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Preclinical report

Effects of KR-30035, a novel multidrug-resistance modulator, on the cardiovascular system of rats *in vivo* and on the cell cycle of human cancer cells *in vitro*

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The present study was performed to evaluate the adverse effects of KR-30035, a multidrug-resistance modulator, on the cardiovascular system *in vivo*, along with its effect on paclitaxel-induced cell cycle arrest in cultured cancer cells. In anesthetized rats, KR-30035 was about 10-fold less potent than verapamil in lowering blood pressure (i.v. ED_{20}: 0.320 ± 0.052 and 0.034 ± 0.005 mg/kg, respectively) and in producing electrocardiogram changes. In conscious spontaneously hypertensive rats, verapamil caused a significant antihypertensive effects at the doses tested (p.o. ED_{20}, 7.8 ± 4.0 mg/kg), whereas KR-30035 did not significantly change either the blood pressure or the heart rate at any doses tested (up to 100 mg/kg). The estimated i.v. LD_{50} values in mice were 5.9 and 48.9 mg/kg for verapamil and KR-30035, respectively. In the presence of 10 μM KR-30035, paclitaxel (1 μM) when added to cultures of HCT15/CL02 human cancer cells greatly shifted the cell population from the G_0/G_1 phases towards G_2/M phases (from 42.4, 30.3 and 27.3 to 14.6, 21.5 and 63.9% for the G_0/G_1, S and G_2/M phases, respectively), with a similar magnitude to that of 10 μM verapamil (14.0, 15.7 and 70.3%, respectively). These results suggest that KR-30035 has weaker *in vivo* effects on the cardiovascular system compared with verapamil, while potentiating the G_2/M arresting effect of paclitaxel on the cell cycle. [© 2000 Lippincott Williams & Wilkins.]

Key words: Cardiac toxicity, cell cycle, KR-30035, multidrug resistance.

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Introduction

A major mechanism by which tumor cells can develop resistance to anticancer drugs is by decreasing the intracellular bioavailability of the anticancer drug. Such a multidrug-resistance (MDR) phenotype of tumor cells is usually mediated by the overexpression of a membrane protein, P-glycoprotein (P-gp), which directly binds to cytotoxic compounds and reduces intracellular drug accumulation through an energy-dependent drug efflux mechanism.[1,2] MDR caused by the overexpression of P-gp could be reversed or modulated by some agents such as verapamil that decrease the efflux of anticancer drugs out of cells through P-gp on the cell membrane.[3] To date, the usefulness of MDR-reversal agents has been limited since plasma concentrations required to reverse MDR result in cardiac toxicity or immunosuppression.[4–6] Accordingly, considerable effort has been directed towards the development of compounds that inhibit P-gp, reverse the MDR phenotype and sensitize cancer cells to conventional chemotherapy without undesired toxicological effects. KR-30035 (5,6-dimethoxy-1-[3-[[2-(3,4,5-trimethoxyphenyl) ethyl]methylamino]propyl]-1,2,3,4-tetrahydronaphthalene-1-carbonitrile) is an arylalkylamine derivative closely related to verapamil and was synthesized at the Korea Research Institute of Chemical Technology (KRICT, Taejon, Korea). It was shown that KR-30035 was a potent modulator of MDR with minimal cardiovascular toxicity *in vitro*.[7] The aim of the present study was to evaluate and compare the possible cardiovascular adverse effects of KR-30035 *in vivo* with verapamil, with corresponding studies of the

effects of KR-30035 on paclitaxel-induced cell cycle arrest of cultured cancer cells.

Materials and methods

Drugs and chemicals

Verapamil, cremophor, paclitaxel, propodium iodide, RNase and sodium citrate were purchased from Sigma (St Louis, MO). KR-30035 was synthesized at the Bio-Organic Science Division, KRICT. Sodium pentobarbital was purchased from Hanlim Pharmaceuticals (Seoul, Korea) and ketamine hydrochloride from Yuhan (Seoul, Korea). RPMI 1640 cell growth medium, trypsin and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY) and Triton X-100 from BioRad (Hercules, CA). Verapamil was dissolved in 0.9% saline and distilled water for i.v. and oral administration, respectively. KR-30035 was dissolved in a combination of 10% ethyl alcohol and 10% cremophor, and serially diluted with 0.9% saline and distilled water for i.v. and oral administration, respectively. Verapamil, KR-30035 and paclitaxel were also dissolved in dimethylsulfoxide (DMSO) for the cell cycle study and diluted with cell culture media (final concentration of DMSO: 0.5%). All drugs and agents were prepared just before use.

Cancer cells

HCT15/CL02 cells were established from human colorectal HCT15 cancer cells by continuous and stepwise exposure to doxorubicin at KRICT.[8] The cells were cultured with RPMI 1640 medium supplemented with 5% FBS as previously reported.[7]

Blood pressure lowering effects in anesthetized rats

Male Sprague-Dawley rats (350–450 g; KRICT) were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and prepared as previously described.[9] Arterial blood pressure was continuously monitored via an Isotec pressure transducer (Hugo Sachs, Freiburg, Germany) connected to a physiograph (WR 3300 Linearcorder; Graphtec, Tokyo, Japan). Electrocardiogram (ECG) and heart rate (HR) were measured by Lead II using an ECG/rate coupler (Type 576; Hugo Sachs), both parameters being analyzed by the AcqKnowledge computer program (Biopac Systems, Goleta, CA) via a signal interface (Model MP100; Biopac Systems). Forty minutes after surgery, verapamil or KR-30035 were i.v. administered at 5 min intervals. Results are expressed as percentage changes of mean arterial pressure (MAP) from baseline values.

Antihypertensive effects in conscious spontaneously hypertensive rats (SHRs)

The blood pressure was measured in conscious male SHRs (12–14 weeks old; Charles River, Yokohama, Japan) as previously described.[10] After recovery from the surgical procedures, blood pressure was recorded continuously via a pressure transducer (CDX-III; Modular, Malvern, PA) coupled to a physiograph (Modular 8000 signal processor; Modular) and HR was derived from the blood pressure pulse, and both parameters were stored and analyzed by the Biowindow computer program (Modular). MAP and HR were monitored for 6 h after oral administration of verapamil and KR-30035. Results are expressed as percentage changes of MAP from baseline values.

Acute toxicity

To evaluate the acute toxicities of verapamil or KR-30035, the LD_{50} values (a dose that resulted in the deaths of 50% of animals treated) were obtained using mice. Verapamil (3.8–8.3 mg/kg, four groups) and KR-30035 (38–83 mg/kg, four groups) were i.v. (tail vein) administered to mice (female BDF_1 mice, 19–21 g; KRICT).

Cell cycle analyses

The cell cycle was analyzed in terms of the DNA content according to a propidium iodide staining method with some minor changes as described previously.[11] Briefly, human cells were seeded in flat-bottom six-well plates (Falcon; Becton Dickinson, Lincoln park, NJ) and incubated for 1 or 2 days. Before the cells reached confluence, paclitaxel was added to the cells in the presence or absence of either KR-30035 or verapamil (10 μM), followed by a 12 or 24 h incubation. Then, the cells were trypsinized and washed twice with cold phosphate-buffered saline (PBS) by centrifugation at 500 *g* for 8 min. After an overnight incubation with 70% ethanol at -20°C, the cells were washed again with PBS. Then, the final cell pellets were resuspened by gentle vortexing into 1.0 ml propidium iodide solution (composition: 50 mg propidium iodide, 4 mM sodium citrate, 450 mg RNase A and 1% Triton X-100 per 1 l of double-distilled water). Cells were incubated for at least 30 min in the darkness and then analyzed by flow cytometry (FACSort; Becton Dickinson, San Jose, CA). The cell cycle was analyzed using CellQuest software

(Becton Dickinson), and the populations of cells in the G_0/G_1, S and G_2/M phases were calculated from their histograms using Cellfit software (Becton Dickinson).

Statistical analysis

All values are expressed as mean ± SEM. Data were analyzed by the paired Student's *t*-test and one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons (Sigma Stat; Jandel, San Rafael, CA) as appropriate. In all comparisons, the difference was considered to be statistically significant at $p < 0.05$. The ED_{20} values (a dose that decreased MAP by 20%) were obtained from a linear regression of effects versus log-dose plotting. LD_{50} values in mice were calculated using the Probit method.

Results

Blood pressure lowering effects in anesthetized rats

The effects of i.v. administered verapamil and KR-30035 on the blood pressure of anesthetized rats are shown in Figure 1. The mean predose values of MAP were 132 ± 5 and 136 ± 10 mmHg for verapamil and KR-30035, respectively. Both verapamil and KR-30035 produced a dose-dependent reduction in MAP with 10 s taken to exert their action and 30–60 s to reach the plateau. While verapamil caused a significant reduction in MAP at all doses starting with 0.01 mg/kg, a significant reduction in MAP was observed to occur at 10-fold higher dose of 0.1 mg/kg of KR-30035. The ED_{20} values for verapamil and KR-30035 were 0.034 ± 0.005 and 0.320 ± 0.052 mg/kg, respectively, with a difference in the maximal hypotensive effects (83.4 ± 1.9 and 60.0 ± 2.7%, respectively).

The effects of i.v. administered verapamil on the ECG and HR in anesthetized rats are shown in Table 1. Verapamil produced a dose-dependent decrease in HR, R-wave height (R-H) and T-wave height (T-H), while prolongating the P–R interval (PR-I) dose dependently. Verapamil caused a significant decrease in R-H at doses ⩾0.1 mg/kg, in T-H at doses ⩾1.0 mg/kg and in HR at doses ⩾0.3 mg/kg. The significant prolongation of PR-I by verapamil was shown at doses ⩾1.0 mg/kg. Additionally, arrhythmia was frequently shown in verapamil-treated rats (seven out of eight rats; three, two and two rats at 0.1, 0.3 and 1.0 mg/kg, respectively). Furthermore, some rats treated with verapamil (five of eight rats) died after the highest dose of verapamil (10 mg/kg). On the other hand, KR-30035

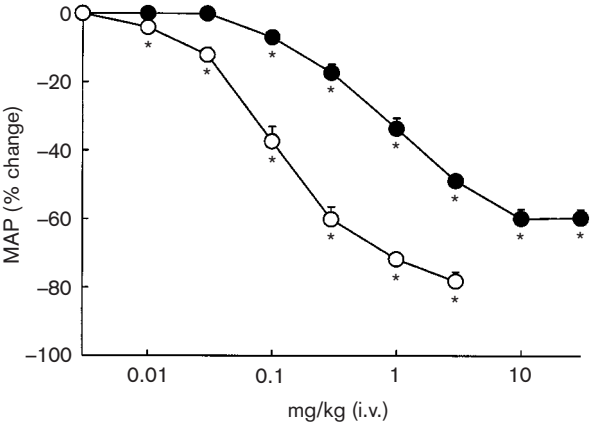

Figure 1. Effects of i.v. administered verapamil (○) or KR-30035 (●) on MAP in anesthetized rats. The data points represent mean percentage change from the control ± SEM (n=5–8). *$p < 0.05$, as compared with the value before administration.

Table 1. ECG changes after i.v. administration of verapamil and KR-30035 in anesthetized rats

Compounds	Dose [cumulative i.v. (mg/kg)]	R-H	T-H	HR	QRS	PR-I	QT-I
Verapamil	control	0.655 ± 0.033	0.123 ± 0.010	466 ± 10.3	17.9 ± 0.6	38.9 ± 1.5	45.7 ± 1.4
	0.1	0.596 ± 0.025[a]	0.119 ± 0.011	442 ± 19.8	18.4 ± 0.8	40.3 ± 0.7	45.3 ± 1.6
	0.3	0.600 ± 0.028[a]	0.118 ± 0.012	427 ± 22.5[a]	18.7 ± 0.7	41.0 ± 1.2	45.4 ± 1.4
	1.0	0.549 ± 0.035[a]	0.103 ± 0.010[a]	351 ± 18.8[a]	19.7 ± 0.9	48.6 ± 2.5[a]	48.9 ± 1.8
	3.0	0.564 ± 0.030[a]	0.108 ± 0.012[a]	217 ± 10.6[a]	20.1 ± 1.1	54.6 ± 3.2[a]	63.7 ± 8.3
KR-30035	control	0.634 ± 0.121	0.113 ± 0.016	483 ± 7.5	18.0 ± 1.0	39.5 ± 1.0	40.3 ± 1.0
	1.0	0.614 ± 0.123	0.109 ± 0.017	459 ± 6.6	18.8 ± 0.8	41.0 ± 1.0	39.5 ± 0.5
	3.0	0.540 ± 0.112[a]	0.116 ± 0.023	414 ± 4.7[a]	19.8 ± 0.6	43.0 ± 0.6	42.5 ± 0.5
	10.0	0.499 ± 0.146[a]	0.101 ± 0.016	288 ± 14.9[a]	20.5 ± 1.3	43.8 ± 1.4	46.0 ± 2.4
	30.0	0.398 ± 0.101[a]	0.104 ± 0.017	246 ± 9.4[a]	23.3 ± 1.8	45.0 ± 2.1	51.0 ± 6.6

The ECG changes were measured at 1 min after administration of verapamil or KR-30035. Values are mean ± SEM (n=5–8).
*$p < 0.05$, significantly different from control.
R-H, R-wave height (mV); T-H, T-wave height (mV); HR, heart rate (beats/min); QRS, QRS complex (ms); PR-I, P–R interval (ms); QT-I, Q–T interval (ms).

produced a dose-dependent decrease in HR and R-H, both parameters being significantly decreased at doses $\geqslant$3.0 mg/kg without significant changes of PR-I. Interestingly enough, KR-30035 did not cause any arrhythmia or death at any doses tested (up to 30 mg/kg).

Antihypertensive effects in conscious SHRs

The effects of orally administered verapamil and KR-30035 on blood pressure in conscious SHRs are shown in Figure 2. The mean predose values of MAP and HR were 163$\pm$2.5 mmHg and 302$\pm$9.7 beats/min. Verapamil dose-dependently decreased MAP with a rapid onset of action (less than 10 min) and reached its maximum effects within 30 min (E_{max}: 12.7$\pm$3.4 and 22.0$\pm$1.3% at 3 and 10 mg/kg, respectively). The antihypertensive effects of verapamil (p.o. ED_{20}: 7.8$\pm$4.0 mg/kg) persisted at a significant level at 3–6 h after dosing (3 and 10 mg/kg), but without any significant change in HR. KR-30035 did not significantly change MAP and HR at any doses tested (up to 100 mg/kg).

Acute toxicity

The estimated i.v. LD_{50} values of verapamil and KR-30035 in mice were 5.9 and 48.9 mg/kg, respectively.

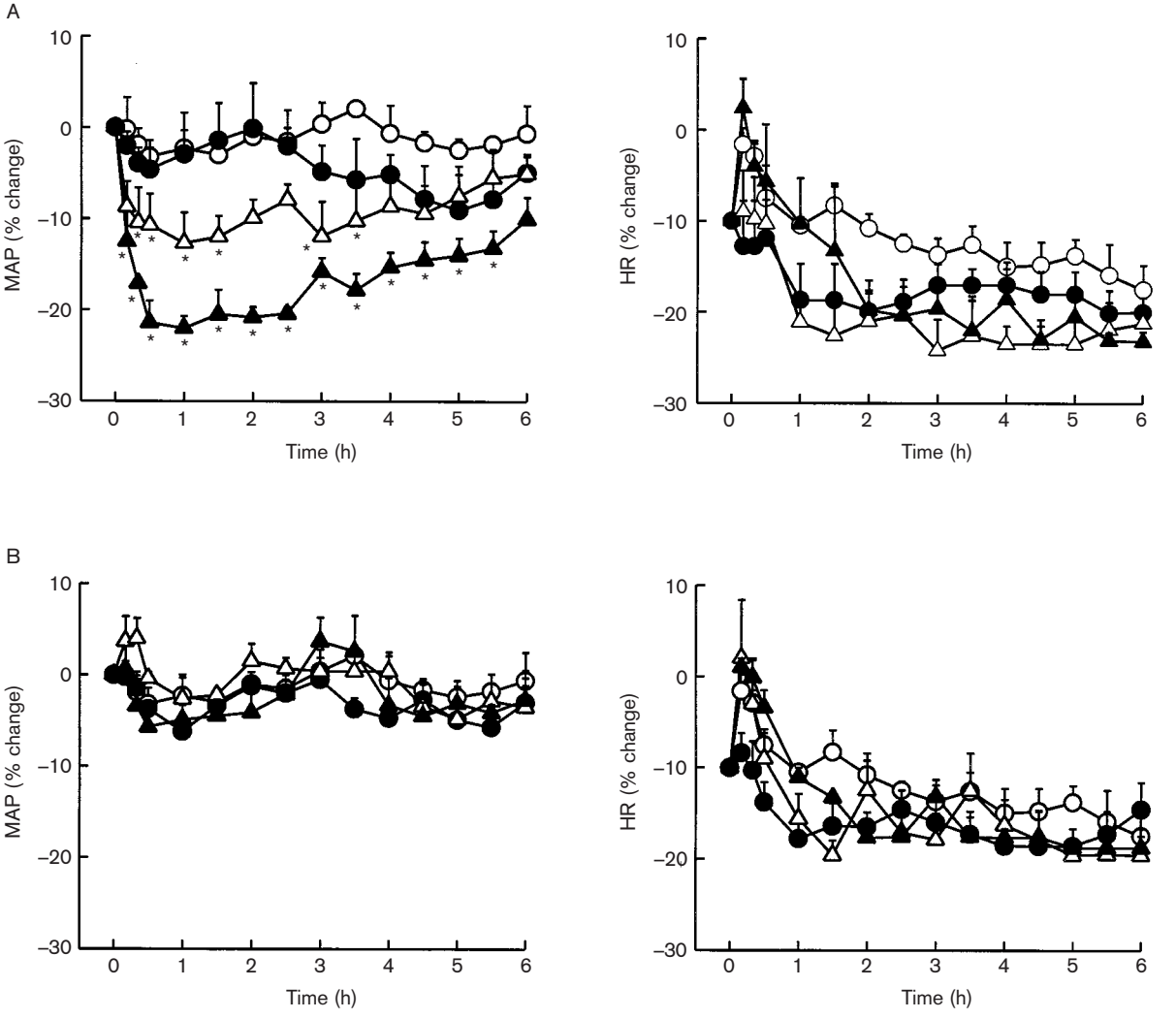

Figure 2. Effects of orally administered verapamil (A) or KR-30035 (B) on MAP and HR in conscious SHRs. Verapamil: vehicle (○), 1 (●), 3 (△) and 10 (▲) mg/kg. KR-30035: vehicle (○), 10 (●) 30 (△) and 100 (▲) mg/kg. The data points represent mean percentage change from the control$\pm$SEM (n=4–6). *$p<0.05$, significantly different from the control.

Cell cycle analyses

Asynchronously growing HCT15/CL02 human cancer cells were incubated for 12 or 24 h in the presence of paclitaxel with or without KR-30035. After 12 h, control cells (in the absence of both paclitaxel and KR-30035) grew exponentially and the ratio of cell populations in the G_0/G_1 (first main peak in Figure 3), S (between first peak and second peak) and G_2/M (second relatively small peak) phases were 51.7, 37.0 and 11.2%, respectively. Paclitaxel (1 μM) in the absence of KR-30035 and verapamil slightly shifted the cell populations from the G_0/G_1 phases towards the G_2/M phases (42.4, 30.3 and 27.3% for G_0/G_1, S and G_2/M phase, respectively). In the presence of 10 μM KR-30035, paclitaxel (1 μM) induced a great shift in the cell populations from the G_0/G_1 phases towards the G_2/M phases (14.6, 21.5 and 63.9%,

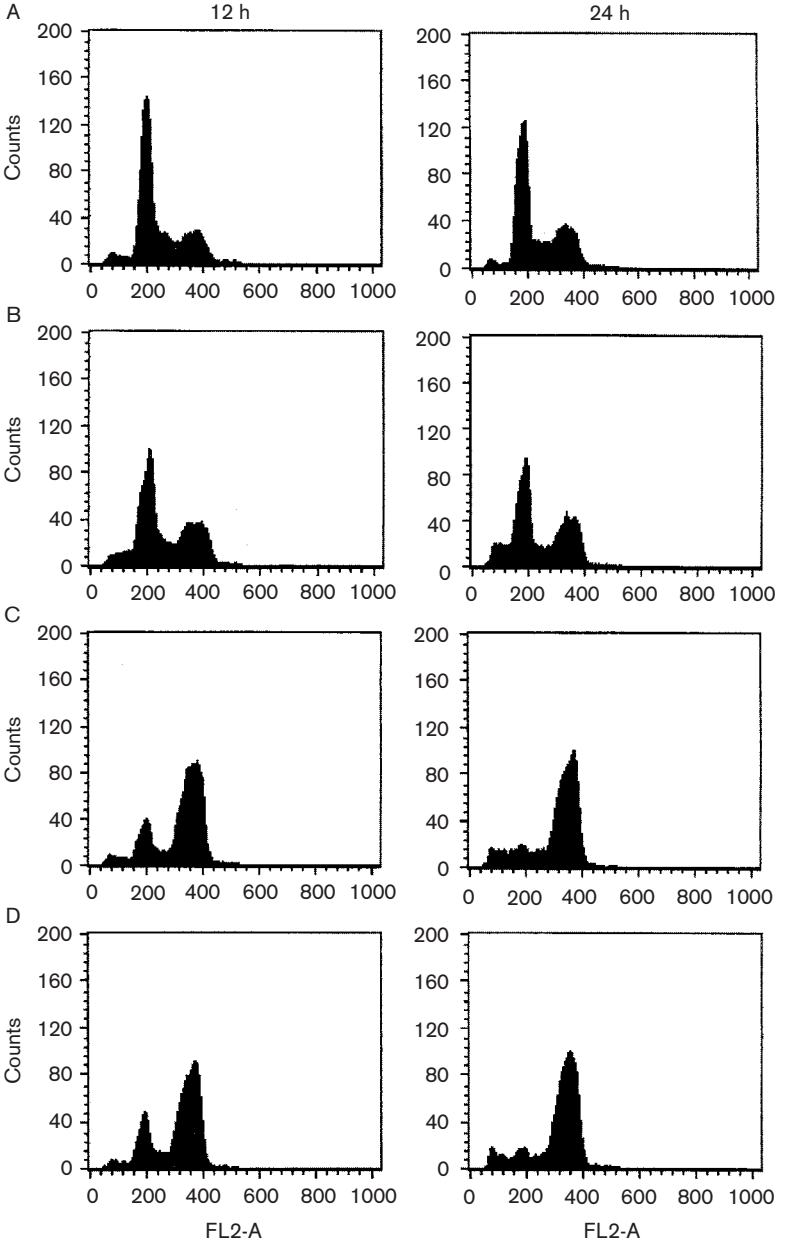

Figure 3. Effects of verapamil or KR-30035 on paclitaxel-induced HCT15/CL02 cell cycle arrest. The cells were cultured with vehicle (A) and paclitaxel (1 μM, B) in the absence or presence of verapamil (10 μM, C) or KR-30035 (10 μM, D) for 12 (left panel) and 24 (right panel) h. Then, the cell cycle was analyzed by propodium iodide-stained DNA content using flow cytometry. These results are a representative of three separate experiments.

respectively). Similarly, paclitaxel (1 μM) greatly shifted the cell populations (14.0, 15.7 and 70.3%, respectively) in the presence of 10 μM verapamil. The results obtained after 24 h incubation were similar to those for 12 h incubation.

Discussion

The results from this study indicate that KR-30035, a structurally novel MDR-reversal modulator, has weaker effects on the cardiovascular system *in vivo* than verapamil along with the property to potentiate the effect of paclitaxel-induced cell cycle arrest of cancer cells. In anesthetized rats, KR-30035 was about 10-fold less potent in lowering blood pressure than verapamil. Verapamil induced a significant decrease in HR at doses $\geqslant$0.3 mg/kg and significant prolongation of PR-I at doses $\geqslant$1.0 mg/kg in anesthetized rats. However, KR-30035 produced a significant decrease in HR at doses $\geqslant$3.0 mg/kg, but without significant prolongation of PR-I (up to 30 mg/kg). Moreover, verapamil caused arrhythmia and death in most of the rats (seven and five out of eight rats, respectively), whereas KR-30035 did not cause any arrhythmia or death at any doses tested (up to 30 mg/kg). In conscious SHRs, although verapamil had a significant antihypertensive effects (p.o. ED_{20}: 7.8 mg/kg), KR-30035 did not significantly change MAP and HR at any doses tested (up to 100 mg/kg). Additionally, the acute toxicity in mice was also about 8-fold lower for KR-30035 than for verapamil (LD_{50}: 48.9 and 5.9 mg/kg, respectively). Previously, it was shown that the vasorelaxant effects on isolated rat aorta and the cardiodepressant effects of LVP on isolated rat heart were 20- and 12-fold smaller for KR-30035 than for verapamil, respectively.[7] All these results from the present and previous studies suggest the possibility that cardiac toxicity with KR-30035 would be much lower than that with verapamil.

It has been reported that KR-30035 was more potent than verapamil in enhancing paclitaxel-induced cytotoxicity to HCT15 cells, as previously confirmed in our laboratory by the results from rhodamine accumulation.[7] The effect of KR-30035 on paclitaxel-induced cell cycle arrest was evaluated in HCT15/CL02 human cancer cells. Paclitaxel alone (1 μM) shifted the cell population from the G_0/G_1 phase towards the G_2/M phase in HCT15/CL02 cells, in line with previous reports.[12,13] KR-30035 (10 μM) potentiated paclitaxel (1 μM)-induced G_2/M arrest to a similar magnitude to that of verapamil (10 μM). Neither KR-30035 nor verapamil (10 μM) showed any effects on the cell cycle in our experiments when tested alone, although some calcium channel blockers such as isradipine and SK&F 96365 might be able to inhibit cell proliferation by a cell cycle arrest in the G_0/G_1[14] or G_2/M phase.[15] These results indicate that KR-30035 potentiated the G_2/M arresting effect induced by paclitaxel in HCT15/CL02 cells without modifying the cell cycle effects of paclitaxel and indirectly confirm our previous report that KR-30035 enhanced the cytotoxicity of cancer drugs via modulation of P-gp.

In conclusion, the results from this study indicate that KR-30035 has about 10-fold lower adverse effects than verapamil on the cardiovascular system *in vivo* and potentiates the effect of paclitaxel-induced cell cycle arrest in G_2/M. These results suggest that KR-30035 might be used as a MDR modulator with less likelihood of inducing cardiac toxicity for the treatment of cancer than verapamil.

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