

Tumour-specific Cytotoxicity and MDR-reversal Activity of Dihydropyridines

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Abstract. The ability of 41 1,4-diphenyl-1,4-dihydropyridine derivatives to inhibit the transport activity of P-glycoprotein were studied by flow cytometry in a multidrug-resistant human colon cancer cell line (COLO320) and in human *mdr1* gene-transfected mouse lymphoma cells (L 5178 Y). The cytotoxicities of these compounds were also examined against human normal and cancer cell lines. The majority of the tested compounds proved to be effective inhibitors of rhodamine 123 outward transport, but their cytotoxicities were not negligible. Some dihydropyridine derivatives displayed cytotoxic activity against four human oral tumour cell lines and against three normal human oral cell lines. There was no clear-cut relationship between the multidrug-resistance activity or cytotoxicity and the chemical structures of the compounds. New ring substituents could prevent the oxidation of the ring of the aromatic compound.

The use of anticancer drugs as part of the treatment strategy has greatly improved the overall prognosis of cancer (1). However, human cancer cells have developed various biological mechanisms that provide a defence against the cytotoxic attack by chemotherapeutic agents.

During the treatment of malignant tumours, the neoplastic cells are often found to be refractory to a variety of drugs with different structures and functions. This phenomenon has been termed multidrug-resistance (MDR). Various MDR phenotypes have been described (1-4).

One of the main causes of cellular drug resistance in cancer cells is the expression of a 170 kDa plasma membrane polypeptide known as the multidrug transporter or P-glycoprotein (P-gp), encoded by the MDR1 gene in humans (5, 6). P-gp is a transmembrane ATP-dependent protein that is able to pump cytotoxic compounds out of the cells, thereby decreasing drug accumulation and enabling the tumour cells to survive (7).

The search for new compounds to reverse MDR in tumours is an urgent task. The development of potential MDR-reversal agents has long been performed in many laboratories, and 1,4-dihydropyridine derivatives, such as Nifedipine and Nicardipine (8), are known to overcome MDR (9). However, the clinical use of such calcium-antagonists remains a therapeutic problem because of their strong vasodilator activity, and new drugs without calcium-antagonistic activity are required to overcome MDR in cancer patients.

In the past few years, much attention has been focused on the development of safer MDR inhibitors characterized by appropriate potency, selectivity and pharmacokinetics (10). 1,4-Dihydropyridines are promising compounds that play important roles in synthetic, therapeutic and bio-organic chemistry. Most of the 1,4-dihydropyridines exhibit pharmaceutical activity, due to the presence of the dihydropyridine ring. Because of their light sensitivity, especially in the solution phase, protection from light during

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their preparation has been recommended to prevent photo-oxidation of the pyridine ring (11).

The aim of the present study was to survey the effective modulators of MDR1 transporters among newly synthesized 1,4-diphenyl-1,4-dihydropyridine derivatives and other 1,4-dihydropyridines, using a multidrug-resistant human colon cancer cell line expressing MDR1/LRP and a human *mdr1* gene-transfected mouse lymphoma cells, and to investigate their tumour-specific cytotoxicity against human tumour and normal cell lines.

Materials and Methods

Compounds. Forty-one substituted 1,4-diphenyl-1,4-dihydropyridine derivatives and other 1,4-dihydropyridines were tested for MDR-reversal ability (Figures 1 and 2).

Materials. The following were used in cultures and assays: doxorubicin hydrochloride (Wako Pure Chem., Ind., Osaka, Japan); rhodamine 123 (R123) (Sigma, St. Louis, MO, USA); verapamil (EGIS, Hungarian Pharmaceutical Company, Budapest, Hungary); Dulbecco's modified Eagle medium (DMEM); RPMI1640 medium (Gibco BRL, Gland Island, NY, USA); fetal bovine serum (FBS) (JRH Biosci, Lenexa, KS, USA); horse serum (Gibco, Auckland, New Zealand); PBS (phosphate-buffered saline); MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma). Stock solutions of rhodamine 123 and verapamil were prepared in water. All dihydropyridine derivatives were dissolved in DMSO (dimethyl sulfoxide).

Cell cultures. Normal human gingival fibroblast (HGF), pulp cells (HPC) and periodontal ligament fibroblast (HPLF) were obtained from human periodontal tissue after informed consent, according to the guidelines of the Meikai University Ethical Committee (No. 0206). Since normal cells have a limited life span, cells at 6-8 population doubling level were used for the present study.

Human squamous cell carcinoma cell lines (HSC-2, HSC-3 and HSC-4) were supplied by Prof. Masao Nagumo, Showa University, Japan. Human promyleocyte leukaemia (HL-60) cells were supplied by Prof. Kazuyasu Nakaya, Showa University. HL-60 cells were maintained at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated FBS in a humidified 5% CO₂ atmosphere. Other cells were cultured as monolayer cultures at 37°C in DMEM supplemented with 10% heat-inactivated FBS in a humidified 5% CO₂ atmosphere, and subcultured by trypsinization.

L 5178 Y mouse T-cell lymphoma cells (obtained from Prof. Gottesmann, NCI and FDA, USA) were transfected with pHA MDR1/A retrovirus, as previously described (12). *mdr1* gene-expressing cell lines were selected by culturing the infected cells with 60 ng/ml colchicine to maintain the expression of the MDR phenotype. L 5178 Y (parent) mouse T-cell lymphoma cells and the human *mdr1* gene-transfected subline were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics.

Human colon cancer cells (COLO320) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM Na-pyruvate and 100 mM Hepes. All adherent cells were detached with 0.25% trypsin and 0.02% EDTA for 5 min at 37°C.

R123 uptake assay. The cells were adjusted to a density of 2x10⁶/ml, resuspended in serum-free McCoy's 5A medium and distributed in 0.5-ml aliquots into Eppendorf centrifuge tubes. The tested compounds were added at various concentrations in different volumes (2.0-20.0 µl) of the 1.0 mg/ml stock solutions, and the samples were incubated for 10 min at room temperature (20°C). Ten µl R123 indicator was then added and the samples were incubated for a further 20 min at 37°C. After washing twice and re-suspension in 0.5 ml PBS, the fluorescence of the cell population was measured by flow cytometry, using a Beckton Dickinson FACScan instrument (cell sorter, Oxford, UK). Verapamil was used as a positive control. The percentage mean fluorescence intensity was calculated for the treated MDR and parental cell lines as compared with the untreated cells. An activity ratio, FAR, was calculated using Equation 1 (13), on the basis of the measured fluorescence values:

$$FAR = \frac{MDR\ treated/MDR\ control}{parental\ treated/parental\ control} \quad (\text{Equation } 1)$$

Cytotoxicities of the compounds on normal and cancer cell lines. Near confluent cells were incubated for 24 h with or without various concentrations of each compound, and the relative viable cell number (absorbance at 540 nm of the MTT-stained cell lysate) was determined using the MTT method. The MTT assay is based on the reduction of MTT, by mitochondrial dehydrogenase of metabolically active cells, to a blue formazan, which can be measured spectrophotometrically. The viability of HL-60 cells was determined using trypan blue exclusion. The 50% cytotoxic concentration (CC₅₀) was determined from the dose-response curve. Each value is the mean of duplicate determinations. Variation between two samples was usually less than 10%.

Tumour specificity (TS) was determined using Equation 2:

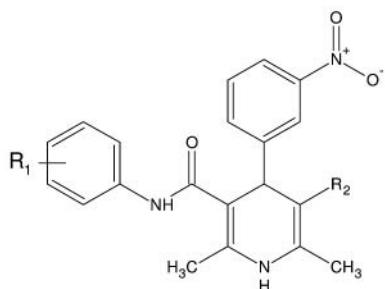
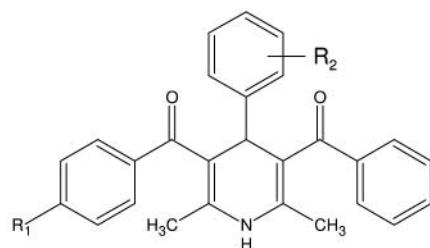
$$TS = [CC_{50}(\text{HGF}) + CC_{50}(\text{HPLF}) + CC_{50}(\text{HPC}) / CC_{50}(\text{HSC-2}) + CC_{50}(\text{HSC-3}) + CC_{50}(\text{HSC-4}) + CC_{50}(\text{HL-60})] \times 4/3 \quad (\text{Equation } 2)$$

Results

Reversal of MDR in tumour cells. The effects of the forty-one substituted 1,4-diphenyl-1,4-dihydropyridine derivatives on MDR reversal were investigated by treating the mouse lymphoma L 5178 Y cells transfected with the human *mdr1* gene and the human colon cancer COLO320 cell line with different concentrations of each compound. The experimental data are listed in Tables I, II and III.

Some dihydropyridines at the higher concentration of 40 µg/ml used were found to be toxic: the cell size and the intracellular structures of the cells were changed during the short-term experiments (data not shown). A non-toxic concentration (0.4 µg/ml) was used for experiments on R123 accumulation. At some representative samples (at therapeutically rational concentrations) the results were statistically evaluated (Table IV).

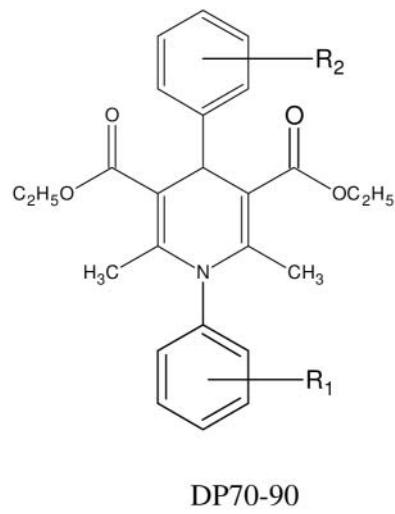
The majority of the test compounds were shown to enhance the drug retention in the cells by inhibiting the



Compound	R ¹	R ²
DL1	4-SCH ₃	3-OPh
DL2	4-SCH ₃	2-Cl
DL3	4-SCH ₃	3-Cl
DL4	4-OCH ₃	3-OPh
DL5	4-CH ₃	3-OPh
DL6	2-Cl	CN
DL7	2-Cl	COOCH ₃
DL8	2-Cl	COOC ₂ H ₅
DL9	2-Cl	COCH ₃
DL10	2-CH ₃	CN
DL11	2-CH ₃	COOCH ₃
DL12	2-OCH ₃	CN
DL13	2-OCH ₃	COOCH ₃
DL14	2-OCH ₃	COOC ₂ H ₅
DL15	2-OCH ₄	COCH ₃
DL16	2,4-diCH ₃	COOCH ₃
DL17	H	COOCH ₃
DL18	H	CN
DL19	2,3-diCH ₃	COOCH ₃
DL20	2,3-diCH ₃	CN

Figure 1. Chemical structure of dihydropyridine derivatives (DL1-20).

efflux-pump activity. Among them, DL8-10, DL12, DL13 and DL15 were found to be the most effective MDR modulators. These derivatives caused a dose-dependent



Compound	R ¹	R ²
DP70	H	H
DP71	H	4-SCH ₃
DP72	H	4-OCH ₃
DP73	H	3-NO ₂
DP74	H	4-Cl
DP75	3-CH ₃	4-SCH ₃
DP76	3-CH ₃	3-NO ₂
DP77	3-CH ₃	4-Cl
DP78	4-OCH ₃	4-Cl
DP79	4-Cl	4-Cl
DP80	4-OCH ₃	3-NO ₂
DP81	4-Cl	3-NO ₂
DP82	4-OCH ₃	H
DP83	4-OCH ₃	4-SCH ₃
DP84	4-OCH ₃	4-OCH ₃
DP85	4-Cl	4-SCH ₃
DP86	H	3-OCH ₃
DP87	2-CH ₃	4-SCH ₃
DP88	H	4-OH, 3-OCH ₃
DP89	4-OCH ₃	4-OH, 3-OCH ₃
DP90	3-CH ₃	4-OH, 3-OCH ₃

Figure 2. Chemical structure of dihydropyridine derivatives (DP70-90).

inhibition of the MDR P-gp. On the other hand, certain compounds, such as DL7, DL8, DL20 and DP85, were ineffective in both cell lines.

The dihydropyridines were less effective in COLO320 cells: a majority of the compounds displayed lower FAR values in COLO320 cells than in L 5178 Y cells; exceptions were DL7, DL11-14, DL16, DL19, DL20, DP70, DP71, DP84, DP87 and DP90.

Table I. Effect of dihydropyridines (DL1-5) on the MDR of mouse lymphoma cell line (L 5178 Y) and human colon cancer cell line COLO320.

Compound	Fluorescence activity ratios (FARs) at different concentrations* ($\mu\text{g/ml}$)					
	L 5178 Y			COLO320		
	0.4	4	40	0.4	4	40
DL1	9.53	24.50	12.09	4.26	12.52	18.32
DL2	11.01	47.34	33.88	6.33	21.89	26.08
DL3	20.80	49.81	30.27	4.13	27.79	24.51
DL4	16.43	45.51	28.70	6.69	23.34	28.36
DL5	5.33	20.22	10.31	2.57	14.38	17.81

*The FARs of the reference compound verapamil were 12.77 and 14.69 for L 5178 Y and COLO320 cells, respectively, when a verapamil concentration of 10 $\mu\text{g/ml}$ was used.

Table II. Reversal of MDR by dihydropyridines (DL6-20) on mouse lymphoma cell line L 5178 Y and human colon cancer cell line COLO320.

Compound	Fluorescence activity ratios (FARs) at different concentrations* ($\mu\text{g/ml}$)					
	L 5178 Y			COLO320		
	0.4	4	40	0.4	4	40
DL6		6.42	53.32		4.66	46.69
DL7		0.84	0.67		2.15	2.23
DL8	10.00	115.67	178.03	6.59	60.27	76.13
DL9		54.49	104.58		40.63	69.04
DL10		4.99	139.61		4.69	140.12
DL11		2.34	8.89		2.77	12.82
DL12		7.50	77.03		3.34	109.35
DL13		12.64	91.07		18.18	144.09
DL14		2.07	6.82		4.56	14.81
DL15	5.69	144.00	158.00	2.5	44.45	64.82
DL16		2.48	4.57		7.43	27.29
DL17		2.23	13.03		1.53	9.46
DL18		1.22	1.61		1.24	2.98
DL19		1.40	8.57		2.88	24.35
DL20		1.28	2.01		2.15	4.92

*The FARs of the reference compound verapamil were 12.77 and 14.69 for L 5178 Y and COLO320 cells, respectively, when a verapamil concentration of 10 $\mu\text{g/ml}$ was used.

Cytotoxicities of the compounds on normal and cancer cell lines. The cytotoxicities of these compounds were also examined on normal and cancer cell lines. All of the compounds were evaluated against the four neoplastic cell lines HSC-2, HSC-3, HSC-4 and HL-60, and against the non-malignant lines HGF, HPC and HPLF (Tables V and VI).

Table III. Reversal of MDR by dihydropyridines (DP70-90) on mouse lymphoma cell line L 5178 Y and human colon cancer cell line COLO320.

Compound	Fluorescence activity ratios (FARs) at different concentrations* ($\mu\text{g/ml}$)					
	L 5178 Y			COLO320		
	0.4	4	40	0.4	4	40
DP70	5.09	40.00	38.89	3.56	33.26	40.33
DP71	2.24	8.01	13.88	1.43	10.33	14.86
DP72	2.67	17.38	27.99	2.10	15.78	20.05
DP73	7.67	34.00	40.21	1.80	18.97	17.72
DP74	1.3	8.22	23.48	1.49	7.75	10.48
DP75	2.67	9.45	8.94	1.95	4.23	8.05
DP76	4.65	13.95	16.05	1.58	9.78	10.77
DP77		4.3	14.68		8.45	9.59
DP78	8.76	25.91	30.66	1.89	18.08	24.67
DP79		3.57	5.62		5.78	7.13
DP80	16.93	41.62	40.91	2.55	36.22	27.8
DP81	6.22	27.01	24.69	0.98	12.84	20.83
DP82	8.03	52.08	19.73	2.14	31.04	36.73
DP83		9.71	16.58		10.68	15.39
DP84	7.49	21.46	7.27	5.81	33.15	25.61
DP85		0.99	0.85		1.69	1.30
DP86		1.45	1.14		4.87	10.77
DP87		2.70	4.59		12.14	22.33
DP88	4.73	13.39	2.40	5.03	18.79	17.03
DP89	1.07	50.14	8.14	10.73	36.38	29.37
DP90	1.05	13.91	1.86	4.23	18.63	9.15

* The FARs of the reference compound verapamil were 12.77 and 14.69 for L 5178 Y and COLO320 cells, respectively, when a verapamil concentration of 10 $\mu\text{g/ml}$ was used.

Of the 1,4-dihydropyridine derivatives DL1-20, DL14 and DL16 exhibited the highest cytotoxic activities against the human tumour cells HSC-2, HSC-3, HSC-4 and HL-60, followed by DL6, DL19, DL20, DL10, DL13 and DL11. As may be seen in Table V, these four human tumour cell lines showed similar sensitivities to these compounds. On the other hand, three normal cells (HGF, HPC and HPLF) were relatively resistant to these derivatives. In order to assess the preferential toxicity for malignant cells, TS was calculated for each compound.

The TS values indicated that DP80 (TS>2.8) and DP88 (TS>2.7) had the highest tumour-specific cytotoxic activities among the DP group of dihydropyridines, although these TS values were much lower than that of doxorubicin, an anthracycline antibiotic used as positive control (TS>14.6) (Tables V and VI). On the other hand, DP70, DP81, DP82 and DP90 had no cytotoxic activity when tested using the cytotoxic activity assay.

The normal human oral cells exhibited a higher resistance to all of these compounds as compared with the oral tumour cell lines, which resulted in an elevation of the TS of some derivatives.

Table IV. Statistical analysis of FAR values of some dihydropyridines measured on mouse lymphoma L 5178 Y and human colon cancer COLO320 cell lines.

Compound	Fluorescence activity ratios ($\mu\text{g/ml}$)*	
	L 5178 Y	COLO320
Compound	4	4
DL1	24.50 \pm 3.463	12.52 \pm 0.244
DL2	47.34 \pm 4.705	21.89 \pm 0.998
DL3	49.81 \pm 4.657	27.79 \pm 1.767
DL4	45.51 \pm 5.289	23.34 \pm 3.179
DL5	20.22 \pm 3.012	14.38 \pm 0.688
DL8	115.67 \pm 2.812	60.27 \pm 9.074
DL15	144.00 \pm 9.549	44.45 \pm 3.706
DP70	40.00 \pm 7.042	33.26 \pm 3.876
DP71	8.01 \pm 0.996	10.33 \pm 1.435
DP72	17.38 \pm 3.955	15.78 \pm 1.954
DP73	34.00 \pm 4.143	18.97 \pm 0.597
DP74	8.22 \pm 2.028	7.75 \pm 0.653
DP75	9.45 \pm 0.633	4.23 \pm 0.770
DP76	13.95 \pm 0.189	9.78 \pm 0.566
DP78	25.91 \pm 0.009	18.08 \pm 0.399
DP80	41.62 \pm 1.042	36.22 \pm 2.503
DP81	27.01 \pm 1.446	12.84 \pm 0.569
DP82	52.08 \pm 4.922	31.04 \pm 1.161
DP84	21.46 \pm 5.160	33.15 \pm 1.716
DP88	13.39 \pm 2.235	18.79 \pm 2.804
DP89	50.14 \pm 8.165	36.38 \pm 0.920
DP90	13.91 \pm 3.756	18.63 \pm 0.164

*Figures are means \pm s.e.m from parallel experiments (n=3-6).

Discussion

The resistance of cancer cells to multiple chemotherapeutic agents remains a major obstacle in cancer therapy. Various compounds have been investigated in different laboratories for the reversal of MDR, including synthetic (14-16) and naturally-occurring, plant-derived compounds (17-20).

Several mechanisms are thought to be involved in drug resistance, including those associated with apoptosis, drug transport and detoxification (1, 3, 21).

Our study was focused on the inhibition of MDR through inhibition of the MDR P-gp in a human *mdrl* gene-transfected mouse lymphoma cell line and in human colon cancer cells by 1,4-dihydropyridine derivatives.

1,4-Dihydropyridines are well known as Ca^{2+} channel blockers and as drugs for the treatment of cardiovascular diseases, including hypertension (22). The 1,4-dihydropyridine heterocyclic ring is a common feature of various bioactive compounds, such as vasodilator, bronchodilator, anti-atherosclerosis, neuroprotective, platelet anti-aggregatory, anti-ischaemic, anti-atherosclerotic and antitumour agents (23-27), and more recently, antitubercular agents (28, 29) and MDR modulators (8, 10, 30, 31).

In this report, we confirmed the MDR-reversal activity of dihydropyridine derivatives (Tables I-III). DL8-10, DL12, DL13 and DL15 displayed a marked inhibition of the MDR of mouse lymphoma cells. The same compounds were also the most effective in elevating drug accumulation in COLO320 cells, but the FAR values in each case were lower than those for the lymphoma cells (except for compounds DL10, DL12 and DL13).

The reason for the lower MDR-reversing effects of the tested compounds in COLO320 cells than in L 5178 Y cells could be the lower P-gp overexpression in COLO320 cells, which was revealed by immunohistology. Human *mdrl* gene-transfected mouse lymphoma cells overexpress the P-gp 170 protein responsible for the drug efflux (6).

The results indicated that the increase in drug accumulation is dose-dependent and the change in maximum fluorescence intensity is characteristic of the great majority of the treated cells.

At higher concentration (40 $\mu\text{g/ml}$), most cells died due to the toxicity of the chemicals. It was shown that at low doses most of the derivatives were able to increase the R123 accumulation without toxic effects.

When the structure-activity relationship was analysed, we could not find a strong structure-activity relationship between the chemical structures and the MDR-reversal activity of the compounds studied.

By using a total of 7 human cell lines, including four tumour cell lines and three normal cell lines, we also confirmed the tumour specificity of some compounds. DL14 had the most noteworthy tumour-specific cytotoxic action, with a TS of 5.9. The dihydropyridine derivatives were more cytotoxic against four oral tumour cell lines as compared with normal cell lines (Tables V and VI). However, the tumour cell lines displayed considerable variation in sensitivity. HL-60 was the most sensitive. On the other hand, the normal cells were all comparable in sensitivity.

In summary, this study has demonstrated that several 1,4-dihydropyridine derivatives exert MDR-reversal activity and tumour-specific cytotoxicity. In connection with their possible future application, especially for cancer therapy, further studies are required to determine the type of cell death induced (apoptosis, autophagy or necrosis) (32) and its mechanism.

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Table V. Cytotoxic activities of 1,4-dihydropyridine derivatives (DL1-20).

Compound	50% cytotoxic concentration (CC ₅₀ µM)*							
	Human tumour cell				Normal cell			TS ^a =CC ₅₀ (normal)/CC ₅₀ (tumour)
	HSC-2	HSC-3	HSC-4	HL-60	HGF	HPC	HPLF	
DL1	>200	>200	>200	96	>200	>200	>200	><1.1
DL2	>200	>200	>200	20	>200	>200	>200	><1.3
DL3	>200	>200	>200	2.8	>200	>200	>200	><1.3
DL4	>200	>200	>200	35	>200	>200	>200	><1.3
DL5	>200	>200	174	17	>200	>200	>200	><1.4
DL6	90	97	78	24	>200	>200	>200	2.8
DL7	>200	>200	>200	>200	>200	>200	>200	><1.0
DL8	>200	>200	>200	10	>200	>200	>200	><1.3
DL9	>200	>200	>200	17	>200	>200	>200	><1.3
DL10	>200	73	55	17	87	>200	143	><1.7
DL11	106	>200	83	24	175	>200	174	><1.8
DL12	>200	>200	93	20	>200	>200	>200	><1.6
DL13	97	75	>200	19	>200	>200	>200	><2.0
DL14	50	41	31	9.4	>200	>200	175	5.9
DL15	>200	>200	95	31	>200	>200	>200	><1.5
DL16	25	46	21	15	41	92	42	2.2
DL17	>200	174	165	41	200	>200	179	><1.3
DL18	>200	191	>200	35	>200	>200	>200	><1.3
DL19	85	94	93	20	96	>200	>200	>2.3
DL20	66	108	111	28	64	71	73	0.9
Doxorubicin	0.38	2.8	0.77	0.18	>20	>20	>20	>14.6

*Each value is the mean of duplicate determinations. Variation between two samples was usually less than 10%.

Table VI. Cytotoxic activities of 1,4-dihydropyridine derivatives (DP70-90).

Compound	50% cytotoxic concentration (CC ₅₀ µM)*							
	Human tumour cell				Normal cell			TS ^a =CC ₅₀ (normal)/CC ₅₀ (tumour)
	HSC-2	HSC-3	HSC-4	HL-60	HGF	HPC	HPLF	
DP70	>500	>500	>500	>500	>500	>500	>500	><1.0
DP71	>500	177	>500	239	>500	>500	>500	><1.4
DP72	>500	284	500	103	>500	>500	>500	><1.4
DP73	>500	>500	>500	44	>500	>500	>500	><1.3
DP74	>500	>500	>500	56	>500	>500	>500	><1.3
DP75	>500	191	>500	500	>500	>500	>500	><1.2
DP76	>500	>500	>500	152	>500	>500	>500	><1.2
DP77	>500	>500	>500	192	>500	>500	>500	><1.2
DP78	>500	>500	>500	49	>500	>500	>500	><1.3
DP79	>500	>500	250	>500	>500	>500	>500	><1.2
DP80	223	122	150	208	471	>500	>500	>2.8
DP81	>500	>500	>500	>500	>500	>500	>500	><1.0
DP82	>500	>500	>500	>500	>500	>500	>500	><1.0
DP83	>500	>500	>500	125	>500	>500	>500	><1.2
DP84	>500	211	>500	89	>500	>500	>500	><1.5
DP85	>500	>500	219	105	>500	>500	>500	><1.5
DP86	>500	>500	>500	238	>500	>500	>500	><1.2
DP87	>500	>500	>500	105	>500	>500	>500	><1.2
DP88	145	135	104	313	>500	440	472	>2.7
DP89	88	77	171	397	168	380	250	1.5
DP90	31	35	40	>500	63	167	125	<0.8

*Each value is the mean of duplicate determinations. Variation between two samples was usually less than 10%.

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