5% CO_2) and maintained at 37°C. The aortic preparations were allowed to equilibrate for 60 min under 2 g of resting tension. Isometric contraction was measured with a force displacement transducer (Grass FT03; Grass, Quincy, MA) and displayed on a chart recorder (Multicorder MC 6625; Hugo Sachs Electronic, Hugstetten-March, Germany). The aortic preparations were precontracted submaximally with 60 mM KCl, washed 3 times for 45 min, and rechallenged with 60 mM KCl to obtain reproducible and stable response. After obtaining a plateau KCl response, the compounds (10^{-8} to 10^{-4} M) were cumulatively added to the tissue bath. The data were expressed as percentage relaxation of the contractile response to KCl and EC_{50} values (molar concentration that induces 50% relaxation of the KCl-induced contraction) were obtained from a linear regression analysis.

Fig. 1



Chemical structure of KR30031.

Langendorff studies in rat hearts

Male SD rats were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) [18]. Heparin (1000 U/kg) was injected into the tail vein, and then the trachea was incubated and artificially ventilated with a rodent ventilator (Model 7025; Ugobasile, Varese, Italy). The hearts were then excised and mounted on a Langendorff apparatus (Hugo Sachs Electronic), where they were perfused with oxygenated modified Krebs–Henseleit bicarbonate buffer containing (in mM) NaCl 116, $NaHCO_3$ 24.9, KCl 4.7, $MgSO_4$ 1.1, KH_2PO_4 1.17, $CaCl_2$ 2.52, glucose 8.32 and pyruvate 2.0 at a constant perfusion pressure (80 mmHg). A water-filled latex balloon attached to a metal cannula was placed in the left ventricle and connected to an Isotec pressure transducer (Hugo Sachs Electronic) for measurement of left ventricular pressure (LVP). After the isolated hearts were allowed to equilibrate for 30 min, the compounds (10^{-7} to 10^{-1} M, 100 μl) were directly injected to the hearts. The data were expressed as percentage decrease in LVP and EC_{50} values (molar concentration that decreases LVP by 50%) were obtained from a linear regression analysis.

Blood pressure lowering effects in anesthetized rats

Male SD rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and were prepared according to previously described procedures [19]. Arterial blood pressure was continuously monitored via an Isotec pressure transducer (Hugo Sachs Electronic) connected to a physiograph (WR 3300 Linearcorder; Graphtec, Tokyo, Japan). Heart rate (HR) was measured by Lead II using an ECG/rate coupler (Type 576; Hugo Sachs Electronic), the parameter being analyzed by AcqKnowledge software (Biopac Systems, Goleta, CA) via a signal interface (MP100; Biopac Systems). Forty minutes after surgery, the compounds were i.v. administered at 5-min

intervals. The data were expressed as percentage decrease in MAP and HR, and ED_{20} values (a dose that decreased MAP by 20%) were obtained from a linear regression analysis.

MDR reversal assay

The ability of modulators to potentiate the cytotoxicity of paclitaxel was evaluated in HCT15/CL02 and MES-SA/DX5 cell lines as previously described.[17] Cells were inoculated over a series of standard 96-well flat-bottom microplates (Falcon) and were then preincubated for 24 h to allow attachment to the microtiter plate. The attached cells were incubated with 0.2 μM paclitaxel (the concentration that does not inhibit cell growth) in the absence or presence of the compounds (0.3–10.0 μM). After continuous exposure to the compounds for 72 h, the culture medium was removed from each well and the cells fixed with 10% cold TCA at 4°C for 1 h. After washing with distilled water, the cells were stained with 0.4% SRB dye and incubated for 30 min at room temperature. The cells were washed again, and then solubilized with 10 mM unbuffered Tris base solution (pH 10.5). The absorbance was measured spectrophotometrically at 520 and 690 nm with a microtiter plate reader (E-max; Molecular Devices, Sunnyvale, CA). To eliminate the effects of non-specific absorbance, the absorbance at 690 nm was subtracted from that at 520 nm. Results were expressed as a percent of control (cell survival fractions) and the IC_{50} values (concentration resulting in 50% inhibition of cell growth). All experiments were performed in triplicate.

Intrinsic cytotoxicity

Cells were seeded into 96-well flat bottom microplates as described above. After 24 h, the compounds (0.3–100 μM) were added, and cells were incubated for an additional 72 h before quantification of cell growth and calculation of IC_{50} values. All experiments were performed in triplicate.

Statistical analysis

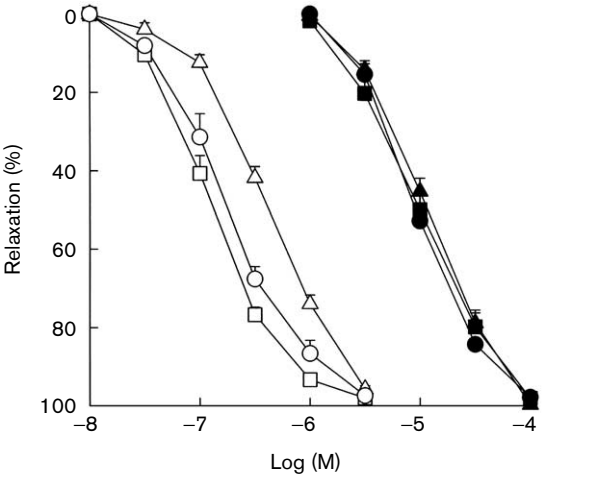
All values are expressed as mean $\pm$ SEM, data were analyzed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for multiple comparisons (Sigma Stat; Jandel, San Rafael, CA) as appropriate. In all comparisons, the difference was considered to be statistically significant at $p < 0.05$.

Results

Vasorelaxant effects on isolated rat aortas

To compare the peripheral vasodilating activities of KR30031 and its optical isomers, their effects on 60 mM KCl-induced aortic constriction were measured, and compared with those of verapamil and its optical isomers. Verapamil, KR30031 and their optical isomers produced concentration-dependent relaxations of the aorta precontracted with KCl (Fig. 2). The relaxant effects of *R*-verapamil on aortic preparations were 3- and 2-fold less potent than those of *S*- and racemic verapamil, respectively (EC_{50}: 0.46 ± 0.04, 0.15 ± 0.01 and 0.21 ± 0.04 μM, respectively; $p < 0.05$; $n = 4$–6). However, the effects of *R*-isomer of KR30031 (*R*-KR30031) on vasorelaxation were similar with those of *S*- (*S*-KR30031) and racemic KR30031 (EC_{50}: 11.82 ± 1.15, 10.23 ± 1.35 and 9.14 ± 0.60 μM, respectively, $n = 4$–6). When comparing *R*-KR30031 and *R*-verapamil as vasorelaxants, the former was approximately 25-fold less potent than the latter (EC_{50}: 11.82 ± 1.15 and 0.46 ± 0.04 μM, respectively; $p < 0.05$).

Fig. 2

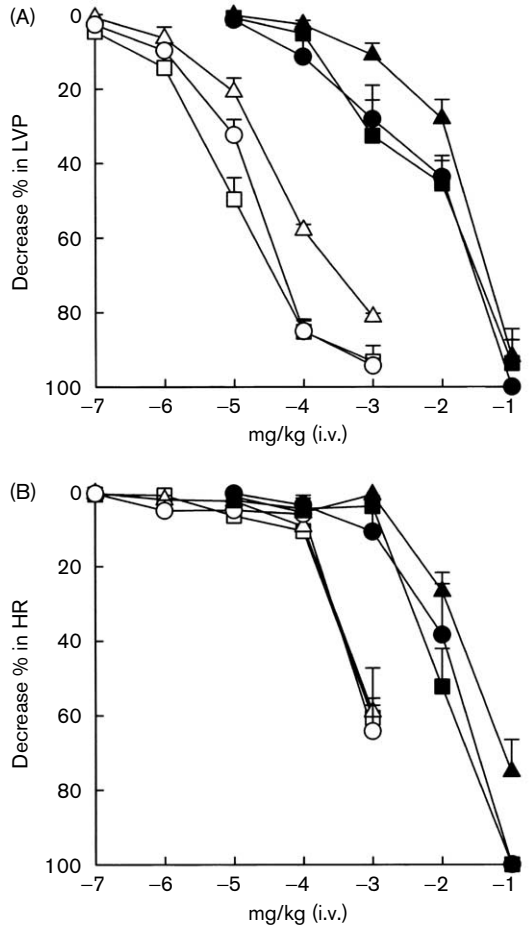


Relaxation of isolated rat aorta precontracted with 60 mM KCl. Racemic verapamil (open circles), *R*-verapamil (open triangles), *S*-verapamil (open squares), racemic KR30031 (filled circles), *R*-KR30031 (filled triangles) and *S*-KR30031 (filled squares). Values are mean percentage change $\pm$ SEM ($n = 4$–6).

Langendorff studies in rat hearts

To compare the direct effects of KR30031 and its optical isomers to the hearts, their inotropic and chronotropic effects in hearts isolated from rats were measured, and compared with those of verapamil and its optical isomers. Verapamil, KR30031 and their optical isomers induced a concentration-dependent decrease in LVP of isolated hearts (Fig. 3A). *R*-verapamil was about 8- and 4-fold less potent than *S*- and racemic verapamil at decreasing LVP in isolated hearts, respectively (EC_{50}: 0.089 ± 0.011, 0.011 ± 0.003 and 0.022 ± 0.008 mM, respectively; $p < 0.05$; $n = 4$–7). The effects of *R*-KR30031 on decrease in LVP were also about 2-fold smaller than those of *S*- and racemic KR30031 (EC_{50}: 23.9 ± 7.8, 9.4 ± 4.0 and 11.6 ± 3.0 mM, respectively, $n = 4$–6), although the potency ratio between isomers of KR30031 (2-fold) was smaller than that of verapamil (8-fold). When comparing the effects *R*-KR30031 and *R*-verapamil in LVP, the

Fig. 3



Concentration–response curve of verapamil, KR30031 and their optical isomers on LVP (A) and HR (B) in isolated rat hearts. Racemic verapamil (open circles), *R*-verapamil (open triangles), *S*-verapamil (open squares), racemic KR30031 (filled circles), *R*-KR30031 (filled triangles) and *S*-KR30031 (filled squares). Values are mean percentage ± SEM ($n = 4$–7).

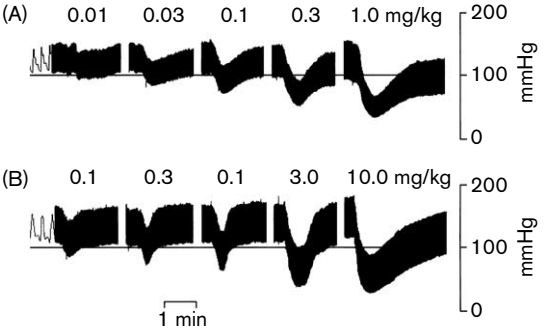
former was 267-fold less potent than the latter (EC_{50}: 23.9 ± 7.8 and 0.089 ± 0.011 mM, respectively; $p < 0.05$).

Verapamil, KR30031 and their optical isomers caused a concentration-dependent decrease in HR of isolated hearts (Fig. 3B). The effects of *R*-verapamil on HR were similar potency with *S*- and racemic verapamil (EC_{50}: 0.68 ± 0.12, 0.50 ± 0.28 and 0.79 ± 0.20 mM, respectively). On the other hand, the effects of *R*-KR30031 on HR were approximately 3-fold smaller than those of *S*- and racemic verapamil (EC_{50}: 41.6 ± 14.2, 11.2 ± 3.5 and 14.0 ± 5.9 mM, respectively).

Blood pressure lowering effects in anesthetized rats

The effects of i.v. administered verapamil, KR30031 and their optical isomers on the blood pressure and HR of anesthetized rats are shown in Figures 4 and 5. The mean

Fig. 4



Dose-dependent decrease by *R*-verapamil (A) and *R*-KR30031 (B) in arterial pressure in anesthetized rats. Quite similar hypotensive profiles were also obtained with other isomers.

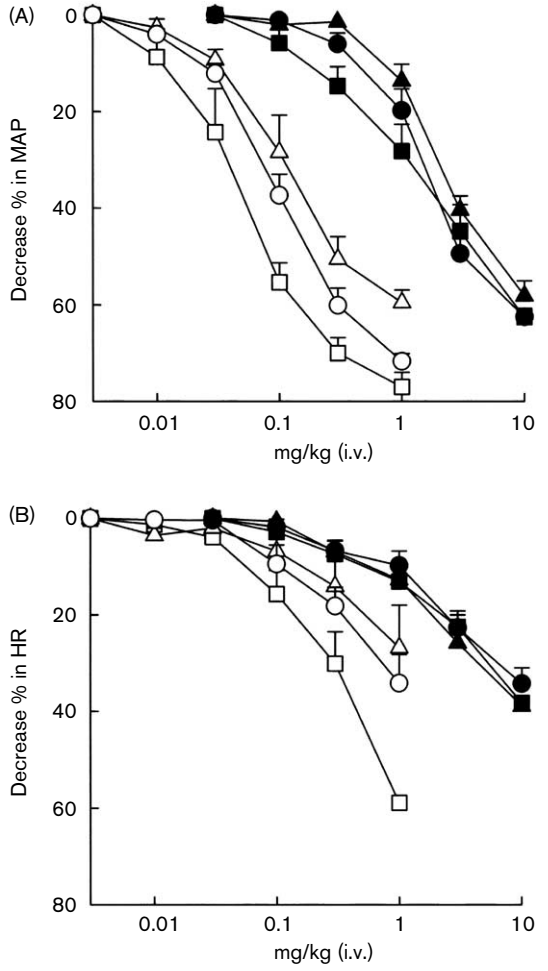
predose values of MAP and HR were 135 ± 3 mmHg and 414 ± 15 beats/min, respectively, and there was no significant difference between predose values prior to administration of each compounds. All compounds of verapamil, KR30031 and their optical isomers produced a dose-dependent reduction in MAP with 10 s taken to exert their action and 30–60 s to reach the plateau (Fig. 4). *R*-verapamil was 2.5-fold less potent than *S*-verapamil and showed similar potency with racemic verapamil in reduction in MAP (ED_{20}: 0.050 ± 0.011, 0.020 ± 0.005, $p < 0.05$, and 0.043 ± 0.007 mg/kg, respectively, $n = 4$–8, Fig. 5A). Similarly, the hypotensive effects of *R*-KR30031 were about 2-fold smaller than those of *S*-KR30031 and similar with those of racemic KR30031 (ED_{20}: 1.152 ± 0.179, 0.597 ± 0.285 and 0.842 ± 0.213 mg/kg, respectively, $n = 4$–6). *R*-KR30031 was 23-fold less potent than *R*-verapamil on blood lowering effects (ED_{20}: 1.152 ± 0.179 and 0.050 ± 0.011, respectively; $p < 0.05$).

Verapamil, KR30031 and their optical isomers dose-dependently decreased HR in anesthetized rats (Fig. 5B). The effects of *R*-verapamil on HR were 4- and 1.6-fold smaller than those of *S*- and racemic verapamil (ED_{20}: 0.501 ± 0.109, 0.124 ± 0.013 and 0.302 ± 0.158 mg/kg, respectively). However, *R*-KR30031 decreased HR with similar potency with *S*- and racemic verapamil (ED_{20}: 1.795 ± 0.445, 1.722 ± 0.213 and 2.588 ± 0.765 mg/kg, respectively).

Modulation of drug resistance in MDR cell lines

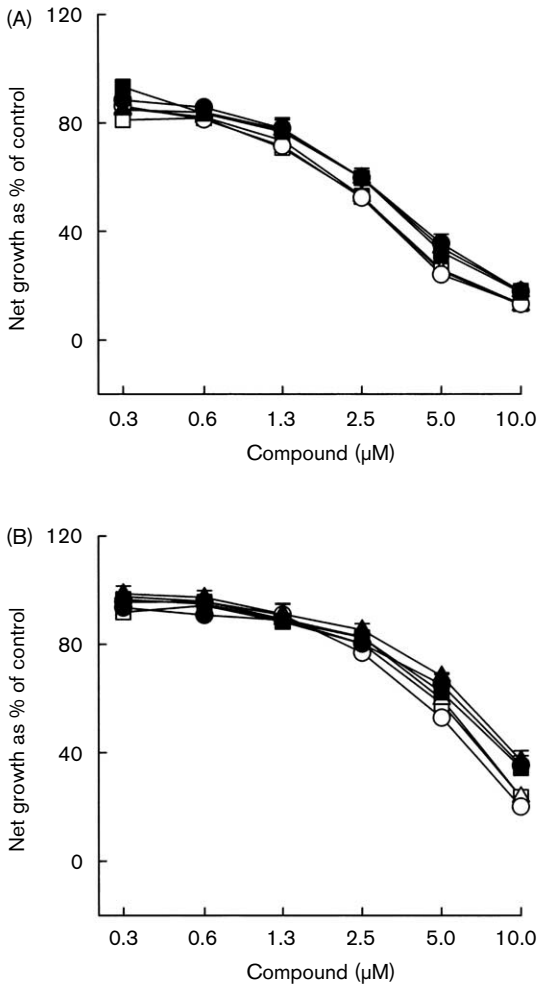
The ability of verapamil, KR30031 and their optical isomers to reverse resistance of the HCT15/CL02 and MES-SA/DX5 cells to paclitaxel is shown in Figure 5. The 0.2 μM paclitaxel used in this study did not inhibit the growth of HCT15/CL02 and MES-SA/DX5 cells (102.3 ± 6.7 and 95.8 ± 4.3%, respectively). In the presence of 0.2 μM paclitaxel, verapamil, KR30031 and their optical isomers produced concentration-dependent

Fig. 5



Effect of verapamil, KR30031 and their optical isomers on MAP (A) and HR (B) in anesthetized rats. Racemic verapamil (open circles), *R*-verapamil (open triangles), *S*-verapamil (open squares), racemic KR30031 (filled circles), *R*-KR30031 (filled triangles) and *S*-KR30031 (filled squares). Values are mean percentage ± SEM ($n = 4–8$).

Fig. 6



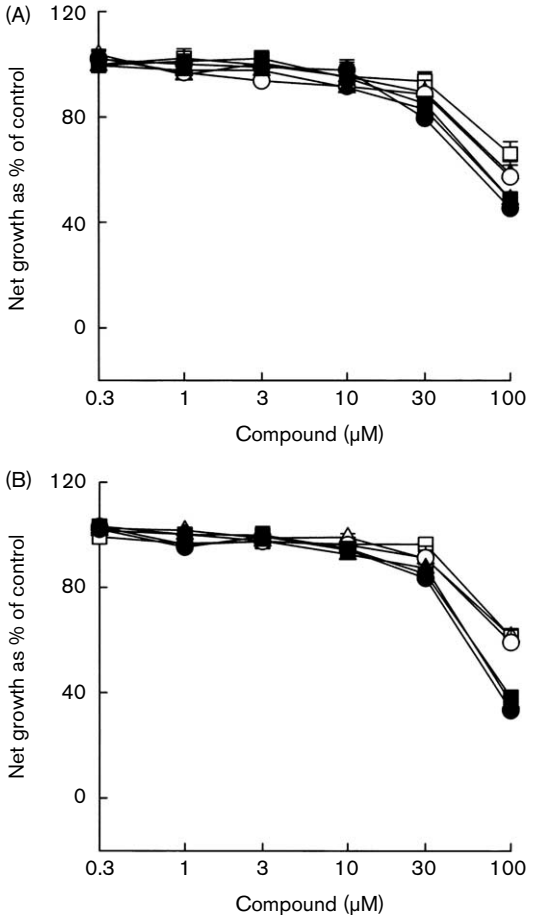
Multidrug resistance reversal effect of verapamil, KR30031 and their optical isomers on the cytotoxicity of paclitaxel (0.2 μM) to HCT15/CL02 (A) and MES-SA/DX5 (B) human cancer cells *in vitro*. Racemic verapamil (open circles), *R*-verapamil (open triangles), *S*-verapamil (open squares), racemic KR30031 (filled circles), *R*-KR30031 (filled triangles) and *S*-KR30031 (filled squares). The experiments were performed at least 3 times (mean ± SEM).

inhibition of cell growth. In HCT15/CL02 cells, the potentiating effect of *R*-verapamil for the paclitaxel-induced cytotoxicity was comparable with that of *S*- and racemic verapamil (IC_{50}: 2.58 ± 0.30, 2.44 ± 0.20 and $2.21 \pm 0.01\,\mu$M, respectively, Fig. 6A). *R*-KR30031 was also equieffective with *S*- and racemic KR30031 in potentiating the paclitaxel-induced cytotoxicity (IC_{50}: 3.11 ± 0.16, 3.04 ± 0.20 and $3.20 \pm 0.38\,\mu$M, respectively). When comparing the effects of *R*-KR30031 and *R*-verapamil for HCT15/CL02 cell growth, there is no significant difference between two groups (IC_{50}: 3.11 ± 0.16 and $2.58 \pm 0.30\,\mu$M, respectively). The potentiating profile and potency of verapamil, KR30031 and their optical isomers for the paclitaxel-induced cytotoxicity in MES-SA/DX5 cells were quite similar to that in HCT15/CL02 cells, although their concentration–response curves were shifted to the right (IC_{50}: 5.52 ± 0.17, 5.33 ± 0.24 and $4.78 \pm 0.20\,\mu$M for *R*-, *S*- and racemic verapamil, respectively; 7.65 ± 0.49, 6.63 ± 0.52 and $7.63 \pm 1.16\,\mu$M for *R*-, *S*- and racemic KR30031, respectively, Fig. 6B).

Intrinsic cytotoxicity

The intrinsic *in vitro* toxicity of verapamil, KR30031 and their optical isomers was evaluated to obtain an indication of the potential therapeutic window, i.e. the ability of the modulator to reverse resistance at non-toxic concentrations. This experiment was performed in HCT15/CL02 (Fig. 7A) and MES-SA/DX5 cell lines (Fig. 7B) using an exposure period equivalent to that used in the MDR reversal assay. The inhibition pattern and potency in cell

Fig. 7



Intrinsic cytotoxicity of verapamil, KR30031 and their optical isomers to HCT15/CL02 (A), MES-SA/DX5 (B) human cancer cells *in vitro*. Racemic verapamil (open circles), *R*-verapamil (open triangles), *S*-verapamil (open squares), racemic KR30031(filled circles), *R*-KR30031 (filled triangles) and *S*-KR30031 (filled squares). The experiments were performed at least three times (mean ± SEM).

growth was similar in all experimental groups. The inhibition in cell growth observed only at the very high concentration of 100 μM was significantly different from control.

Discussion

The major finding of this study is that *R*-KR30031 was 2- and 20- to 250-fold less potent than *S*-KR30031 and *R*-verapamil in cardiovascular adverse activity in rats, respectively, while all compounds were equally effective in reversing drug resistance in cancer cells. First, the cardiovascular adverse effects of verapamil, KR30031 and their optical isomers in rat aorta, in rat heart and in anesthetized rats were measured and compared to each other. *R*-verapamil was 2.5- to 8-fold less potent than *S*-verapamil in relaxing aorta isolated from rat, in decreasing LVP of heart isolated from rat and in lowering MAP in anesthetized rat. These results are in line with the studies indicating that *S*-verapamil was 2- to 15-fold as potent as *R*-verapamil in decreasing MAP and in producing a negative inotropic effect [10–12]. *R*-KR30031 also was less potent than *S*-KR30031 in cardiovascular adverse effect, although the potency difference between the two isomers (2-fold) was smaller than that of verapamil (2.5- to 8-fold). Importantly, the cardiovascular effects of *R*-KR30031 were relatively lower than *R*-verapamil; *R*-KR30031 was 25-, 267- and 23-fold less potent than *R*-verapamil in relaxing isolated aorta, in decreasing LVP of isolated heart and in lowering MAP in anesthetized rat, respectively. These results suggest that the possibility to induce cardiac toxicity with *R*-KR30031 would be much lower than that with *R*-verapamil.

Increasing evidence has suggested that both isomers of verapamil show activity equal to that of racemic verapamil in reversing drug resistance *in vitro* [13,14]. This suggestion was confirmed again by the present study indicating that potency of *R*-verapamil enhancing the paclitaxel-induced cytotoxicity to HCT15/CL02 and MES-SA/DX5 cells was quite similar to that of *S*- and racemic verapamil. KR30031, and its optical isomers also showed patterns similar to that of verapamil in potentiating the paclitaxel-induced cytotoxicity. *R*-KR30031 potently enhanced the paclitaxel-induced cytotoxicity to HCT15/CL02 and MES-SA/DX5 cells (IC_{50}: 3.11 and 7.65 μM, respectively), which was comparable to that of *R*-verapamil (IC_{50}: 2.58 and 5.52 μM, respectively). However, intrinsic cytotoxicity of *R*-KR30031 in HCT15/CL02 and MES-SA/DX5 cells was observed only at the very high concentration of 100 μM. In contrast to the modulatory activity in the HCT15/CL02 and MES-SA/DX5 cells that reveals high level of P-gp expression, KR30031 had no significant effect on cytotoxic drug activity in SK-OV-3 cells that does not express P-gp [16]. KR30031 also inhibited the efflux of rhodamine 123 in HCT15/CL02 cells, but not in SK-OV-3 cells [16]. Furthermore, neither did it affect the cytotoxicity of non-P-gp-substrates such as tamoxifen [16]. Consistent with this, recent reports suggested that verapamil potentiates the cytotoxicity to P-gp-expressed cells, but not to P-gp-non-expressed cells [17,20]. All these results from the present and previous studies suggest that KR30031 enhances the cytotoxicity of anticancer drugs via modulation of P-gp.

In summary, our results indicate that *R*-KR30031 was about 2-fold less potent than *S*-KR30031 in cardiovascular adverse effects, such as decreasing LVP and hypotension, while equipotent with *S*-KR30031 in the reversing multidrug resistance. Importantly, *R*-KR30031 was about 20- to 250-fold less potent than *R*-verapamil in cardiovascular adverse effects, although their multidrug resistance reversal activity was comparable to each other.

These results suggest that *R*- and racemic KR30031 are potent modulators of MDR with reduced cardiovascular activity, and could be potentially useful for the treatment of cancer in combination with anticancer agents.

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