CHAPTER IV

RESULTS

Part A: Differential cytotoxicity of Renieramycin M on normal and cancerous cell lines.

1. Cytotoxicity of RM

At 72-hours treatment, RM inhibited the viability of all cell types in this study as determined by an MTT assay. In addition, RM elicited its cytotoxic effect in concentration-dependent manner, and at the low concentration of nanomolar range (Figure 11 and 12). The results suggested that RM was a potent cytotoxic agent. The IC_{50} values of RM against KB, H460, and CC2511 cells were summarized in Table 7. The results showed that H460 and KB, representative cancerous cells, were more sensitive to RM treatment than normal cells, CC2511.

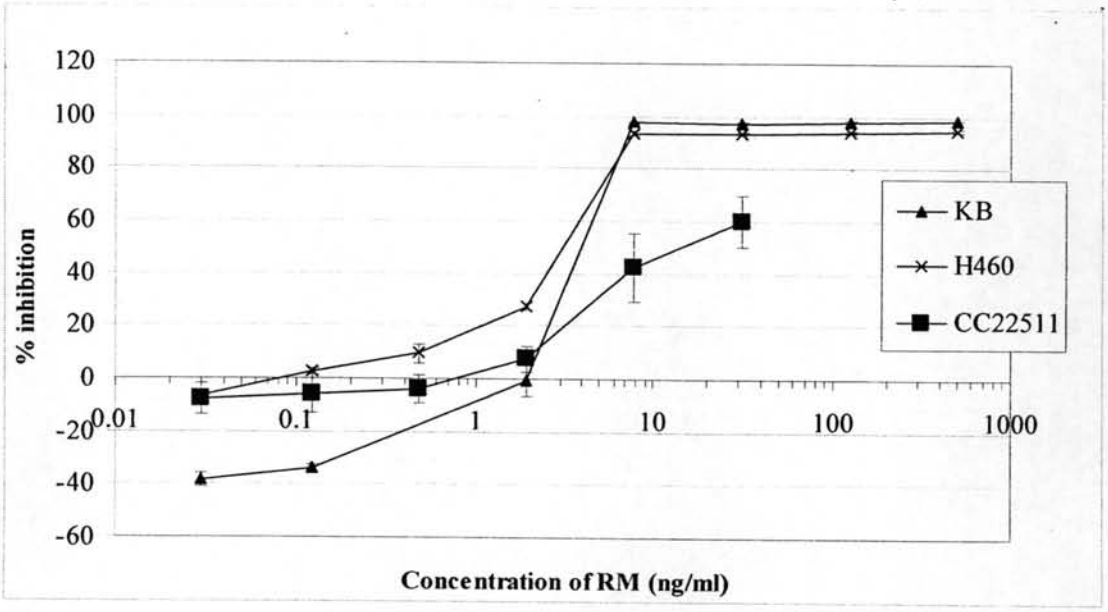

Figure 11 The cytotoxicity of RM in KB, H460, and CC2511 cells, using MTT reduction as the endpoint. Each point represents mean ± S.E. for 3-4 replications.

Table 7 The apparent IC_{50} values of RM on KB, H460, and CC2511 cell lines, as determined by an MTT assay.

Cell lines	IC_{50} value of RM (ng/ml)[a] (nM)
KB	2.19 ± 0.56 (3.81)
H460	2.57 ± 0.43 (4.47)
CC2511	20-30 (34.8-52.2)

[a] All data are expressed as mean ± S.E., n = 3-4 replications.

In this study, the influence of P-gp on the RM induced-cytotoxicity was also determined in P-gp overexpressing cells (LLC-MDR_1) and their parental cells (LLC-PK_1) (Figure 12). As shown in Table 8, the apparent IC_{50} value of RM for LLC-MDR_1 cells were 16-fold less than that for LLC-PK_1 cells, suggesting overexpression of P-gp in the cells significantly increased the sensitivity toward RM treatment. Furthermore, LLC-MDR_1 cells were the most sensitive cells to RM-induced cytotoxicity in this study.

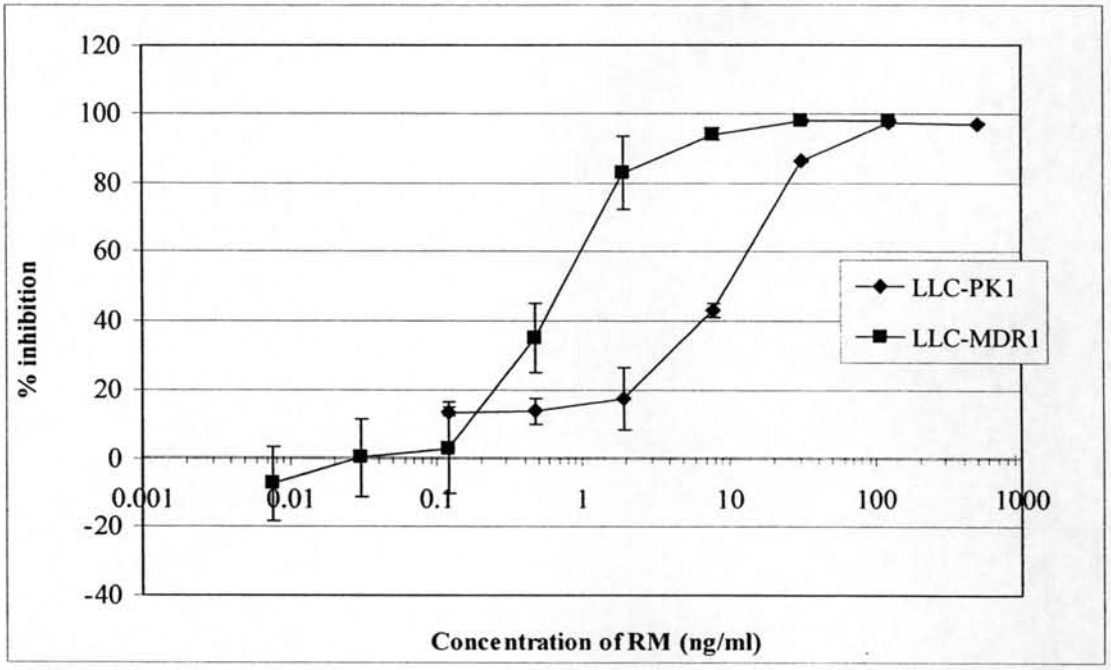

Figure 12 The cytotoxicity of RM in LLC-PK_1 and LLC-MDR_1 cells, using MTT reduction as the endpoint. Each point represents mean ± S.E. for 3-4 replications.

Table 8 The apparent IC_{50} values of RM on LLC-PK_1 and LLC-MDR_1 cell lines, as determined by an MTT assay.

Cell lines	IC_{50} value of RM (ng/ml)[a] (nM)
LLC-PK_1	11.2 ± 0.39 (19.5)
LLC-MDR_1	0.68 ± 0.05 (1.19)

[a] All data are expressed as mean ± S.E., n = 3-4 replications.

2. Type of cell death caused by RM in LLC-PK$_1$ and LLC-MDR$_1$ cell lines

In order to determine the type of cell death caused by RM, the evaluation of membrane integrity and characteristic of cell death were further performed in LLC-PK$_1$ cells and LLC-MDR$_1$ cells by an LDH released assay.

The concentration-dependent cytotoxic effects of RM in LLC-PK$_1$ cells and LLC-MDR$_1$ cells were also demonstrated at 24 hours-treatment. At the low concentration of 1 ng/ml, RM significantly caused the LDH release in the LLC-MDR$_1$ cells with slightly effects on its viability measured by MTT assays (Figure 13). The increase in RM concentration produced the markedly effects on LDH release and cell viability in concentration-dependent manner. In contrast, the cytotoxic effects of RM on LLC-PK$_1$ cells were unnoticeable at the concentration of 1 and 10 ng/ml. RM elicited noticeable cytotoxicity toward LLC-PK$_1$ cells at the high concentration of 100 ng/ml. However, the effects of RM at this concentration on LDH release and cell viability were less observed in the LLC-PK$_1$ cells than in the LLC-MDR$_1$ cells. Hence, these findings supported that LLC-MDR$_1$ cells were more susceptible to RM cytotoxicity than LLC-PK$_1$ cells. It is possible that RM treatment compromised membrane integrity, resulting in cell death. In addition, the degree of RM-induced membrane leakage appeared to be higher in P-gp overexpressing LLC-MDR$_1$ cells than in the parental LLC-PK$_1$ cells.

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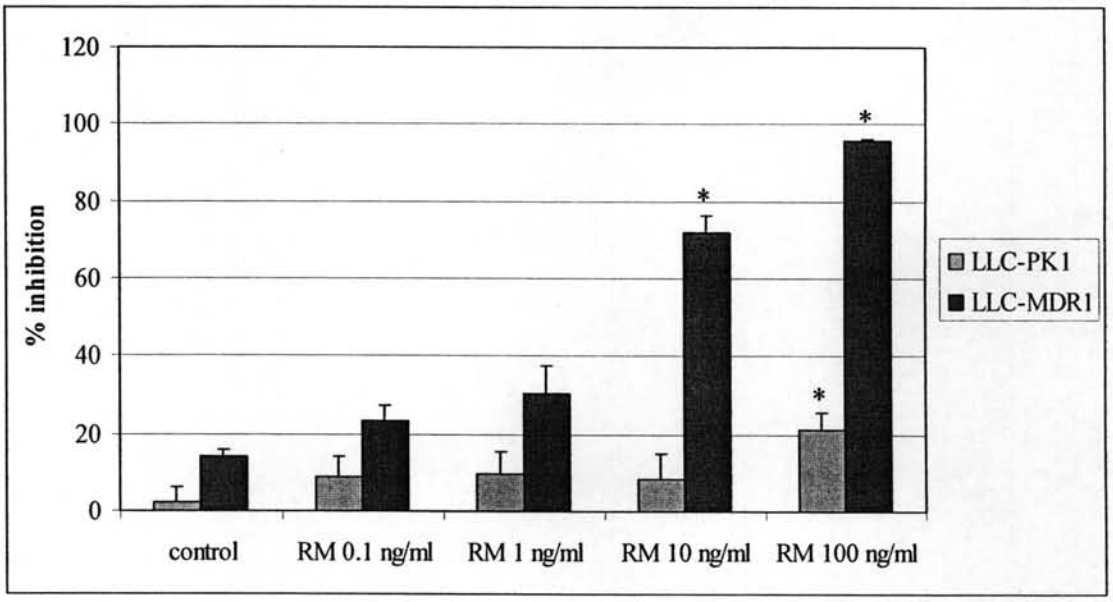

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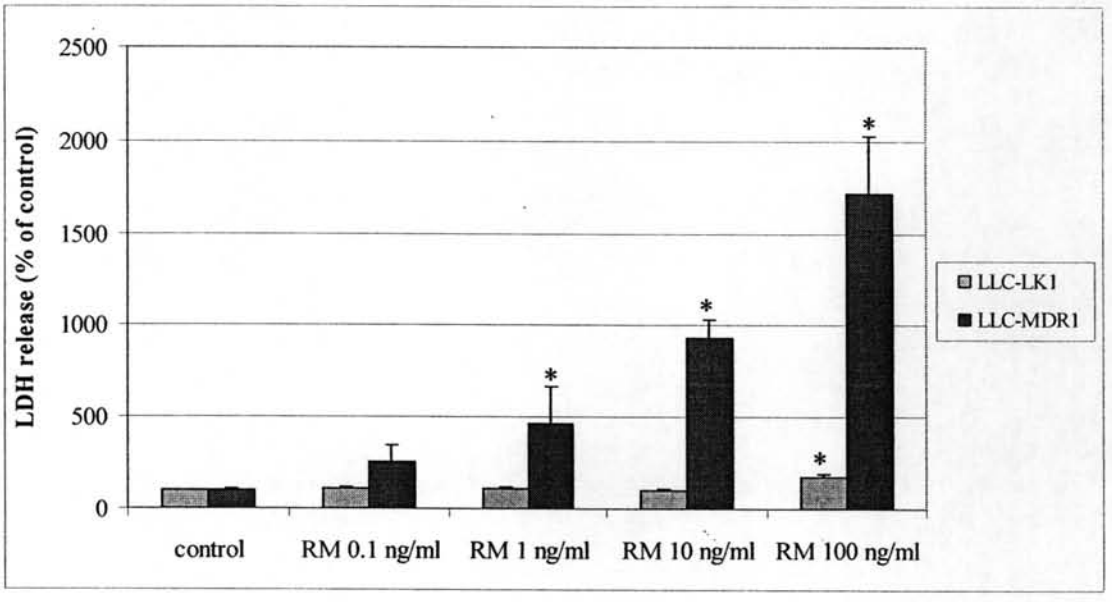


Figure 13 Effects of RM on cell viability as measured by MTT assay (A) and LDH release (B) in LLC-PK_1 cells and LLC-MDR_1 cells after 24- h treatment. Values of LDH release in untreated control cells referred to 100%. Data represent mean ± S.E. of four replicates for each experimental condition. Significantly different from control values, * $P< 0.05$

Part B: Effects of Renieramycin M on P- gp function

1. Characterization of P-gp activity in LLC-PK$_1$ and LLC-MDR$_1$ cells

P-gp activity was evaluated by the different degree of VBL-induced cytotoxicity in LLC-MDR$_1$ cells and their parental sensitive LLC-PK$_1$ cells. In agreement with other literature reports, this study demonstrated that LLC-PK$_1$ cells were more susceptible to VBL treatment than LLC-MDR$_1$ cells (Figure 14). The apparent IC_{50} values of VBL in LLC-PK$_1$ and LLC-MDR$_1$ cells were 1.17 and 4.78 ng/ml, respectively (Table 9). The findings in this study showed that LLC-PK$_1$ and LLC-MDR$_1$ cells were approximately 4.08 fold difference in their VBL sensitivity.

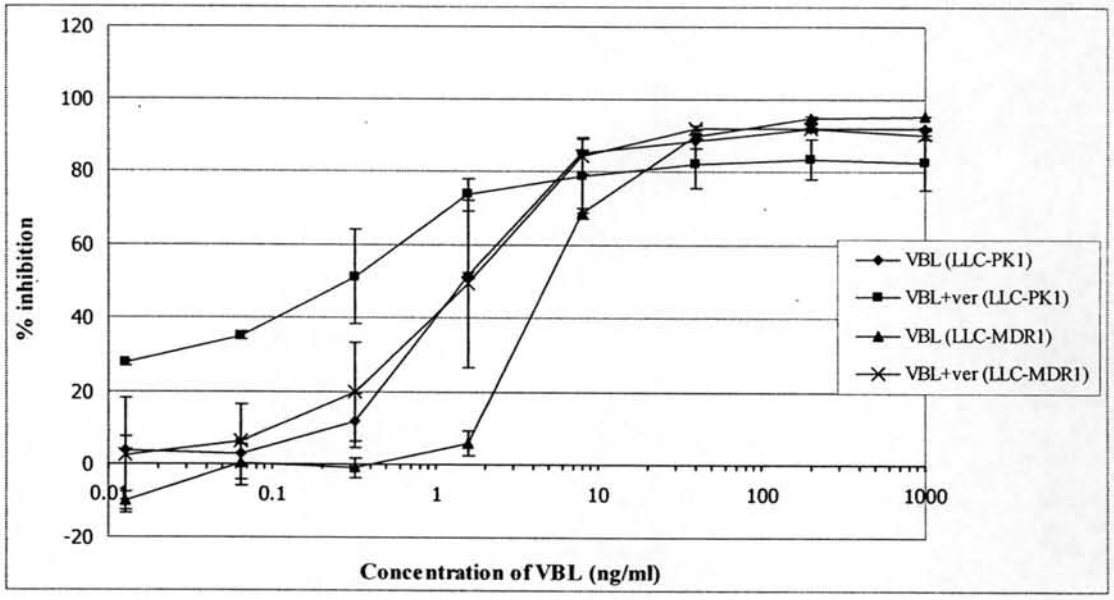


Figure 14 VBL-induced cytotoxicity in LLC-PK$_1$ and LLC-MDR$_1$ cells as determined by the MTT assay. The cells were cultured with a various concentration of VBL with or without verapamil (ver) 20 μM for 72 hours. The results were presented as the means ± S.E. of at least triplicate determinations.

The presence of verapamil, a known P-gp inhibitor, at the concentration of 20 μM could potentiate the VBL-induced cytotoxicity in both LLC-PK_1 and LLC-MDR_1 cells. In other words, verapamil was able to reverse VBL resistance in both cell types. As shown in Figure 14, verapamil significantly shifted the concentration - response curve of VBL-induced cytotoxicity leftward in both cell types with the apparent IC_{50} values as well as folds of MDR reversal shown in Table 9.

Table 9 The apparent IC_{50} values of VBL in the presence and absence of verapamil in LLC-PK_1 and LLC-MDR_1 cells.

Groups	IC_{50} [a] (ng/ml) (Degree of resistance) [b]	Fold reversal of MDR [c]
VBL		
LLC-PK_1	1.17 ± 0.13	
LLC-MDR_1	4.78 ± 0.17 (4.08)	
VBL+ verapamil 20 μM		
LLC-PK_1	0.38 ± 0.20	3.08
LLC-MDR_1	1.38± 1.01*	3.46

[a] IC_{50} (means ± S.E.) values were obtained from at least three independent experiments, using MTT assay after a 72 hours treatment with VBL and VBL co-treatment and verapamil.

[b] The degree of resistance was the ratio between the IC_{50} values of VBL obtained from MDR cells to those obtained from the parental sensitive cells.

[c] The fold reversal of MDR was defined as the ratio of the IC_{50} values for VBL alone to those in VBL and co-treatment with verapamil (the modulating agent)

*$P < 0.05$ vs VBL group

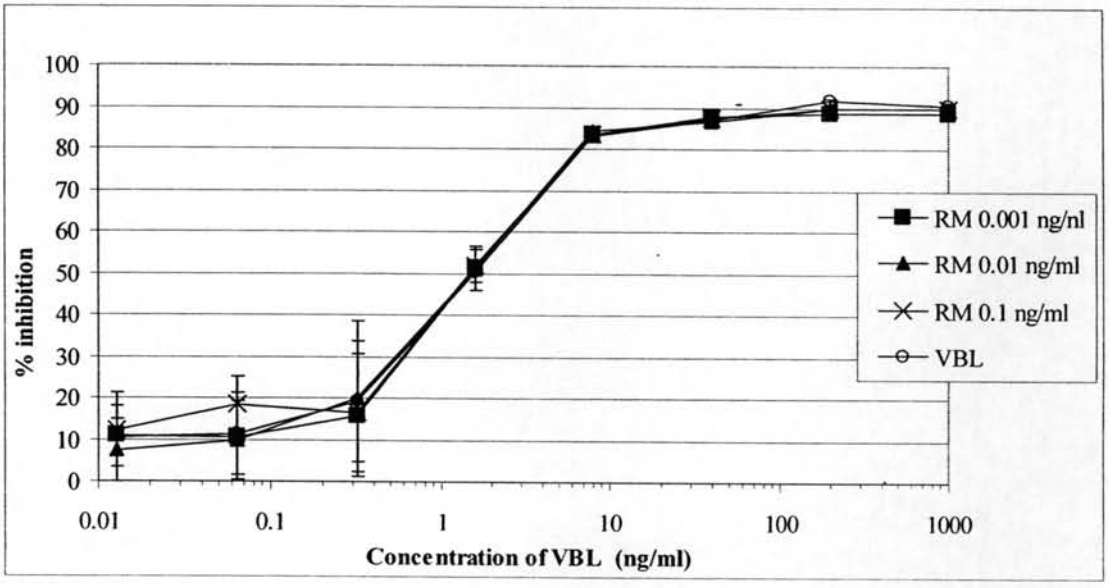
2. Effects of RM on P-gp substrate accumulation

2.1 Effects of RM on VBL-induced cytotoxicity

2.2.1 The pre-treatment studies

In order to determine the effect of RM on inhibition of P-gp expression, the experiments were designed to measure the P-gp function after 24-hours pre-treatment with RM at the non-toxic concentration. The result showed that RM had no significantly influenced on VBL-induced cytotoxicity in either LLC-PK_1 or LLC-MDR_1 cells (Figure 15, Table 10). However, RM at the concentration 0.01 and 0.001 ng/ml slightly decrease VBL toxicity in LLC-MDR_1 cells, as evidenced by an increase in the apparent IC_{50} values of VBL (Table 10). It is possible that pre-treatment of RM at the low concentration for 24 hours could protect P-gp overexpressing cells from VBL-induced toxicity. In addition, the MDR reversal effect of RM decreased when the concentration of RM increased.

A



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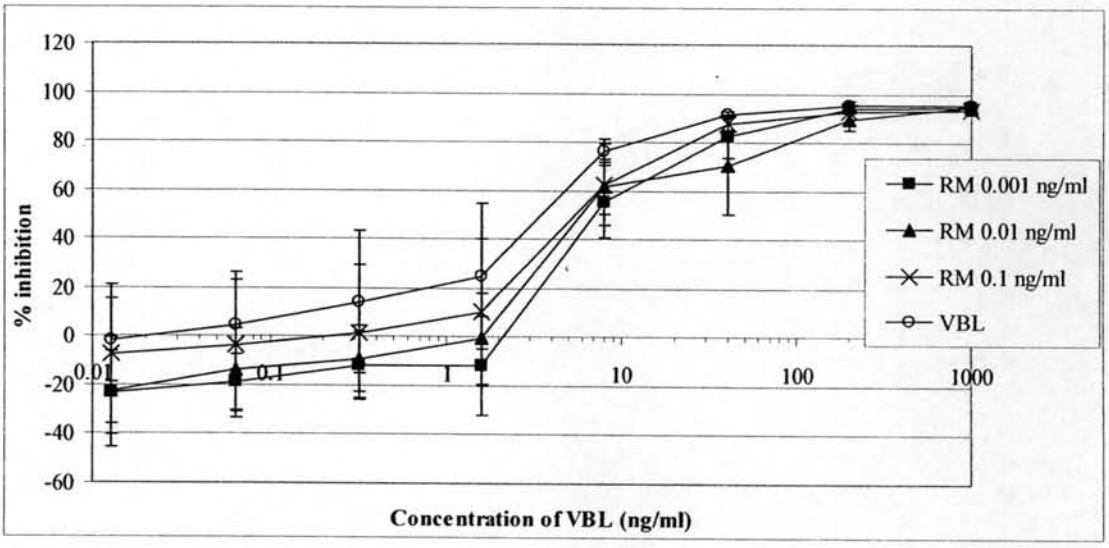

Figure 15 Effects of RM on VBL-induced cytotoxicity in LLC-PK_1 cells (A) and LLC-MDR_1 cells (B). The cytotoxicity was determined by the MTT assay. The cells were cultured with or without RM 24 hours prior to treatment with VBL. The result were presented as means ± S.E. of at least triplicate determinations.

Table 10 The apparent IC_{50} values of VBL in pre-treatment with or without RM at the non-toxic concentrations for 24 hours in LLC-PK_1 and LLC-MDR_1 cells.

Group	concentration (ng/ml)	LLC-PK_1 cells		LLC-MDR_1 cells	
		IC_{50} VBL [b]	Fold reversal of MDR [a]	IC_{50} VBL [b]	Fold reversal of MDR [a]
Control		1.44 ± 0.07		4.60 ± 0.37	
RM	0.001	1.29 ± 0.25	1.12	5.54 ± 1.17	0.83
	0.01	1.21 ± 0.45	1.19	5.20 ± 1.28	0.88
	0.1	1.49 ± 0.12	0.97	4.64 ± 1.23	0.99

Each value is means ± S.E. of at least three independent experiments.

[a] The fold reversal of MDR was defined as the ratio of the IC_{50} values for VBL alone to those in the VBL and RM pre-treatment

[b] IC_{50} values of VBL were presented as mean ± S.E., ng/ml

2.2.2 The co-treatment studies

In order to determine the interaction of RM and P-gp substrate, the experiments were designed to measure the P-gp function in the coexisting conditions between RM and certain P-gp substrates including VBL, puromycin and rhodamine123.

- Effects of RM on VBL induced-cytotoxicity

In this study, the VBL-induced cytotoxicity was determined in the absence and presence of RM at the non-toxic concentration. As shown in Figure 16, the viability of cell obtained from the VBL treatment was comparable to that obtained from the VBL and RM co-treatment. The result suggested that RM had no effect on VBL accumulation in either P-gp overexpressed cells or its parental cells. However, the presence of RM appeared to protect VBL-induced cytotoxicity in both cell types, as evidenced by the increases in the apparent IC_{50} values in the co-treatment groups (Table 11). In addition, RM was able to influence the VBL resistance in LLC-PK_1 cells more than in LLC-MDR_1 cells. This finding suggested that LLC-MDR_1 cells were more sensitive to RM co-treatment than LLC-PK_1 cells.

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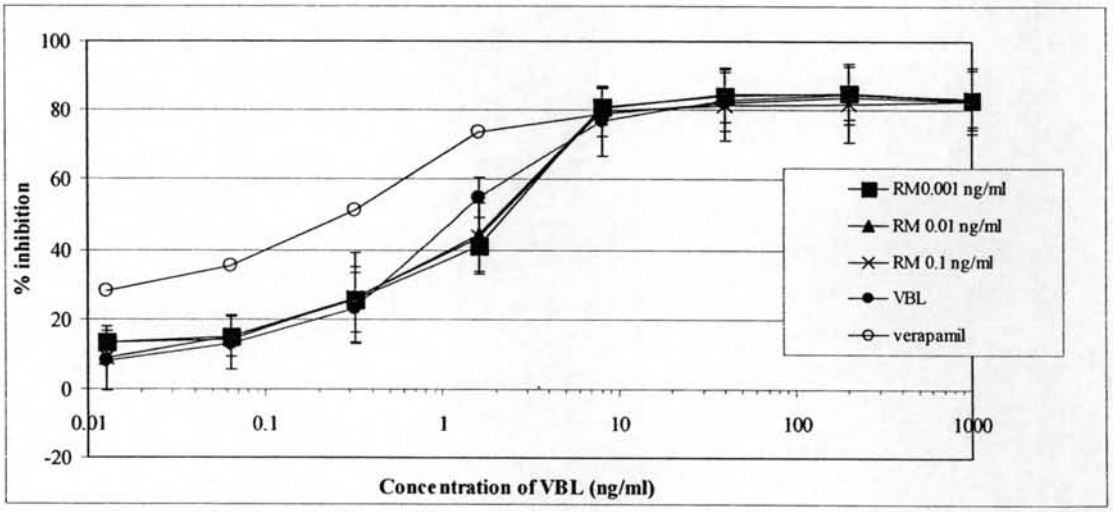

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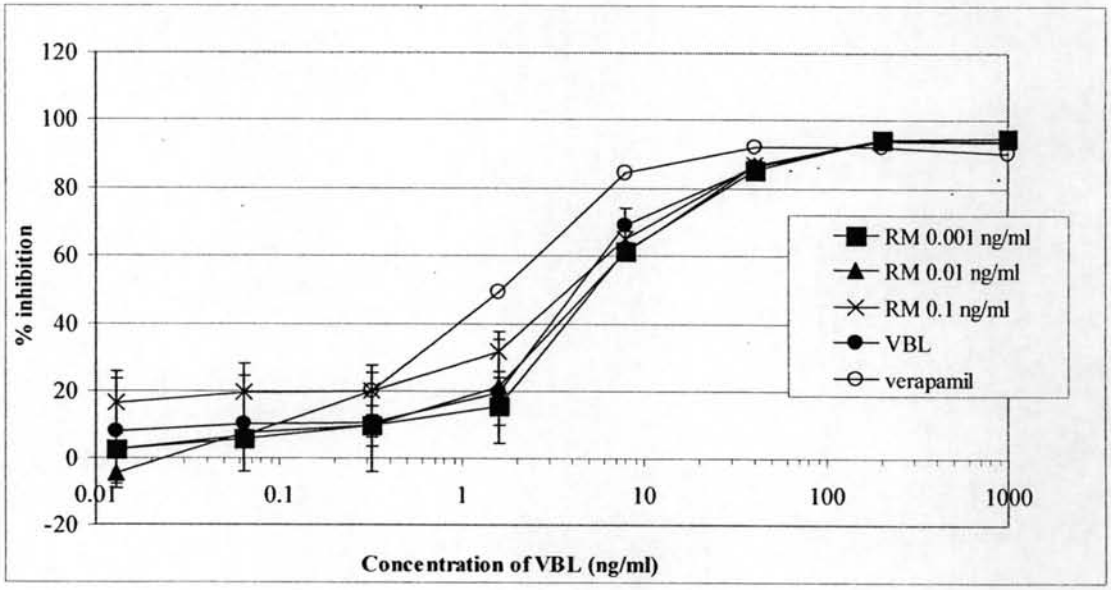


Figure 16 Effects of RM on VBL-induced cytotoxicity in LLC-PK_1 cells (A) and LLC-MDR_1 cells (B), as determined by the MTT assay. The cells were cultured with a full range of concentrations of VBL in the presence or absence RM for 72 hours. The results were presented as mean ± S.E. of at least triplicate determinations.

Table 11 The apparent IC_{50} values of VBL in the absence and presence of RM in LLC-PK1 cells and LLC-MDR1 cells.

Group	concentration (ng/ml)	LLC-PK_1 cells		LLC-MDR_1 cells	
		IC_{50} VBL [b]	Fold reversal of MDR [a]	IC_{50} VBL [b]	Fold reversal of MDR [a]
Control		0.9210 ± 0.0557		4.8322 ± 0.3662	
RM	0.001	1.6113 ± 0.4124	0.57	5.5292 ± 0.2455	0.87
	0.01	1.5878 ± 0.9821	0.58	4.7971 ± 0.1836	1.01
	0.1	1.3066 ± 0.4916	0.70	5.2449 ± 0.7446	0.92
Verapamil	20 μM	0.4852 ± 0.1993*	1.90	1.7653 ± 1.0103*	2.76

Each value represented mean ± S.E. of at least three independent experiments.

[a] The fold reversal of MDR was defined as the ratio of IC_{50} for VBL alone to those in the VBL and RM co-treatment.

[b] IC_{50} of VBL were mean ± S.E., ng/ml

- Effects of RM on puromycin induced-cytotoxicity

The co-treatment between puromycin and RM significantly increased the apparent IC_{50} values of puromycin in LLC-MDR_1 cells ($P<0.05$). As shown in Figure 17, the concentrations-response curve of puromycin shifted rightward in the presence of RM. The results suggested that RM increased puromycin resistance of *MDR1* expressing cells. However, this effect decreased at the higher concentration of RM. In this study, verapamil which is a known P-gp inhibitor was also presented as a positive control study. The presence or absence of verapamil had no effect on the apparent IC_{50} values of puromycin (Table 12), suggesting that the inhibitory effect of verapamil on P-gp activity could not increase an accumulation of puromycin in LLC-MDR_1 cells.

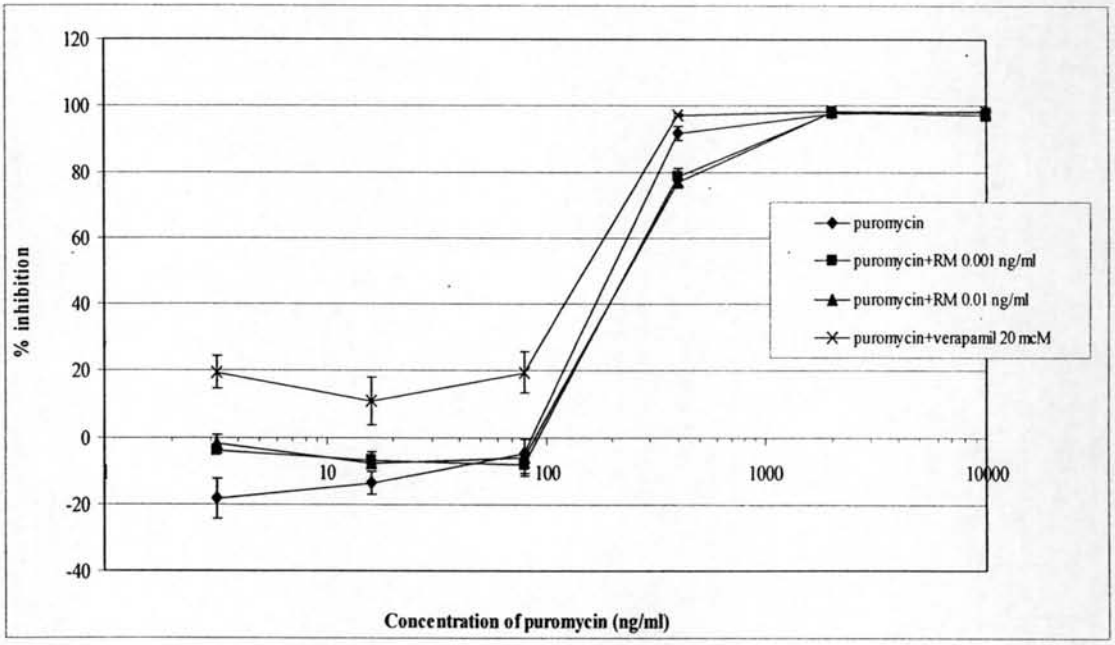


Figure 17 Effects of RM on puromycin-induced cytotoxicity in LLC-MDR_1 cells, as determined by the MTT assay. The cells were cultured with a full range of concentrations of puromycin in the presence or absence RM or verapamil for 72 hours. The result was presented as mean ± S.E. at least triplicate determinations.

Table 12 The apparent IC_{50} values of puromycin in LLC-MDR_1 cells. The values were determined in the co-treatment with RM or verapamil.

Groups	IC_{50} of puromycin (mean ± S.E, ng/ml)	Fold reversal of MDR [a]
Puromycin	166.76 ± 15.9	
Puromycin + RM 0.001 ng/ml	308.27 ± 22.9*	0.54
Puromycin + RM 0.01 ng/ml	272.73 ± 32.1*	0.61
Puromycin + verapamil 20 μM	153.36 ± 5.35	1.09

Each value represented mean ± S.E. of at least three independent experiments.

[a] The fold reversal of MDR was defined as the ratio of the IC_{50} value for puromycin to that for puromycin with the modulating agent.

*$P < 0.05$ vs puromycin group

- Effect of RM on accumulation of rhodamine 123 (Rh123)

In order to investigate the effect of RM on accumulation of Rh123 (a known P-gp substrate), the studies were performed in three culture conditions including in PBS buffer, energy supply medium (glucose added), and energy free medium (NaN_3 added). In each condition, the level of Rh123 in LLC-PK_1 cells was significantly higher than in LLC-MDR_1 cells. The magnitudes of differences were 4.8, 1.9, and 2.5-fold in energy supply, energy free, and PBS medium, respectively (Figure 18). These findings supported that LLC-MDR_1 cells had higher P-gp activity than LLC-PK_1 cells. In addition, the presence of verapamil (20 μM) increased the accumulation of Rh123 in both LLC-PK_1 and LLC-MDR_1 cells (Figure 19). These findings also demonstrated that both LLC-PK_1 and LLC-MDR_1 cells possessed the P-gp activity.

Because the function of P-gp is ATP-dependent, it can be expected that Rh123 accumulated in higher number under the energy deficit condition. In this regard, the

accumulation of Rh123 was more pronounced in both cells being cultured in energy free medium (Figure 18). However, the result showed that number of Rh123 accumulation in LLC-PK_1 cells was greater when the cells were being cultured in energy supply medium, not the energy free medium. It could be postulated that the presence of glucose in energy supply medium affected the accumulation of Rh123 in LLC-PK_1 cells.

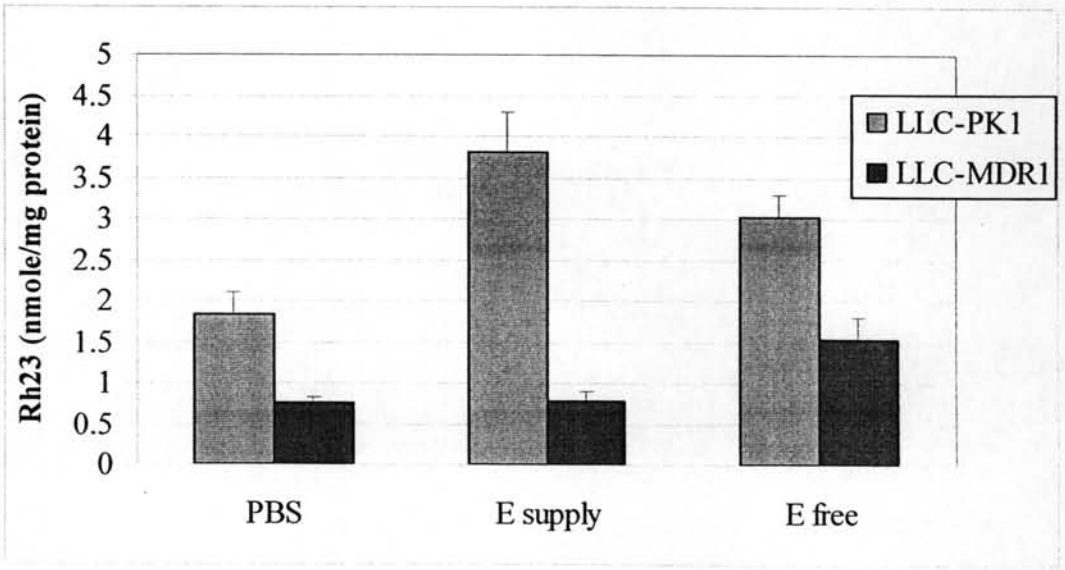
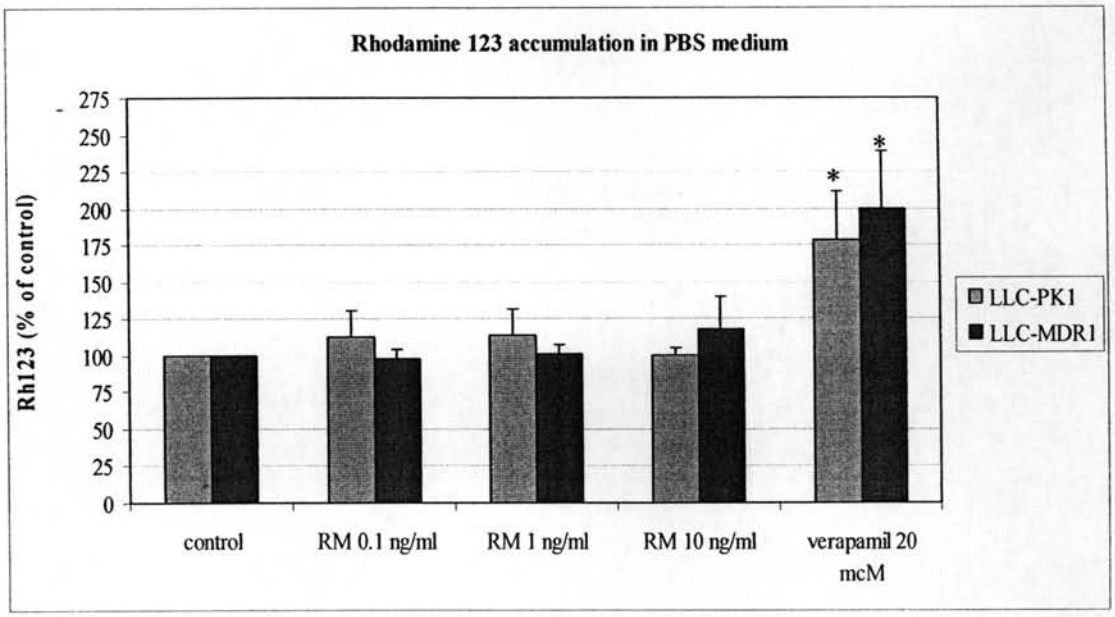
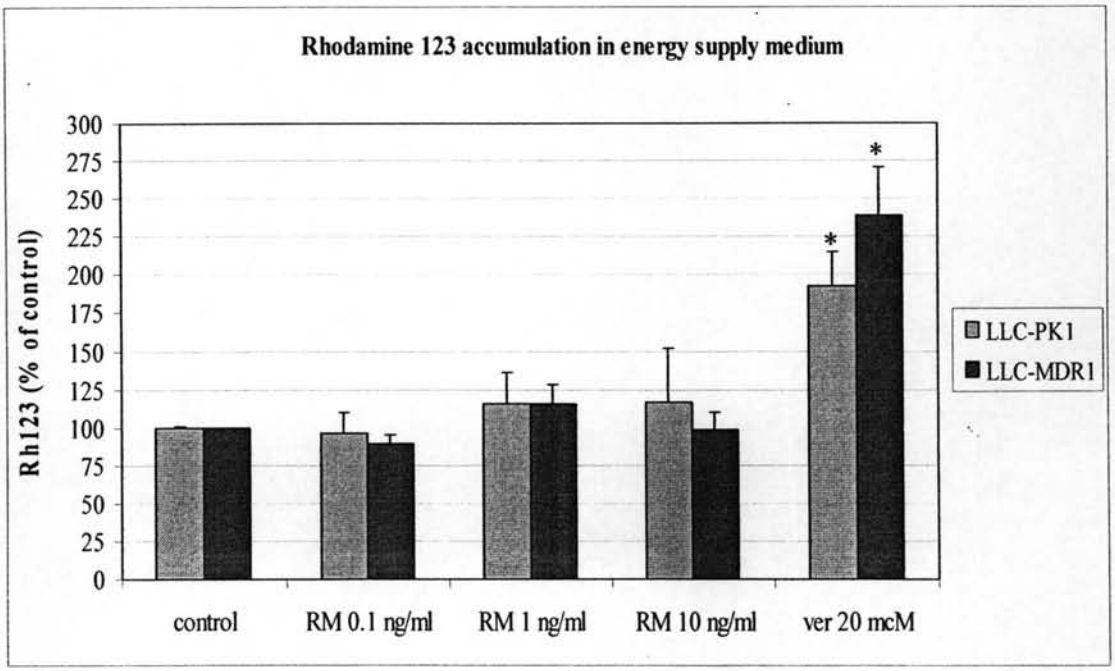


Figure 18 Accumulation of Rh123 in LLC-PK_1 and LLC-MDR_1 cells being cultured in various conditions. The data represented mean ± S.E. of 3-4 independent experiments.

A


Rhodamine 123 accumulation in PBS medium
Rh123 (% of control)
275
250
225
200
175
150
125
100
75
50
25
0
control
RM 0.1 ng/ml
RM 1 ng/ml
RM 10 ng/ml
verapamil 20 mcM
LLC-PK1
LLC-MDR1

B


Rhodamine 123 accumulation in energy supply medium
Rh123 (% of control)
300
275
250
225
200
175
150
125
100
75
50
25
0
control
RM 0.1 ng/ml
RM 1 ng/ml
RM 10 ng/ml
ver 20 mcM
LLC-PK1
LLC-MDR1

C

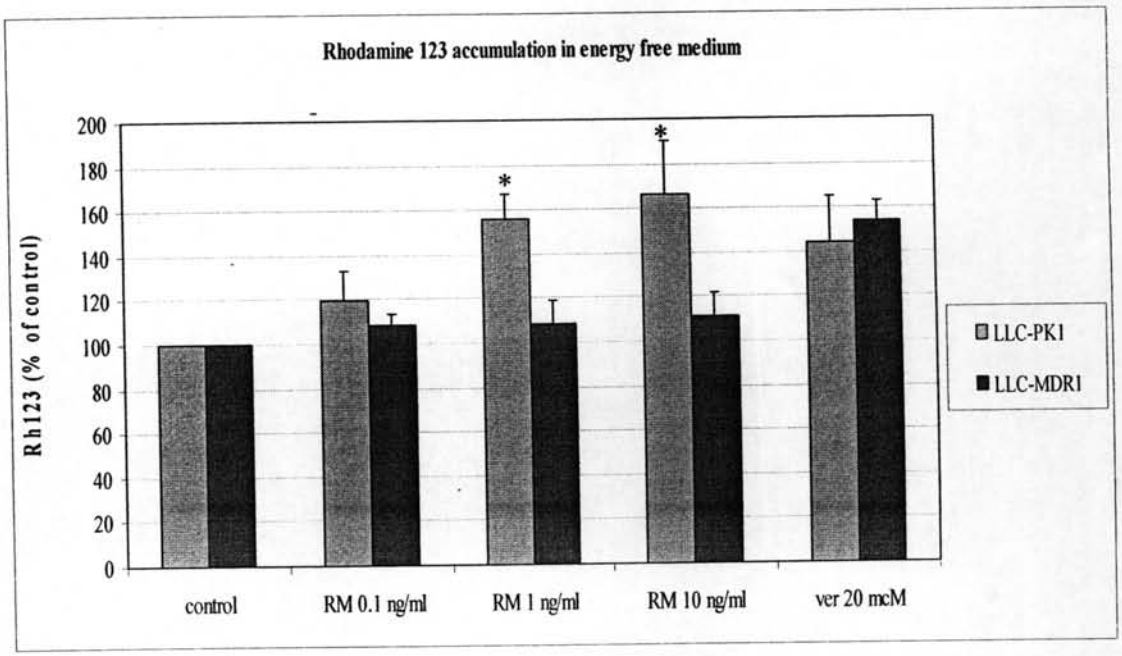


Figure 19 Effects of RM on the accumulation of Rh123 in LLC-PK_1 and LLC-MDR_1 cells. The cells were incubated with Rh123 (10 μM) for 2 hours in PBS (A), energy supply medium (B), and energy free (C) in the presence or absence of RM and verapamil. The data represented as mean ± S.E. (% of control) in 3- 4 independent experiments. * $P< 0.05$ vs control.

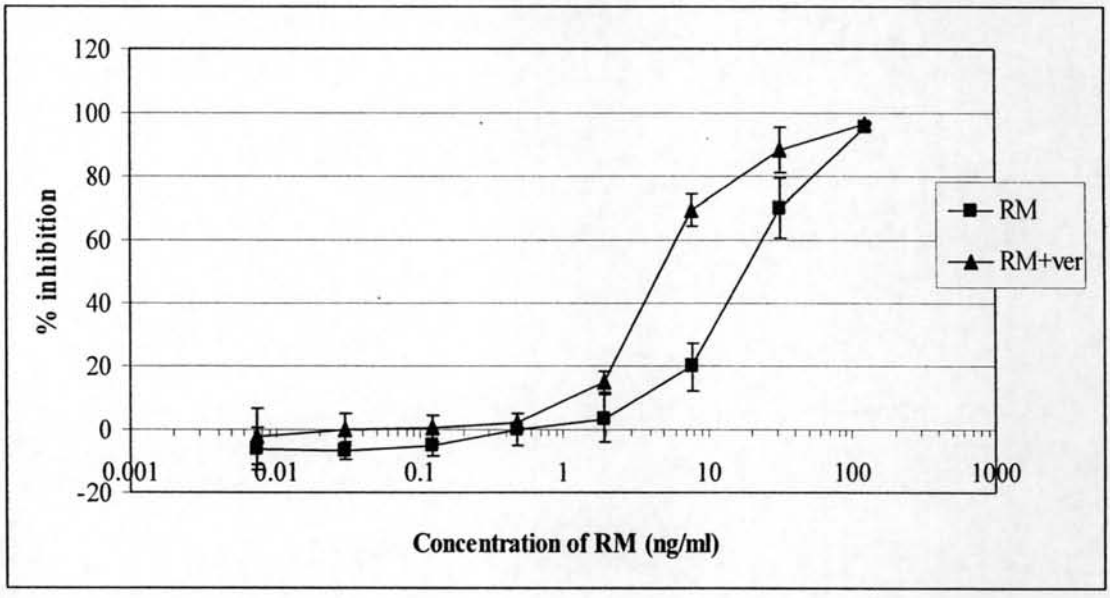
In this study, RM had no significant effects on the intracellular level of Rh123 in LLC-MDR_1 cells being cultured in each experimental condition. Similarly to the observations in LLC-MDR_1 cells, RM had no effects on Rh123 accumulation when the LLC-PK_1 cells were cultured in PBS and medium with energy supply. However, the effects of RM on Rh123 accumulation in LLC-PK_1 cells being cultured in energy deprivation were different from those of LLC-MDR_1 cells. In LLC-PK_1 cells deprived of energy supply, RM at the concentration of 1 and 10 ng/ml significantly increased Rh123 accumulation ($P<0.05$).

3. Effects of P-gp inhibitor on the accumulation of RM

3.1 Effect of verapamil on RM-induced cytotoxicity

Co-treatment of RM with verapamil caused a decrease in the apparent IC_{50} values of RM-induced cytotoxicity of either LLC-PK_1 or LLC-MDR_1 cells. This result suggested that verapamil could restore RM resistance in both cells (Figure 19). The MDR reversals in the presence of verapamil were 3.96- and 3.38-fold in LLC-PK_1 and LLC-MDR_1 cells, respectively (Table 13). It is likely that RM was a substrate of P-gp and its efflux could be inhibited by P-gp inhibitor such as verapamil.

A



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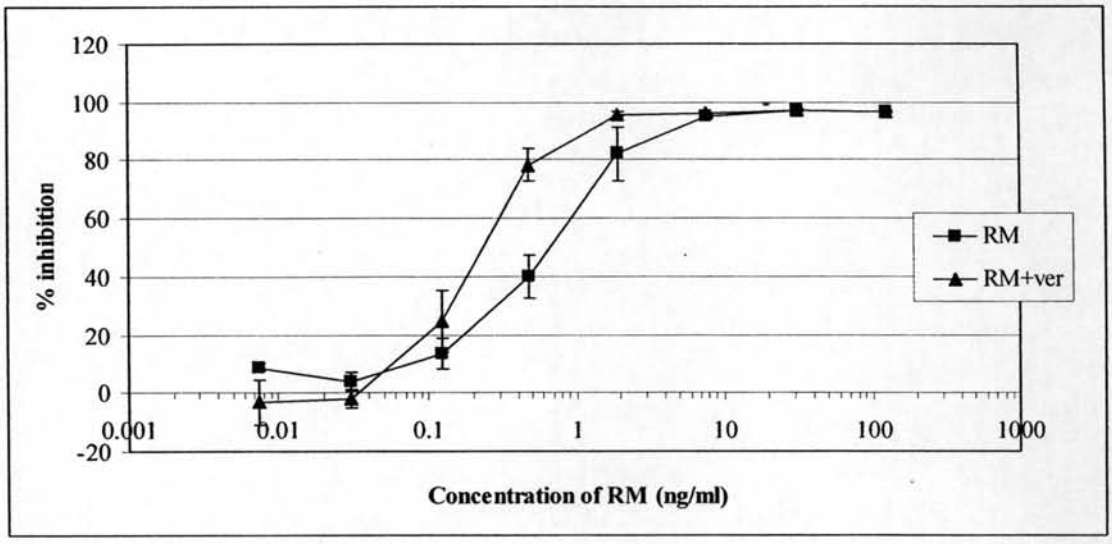

Figure 20 Effects of verapamil on RM induced cytotoxicity in LLC-PK_1 cells (A) and LLC-MDR_1 cells (B). The cytotoxicity was determined by the MTT assay. The cells were cultured with a full range of concentrations of RM in the presence or absence verapamil 20 μM for 72 hours. The results were presented as mean ± S.E. of at least triplicate determination.

Table 13 The apparent IC_{50} values of RM in co-treatment experiments with or without verapamil

Group	concentration (ng/ml)	LLC-PK_1 cells		LLC-MDR_1 cells	
		IC_{50} of RM [b]	Fold reversal of MDR [a]	IC_{50} of RM [b]	Fold reversal of MDR [a]
RM		18.54 ± 5.21		0.71 ± 0.17	
RM + verapamil	20 μM	4.68 ± 0.70	3.96	0.21 ± 0.04*	3.38

Each value represented mean ± S.E. from at least three independent experiments

[a] The fold reversal of MDR was defined as the ratio of IC_{50} values for RM alone to those of RM with the modulating agent

[b] IC_{50} of RM were mean ± S.E., ng/ml

* $P<0.05$ vs RM

In addition, the synergistic effects between RM and verapamil on the accumulation of Rh123 were investigated as shown in Figure 21. The results revealed that the presence of RM decreased effect of verapamil in both cell types. In normal condition (PBS), RM 0.01 ng/ml significantly decreased Rh123 accumulation in LLC-PK_1 cells and this effect decreased when concentration of RM increased (Figure 21A). In energy free condition, the presence of RM did not interfere the effect of verapamil on accumulation of Rh123 in both cell types. Taken together, the results suggested that RM and verapamil might be interfering either the passive uptake of Rh123 into LLC-PK_1 cells or energy-independent efflux of Rh123 in LLC-PK_1.

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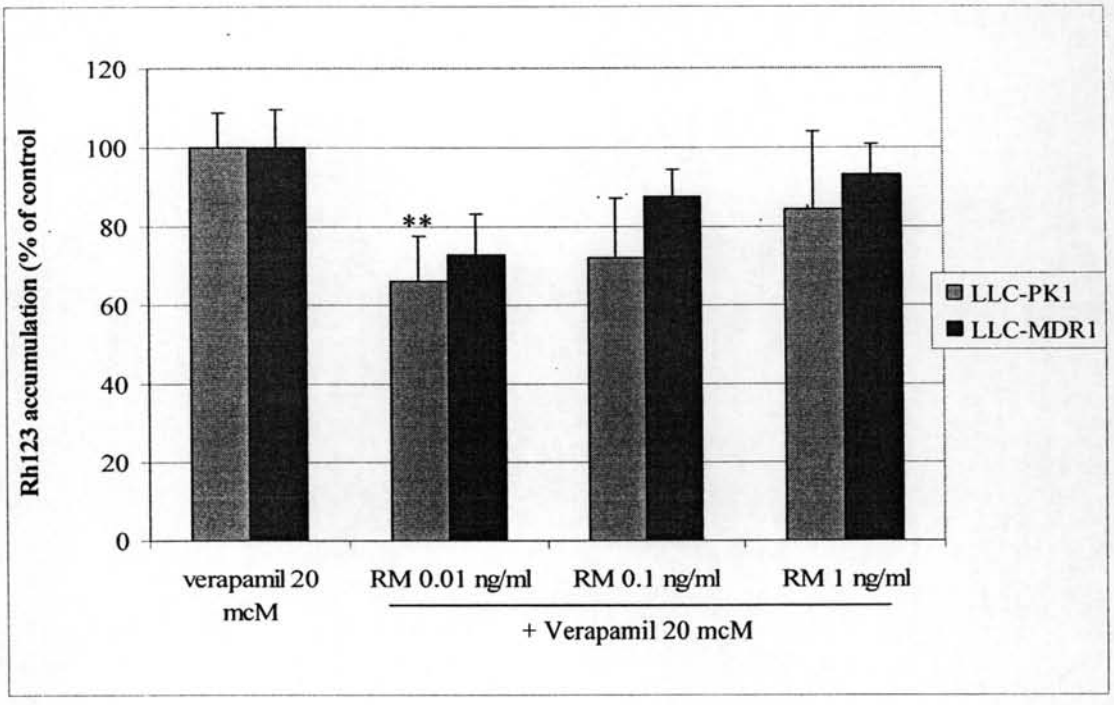

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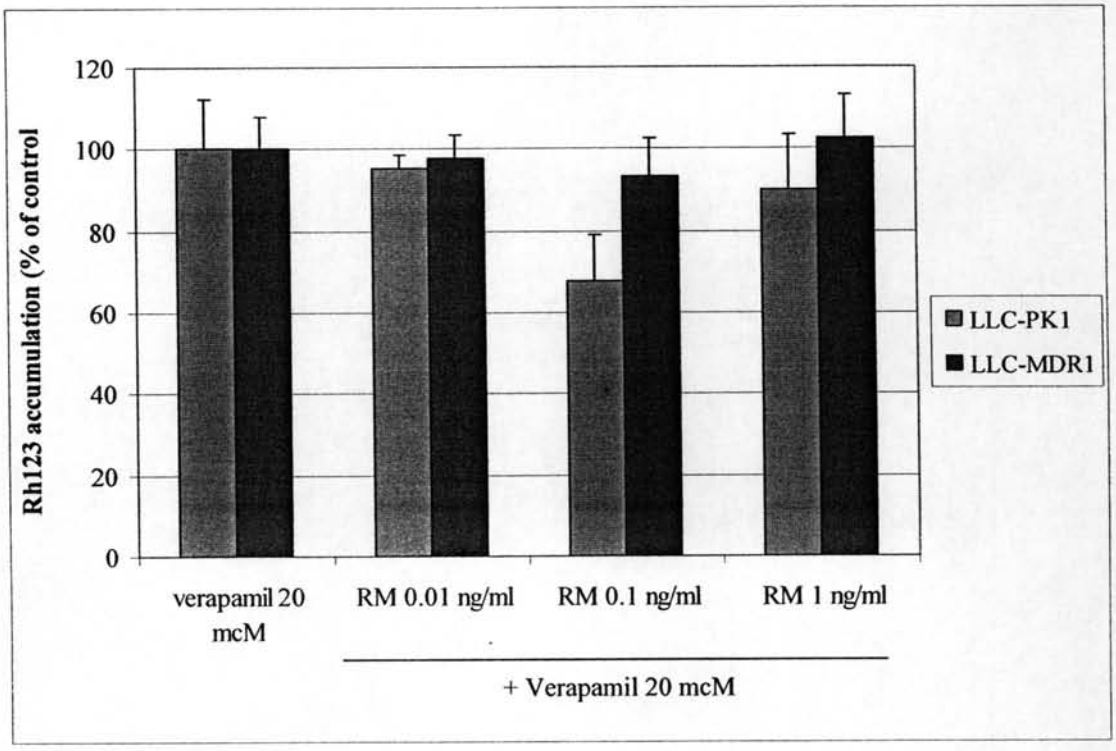

Figure 21 Effects of RM co-treatment with verapamil on the accumulation of Rh123 in LLC-PK_1 and LLC-MDR_1 cells. Rh123 accumulation was measured after incubation with RM 0.01, 0.1, and 1 ng/ml with or without 20 μM verapamil in PBS (A) and energy free medium (B) (bars means ± S.E. of 3-4 independent experiments). ** $P< 0.05$ vs verapamil.