POTENTIATION OF VINCRISTINE CYTOTOXICITY BY RUBIGINONE B1 AND PIPERAFIZINE A IN HUMAN MOSER AND K562 CELLS—MODE OF ACTION

MASAMI OGASAWARA, MASAMI HASEGAWA, YASUTARO HAMAGISHI*, HIDEO KAMEI and TOSHIKAZU OKI

Bristol-Myers Squibb Research Institute, 2-9-3 Shimo-meguro, Meguro-ku, Tokyo 153, Japan

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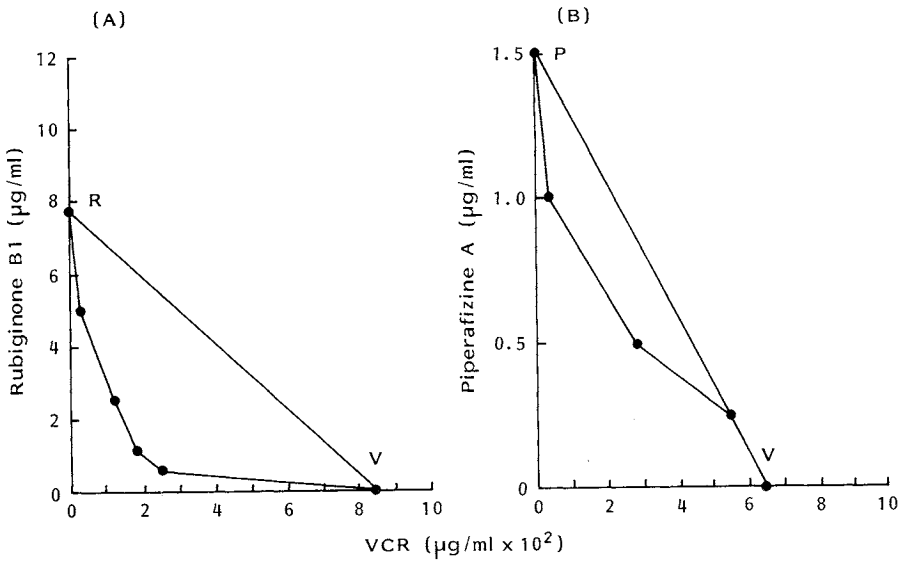
Rubiginones and piperafizines were isolated from the fermentation broths of *Streptomyces griseorubiginosus* Q144-2[1] and *Streptoverticillium aspergilloides* Q576-2[2], respectively, in our screening program for potentiators of vincristine (VCR)-induced cytotoxicity against human colorectal carcinoma Moser cells *in vitro*. Among their congeners rubiginone B1 and piperafizine A were determined to be the most potent in combination with VCR. However, their mechanism of action still remains to be unelucidated. This note deals with the modes of action of rubiginone B1 and piperafizine A in Moser cells as well as in doxorubicin (ADM)-resistant human leukemia K562 (kindly provided by Prof. T. TSURUO of Institute of Applied Microbiology, The University of Tokyo).

First of all, to examine whether their potentiations of VCR cytotoxicity are due to synergistic or additive effects, an isobole analysis was conducted in Moser cells according to the method of BERENBAUM[3]. The results are shown in Fig. 1. Each point in the figure represents the concentration at which 50% cell-growth inhibition was observed for the compound alone or in combination. If there is no effect between the compound and VCR in combination, all the points will lie on a straight line, R-V line in Fig. 1(A) and P-V line in Fig. 1(B). On the other hand, if they have synergistic effects, the points will be on a concave-up line. The converse will be expected when their effects are antagonistic. The results showed a typical concave-up line for both compounds and thereby suggested that their effects on VCR cytotoxicity in Moser cells are synergistic.

Fig. 1. IC_{50}-isobologram analysis of interaction between VCR and rubiginone B1(A) or piperafizine A(B) in Moser cells.



Moser cells were cultured with compound alone or with compound plus VCR in EAGLE's MEM (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% fetal calf serum and 60 μg/ml kanamycin and the cell viability was determined by DNA fluorimetric assay using Hoechst 33342 dye[9,10] after 3-day cultivation. VCR was purchased from Sigma Chemical Co. and used directly without purification. The purities of rubiginone B1 and piperafizine A used in this note were more than 95%. DMSO was used as a vehicle at the final concentration less than 1% in all experiments.

Table 1. Enhancement of VCR accumulation in Moser and K562/ADM cells by rubiginone B1, piperafizine A and verapamil.

Cell	Compound	Concentration (μg/ml)	Intracellular level of VCR[a] (dpm/5×10^5 cells)	Relative amount (%)
Moser	None		2,796	100
	Rubiginone B1	5	7,870	282
		10	7,806	279
	Piperafizine A	5	4,333	155
		10	5,056	181
	Verapamil	5	6,222	223
		10	6,634	237
K562/ADM	None		3,786	100
	Rubiginone B1	1	4,965	131
		5	7,519	198
		15	12,619	333
	Piperafizine A	1	3,892	103
		5	5,788	153
		20	14,095	372
	Verapamil	10	14,935	394

Experimental conditions and the culture medium for Moser cells are described in text and the legend of Fig. 1, respectively. K562/ADM cells were cultured in RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd.) supplemented with 5% fetal calf serum and 100 μg/ml kanamycin.

[a] Each figure is the mean of duplicate determinations.

To clarify their synergism in detail, the effects of two compounds on the cellular uptake of VCR were examined in Moser cells according to the method of SUGIMOTO *et al.*[4] with a minor modification. The trypsinized Moser cells (5×10^5) were incubated with 0.1 μCi [^{3}H]VCR (3 Ci/mmol; Amersham Co.) for 60 minutes at 37°C in 100 μl of the culture medium in the presence or absence of rubiginone B1 or piperafizine A and then the intracellular concentration of [^{3}H]VCR was determined as follows: The whole reaction mixture (100 μl) was transferred into a Eppendorf tube containing 200 μl of mixture of silicon oil SH550 and liquid paraffin (80 : 20). The cells were collected by centrifugation, then dissolved in 100 μl 1% SDS and their radioactivity was measured in a scintillator Biofluor (NEN Research Products Co.). The results are summarized in Table 1. Rubiginone B1 and piperafizine A markedly elevated the intracellular level of VCR. In the presence of 5 and 10 μg/ml rubiginone B1, the concentration of [^{3}H]VCR in Moser cells was about 3-fold more than in the absence of rubiginone B1. In the presence of 5 and 10 μg/ml piperafizine A, 1.6 and 1.8-fold increases in intracellular, accumulation of [^{3}H]VCR were observed, respectively. These results suggested that their synergistic potentiation of VCR cytotoxicity are due at least partly to elevation of intracellular accumulation of VCR in Moser cells.

How do they increase the intracellular level of VCR—by enhancement of cellular uptake or by inhibition of efflux of VCR once taken up into the cells or by both actions? To answer this question, their effects on the leaking process of VCR from Moser cells were examined. Moser cells (5×10^5) accumulated intracellularly about 3.7% of the added radioactivity after incubation with 0.1 μCi [^{3}H]VCR (3 Ci/mmol) for 60 minutes at 37°C in 100 μl of the culture medium in the presence of 10 μg/ml verapamil (Sigma Chemical Co.), a well-known inhibitor of ATP-dependent efflux-pump of the mammalian cells. By removing verapamil from the medium, the cells readily released the pooled [^{3}H]VCR into the medium with about 40% of the radioactivity released within 120 minutes at 37°C (Table 2). Interestingly, the efflux of VCR from the cells were markedly reduced in the presence of rubiginone B1 or piperafizine A. Five μg/ml of rubiginone B1 (15.7 μM) and piperafizine A (17.1 μM) caused 38.4 and 66.3% inhibitions, respectively, while verapamil itself prevented VCR efflux by 63.1% at 5 μg/ml (10.2 μM) (Table 2). These results suggested that the elevation by rubiginone B1 or piperafizine A of intracellular concentration of VCR in Moser cells resulted from reduction of efflux rate of VCR from the cells.

To examine this possibility further, the compounds were studied in ADM-resistant K562 cells

Table 2. Effects of rubiginone B1, piperafizine A and verapamil on intracellular retention of VCR by Moser and K562/ADM cells.

Cell	Compound	Concentration (μg/ml)	Intracellular retention of VCR[a]	Relative amount	Inhibition of VCR decrease (%)
Moser	None				
	0 minute		8,080[b]	100	
	120 minutes		4,638[b]	57	
	Rubiginone B1	5	5,959[b]	74	38.4
		10	6,582[b]	81	56.5
	Piperafizine A	5	6,921[b]	86	66.3
		10	6,460[b]	80	52.9
	Verapamil	5	6,810[b]	84	63.1
		10	7,508[b]	93	83.4
K562/ADM	None				
	0 minute		6,651[c]	100	
	120 minutes		2,043[c]	31	
	Rubiginone B1	5	3,209[c]	48	25.3
	Piperafizine A	5	3,947[c]	59	41.3
	Verapamil	5	5,308[c]	80	70.9

Experimental conditions are described in text.
[a] Each figure is the mean of duplicate determinations.
[b] dpm/5×10^5 cells, [c] dpm/2×10^5 cells.

(K562/ADM) which express higher activity of ATP-dependent efflux-pumping when compared with the ADM-sensitive parental cell line K562/S[5,6]. Rubiginone B1 and piperafizine A, as expected, markedly enhanced VCR accumulation in K562/ADM cells in a dose-dependent manner at concentrations from 1 to 20 μg/ml (Table 1) although they failed to exhibit such enhancement in K562/S cells even at 15 μg/ml (data not shown). In addition, as in Moser cells, at 5 μg/ml both compounds inhibited efflux of VCR once pooled into K562/ADM cells by verapamil (Table 2). These results apparently indicated that rubiginone B1 and piperafizine A potentiated VCR cytotoxicity by elevation of intracellular accumulation of VCR through inhibition of efflux of VCR after uptake into K562/ADM cells and suggested the possibility that they interact with the ATP-dependent efflux-pumping system in which *mdr* protein gp170 plays a major role[7,8]. On the other hand, rubiginone B1 and piperafizine A seem to have no significant effects on the cell-uptake process of VCR because they did not cause elevation of intracellular VCR accumulation in K562/S cells.

Based on these findings, we expect that rubiginone B1 and piperafizine A can serve as an efflux blocker like verapamil[8] in cancer chemotherapy to treat a variety of *mdr* protein-involved multi-drug resistant tumor cells in combination with a variety of cytotoxic agents such as VCR and doxorubicin.

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