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Selection of three out of 24 anti-cancer agents in poorly-differentiated gastric cancer cell lines, evaluated by the AUC/ΔIC_{50} ratio

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The purpose of this study was to screen 24 anti-cancer drugs, either in use or in clinical study, using four cell lines, all of which originated from poorly-differentiated gastric cancers. The MTT assay was used at 1, 6, 24 or 72 h exposure times as the chemosensitivity test. We also examined P-glycoprotein expression, *mdr-1* gene amplification and the modifier effect of verapamil. All four cell lines generally showed the same chemosensitivity pattern, while GCIY cells showed *mdr-1* gene amplification and P-glycoprotein expression, and KATOIII cells showed the multidrug resistant pattern without P-glycoprotein expression. Both cell lines acquired higher chemosensitivity after verapamil addition. All IC_{50} data (with or without verapamil) were multiplied by exposure time (ΔIC_{50}) and compared with the clinical 'area under the concentration curve (AUC)'. SN-38 with/without verapamil, cisplatin with verapamil and pirarubicin with/without verapamil seemed to be the best candidates for poorly-differentiated gastric cancer chemotherapy. Plant alkaloids could also be candidates. With further experiments, we may be able to deduce commonly effective chemotherapy for poorly-differentiated gastric cancer from these drugs.

Key words: Chemosensitivity test, cisplatin, gastric cancer cells, pirarubicin, SN-38, verapamil.

Introduction

Cancer chemotherapies have provided curative treatments for some 12 categories of cancer.[1] In these diseases, certain combinations were specially effective in specific cancers. Therefore, we can hypothesize that if an effective chemotherapy exists for a certain kind of cancer, those drugs would be commonly effective in the same pathological type of cancer. In other words, drugs which are effective in several cases of the same pathological types of cancer should be generally effective on all of the same pathological types of cancer. On the basis of this hypothesis, in the National Cancer Institute (NCI) in the US, a panel of 60 human tumor cell lines has been organized into subpanels representing leukemia, melanoma, and cancers of lung, colon, kidney, ovary and central nervous system in order to screen new compounds.[2,3] This NCI system, however, does not include cell lines originating from gastric cancers, especially from poorly-differentiated ones.

On the other hand, chemotherapy is the most promising approach for the cure of advanced gastric cancers, especially poorly-differentiated types with disseminated metastases in the abdominal cavity. However, it has not been successful so far.[4] To improve chemotherapy for poorly-differentiated gastric cancer, it may be useful to screen the drugs which are already in commercial use or in phase studies using cells originating from the gastric cancer. These drugs have usually been screened using cells derived from hematological tumors, but not from gastric cancer. In other words, knowledge of the *in vitro* sensitivity distribution of gastric cancer cells to the existing drugs can give us guidance to establish improved chemotherapy. In this study, using a panel of four cell lines (including the GCIY cell line which was established by our group[5]), all of which originated from poorly-differentiated adenocarcinoma of stomach, we intended to select effective drugs out of 24 anti-cancer drugs which already exist in commercial use or in clinical study.

In the selection of effective drugs, resistance, especially classical multidrug resistance (MDR), is an important problem. Although normal gastric mucosa does not express P-glycoprotein, which is the product of the MDR gene (*mdr-1*), overexpression of P-glycoprotein on the xenografts of human gastric cancers was reported.[6] If P-glycoprotein is expressed on the surface of gastric cancer cells, some

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chemicals,[7] especially verapamil,[8] would be expected to enhance the cytotoxicity of MDR-related anti-cancer drugs. There is, however, no report about overexpression of P-glycoprotein in gastric cancer cell lines. Therefore, we also examined P-glycoprotein expression on the cell lines and the modifier effect of verapamil on chemosensitivity.

Materials and methods

Cell lines

The GCIY cell line was established by continuous cultivation after 40 passages of the cells in the ascites from a 39 year old female patient with scirrhous type gastric cancer.[5] The histological examination of the primary cell culture revealed GCIY cells were classified as poorly differentiated adenocarcinoma and also as adenomatous epithelia by Papanicolaou staining. GCIY cells were of moderate size, PAS positive and showed a monolayer arrangement with a mean population doubling time of 55 h. The transplanted cells reproduced the primary tumor in nude mice. Tumor related substrates, such as CA19-9, CEA and α-fetoprotein, were detected in the culture medium of confluent monolayer GCIY cells.

Three other gastric cancer cell lines used in the present study were as follows; JR-St cell line kindly provided by Dr Terano (University of Tokyo),[9] STKM-1 cell line from Dr Yanoma (Kanagawa Cancer Center)[10] and KATOIII from Dr Nishida (University of Tsukuba).[11] These cells were well-established cell lines of scirrhous type poorly-differentiated adenocarcinoma as reported previously. Cancer related antigens, such as CA19-9 and CEA, were also detected in the culture medium of those cells. As negative and positive controls for multi-drug resistant cells, K562 and K562/A cell lines were used in the present study.[12] The K562/A cell line was isolated by continuous exposure of K562 cells to vincristine for more than 1 month followed by treatment with doxorubicin. K562/A cells showed an amplification of the *mdr-1* gene and increased expression of P-glycoprotein.

Cells were cultivated in Eagle's MEM (Nissui, Tokyo, Japan) supplemented with sodium bicarbonate, glutamic acid and 15% fetal calf serum (Flow, Irvine, UK).

Drugs

Twenty-four anti-cancer drugs (Table 1) were employed in this study. All drugs were kindly provided from the company listed. Of 24 drugs examined, 10 drugs from Act-D to VDS on Table 1 and etoposide are drugs associated with MDR (MDR drugs). Verapamil, a calcium channel antagonist, was purchased from Eisai (Tokyo, Japan).

Chemosensitivity test

For the measurement of drug sensitivity, the MTT assay[13] with slight modifications[14] was performed. Briefly, cell suspensions were prepared and dispensed in 100 μl aliquots into 96-well tissue culture plates at a concentration yielding 1×10^4 cells/well. After 3 days in culture, an anti-cancer drug was added to the wells at a concentration of 0.01–10 μg/ml in 100 μl of culture medium. 5-FU and etoposide were used at a concentration of 0.1–100 μg/ml culture medium in consideration of the clinical dosage of these drugs. L-ASP was used at a concentration of 0.01–10 U/ml. After exposure to the drugs (1, 6, 24 or 72 h), cells were washed three times with phosphate buffered saline and recultured in a drug-free media until the fourth day. A volume of 20 μl MTT solution was added to each well and incubated at 37°C for 4 h. Finally the crystalline deposit of MTT formazan was dissolved in 200 μl dimethylsulfoxide and optical density at 540 nm (OD_{540}) was determined. The growth inhibition rates were calculated from the ratios of the OD_{540} to the OD_{540} of the control.

In addition, verapamil was used as a modifier. Verapamil was added at a concentration of 1 μg/ml with anti-cancer drugs. Verapamil showed no cytotoxicity to all cell lines at a concentration from 0.01 to 10 μg/ml (data not shown).

P-glycoprotein and *mdr-1* in tumor cells

An immunofluorescence assay was used to detect the levels of P-glycoprotein in the cancer cell lines. In brief, 1×10^6 cells were incubated for 20 min with anti-P glycoprotein monoclonal antibody (MRK16; kindly provided by Dr Tsuruo, University of Tokyo).[15] Subsequently, the cells were reacted with FITC-conjugated anti-mouse immunoglobulin antibody for 20 min and resuspended in 1% paraformaldehyde. The cells were analyzed with a FACScan flow cytometer according to the instructions of the company (Becton Dickinson, Mountain View, CA).

DNA and RNA were extracted by ultracentrifugation with an RNA extraction kit featuring cesium

Table 1. Anti-cancer drugs

Name of drugs	Abbreviations	Company	Symbol in Fig. 1
Antitumor antibiotics			
neocarcinostatin	NCS	Yamanouchi, Tokyo	A
bleomycin hydrochloride	BLM	Nippon Kayaku, Tokyo	B
pepleomycin sulfate	PEP	Nippon Kayaku, Tokyo	C
micomycin C	MMC	Kyowa, Tokyo	D
actinomycin-D	Act-D	Banyu, Tokyo	E
Anthracyclines and anthraquinone			
doxorubicin hydrochloride	ADM	Kyowa, Tokyo	F
pirarubicin	THP-ADM	Nippon Kayaku, Tokyo	G
epirubicin hydrochloride	FAB	Kyowa, Tokyo	H
daunorubicin hydrochloride	DM	Meiji, Tokyo	I
mitoxantrone hyrochloride	MIT	Lederle (Japan), Tokyo	J
Plant alkaloids			
vincristine sulfate	VCR	Shionogi, Osaka	K
vinblastin sulfate	VBL	Shionogi, Osaka	L
vindesine sulfate	VDS	Shionogi, Osaka	M
SN-38[a]	SN-38	Daiichi, Tokyo	N
etoposide	etoposide	Nippon Kayaku, Tokyo	O
Alkylating agents			
cyclophosphamide[b]	CPM	Shionogi, Tokyo	P
Ifosfamide[b]	IFM	Shionogi, Tokyo	Q
ranimustine	MCNU	Tanabe, Tokyo	R
Platinum analogs			
cisplatin	cisplatin	Nippon Kayaku, Tokyo	S
carboplatin	CBDCA	Bristol-Myers Squibb, Tokyo	T
Antimetabolites			
5-fluorouracil	5-FU	Kyowa, Tokyo	U
methotrexate	MTX	Lederle (Japan), Tokyo	V
cytarabine	ara-C	Nippon Shinyaku, Tokyo	W
Enzyme			
L-asparaginase	L-ASP	Kyowa, Tokyo	X

[a] Active form of CPT 11.
[b] Active forms of CPM and IFM (4-hydroproxy cyclophosphamide and 4-hydroperoxy ifosfamide, respectively) were used.

trifluoroacetate (Pharmacia LKB Biotechnology). Total RNA (2 μg) was electrophoresed and blotted onto nylon membrane (OptiBLOT™; IBI). The membranes were hybridized with ^{32}P-labeled *mdr-1* (kindly provided by Dr Tsuruo, University of Tokyo)[6] as described previously.[16] Total DNA (10 μg/ml) was digested by 5 U/μg of the restriction enzyme *Bam*HI or *Eco*RI at 37° C for 2 h. The digested DNA was electrophoresed and blotted onto nylon membrane (Zetabind, CUNO, CT, USA). The membranes were hybridized with ^{32}P-labeled probe overnight and exposed to X-ray films.

Oligonucleotides were designed from the consensus sequence flanking the 1 kb homologous region of the human *mdr-1* gene.[17] The sequence and location of the sense and antisense primers used in the present study were as follows; the sense primer (26-mer) 5′-CTATTGGACAAGTACTCACTGTATTC-3′; the antisense primer (25-mer) 5′-GACATTTC-CAAGGCATCAATTTCAC-3′. Oligonucleotides were synthesized at INTER TECH (Tokyo, Japan) using DNA synthesizer model 394 (Applied Biosystems). The conditions for polymerase chain reaction were the same as previously reported.[18] The amplified DNA fragments were visualized by agarose gel electrophoresis for the confirmation of the fragment sizes. The amplified DNA fragments were cut out of 1% agarose gel (Pharmacia LKB Biotechnology) and further purified by using Ultra Free C3HV filter (Milipore). These fragments were subsequently subcloned into the TA Cloning™ vector, pCR™ 1000/*Hph*I vector (Invitrogen) and sequenced by dsDNA sequencing System (Gibco BRL).

Data analysis

Survival ratios of each cell line were obtained after a certain interval of exposure to anti-cancer drugs. From each experiment, the concentration of anti-cancer drug to cause 50% inhibition (IC_{50}) was obtained. Then the IC_{50} was multiplied by exposure time to obtain ΔIC_{50} and the lowest ΔIC_{50} was se-

lected in each cell line for each drug to calculate the ΔIC_{50} score. In the case that IC_{50} could not be obtained even under 72 h exposure, a value of 10 μg/ml multiplied by 72 h was used as the lowest IC_{50}. The ΔIC_{50} score of every drug was calculated as follows:

$$\Delta IC_{50}\ \text{score} = -[\log(\text{GCIY}\ \Delta IC_{50}) + \log(\text{JR-St}\ \Delta IC_{50}) + \log(\text{KATOIII}\ \Delta IC_{50}) + \log(\text{STKM-1}\ \Delta IC_{50})]$$

Since the effects of almost all anti-cancer drugs are dependent on AUC, especially the so-called type 1 (cell cycle non-specific) anti-cancer drugs, the results of this study were also assessed by comparing ΔIC_{50} to the clinical AUC using the value of the AUC divided by ΔIC_{50}. Table 2 shows the AUC values which we used. Principally, we used the AUC which was obtained by bolus intravenous injection. We selected the highest value in each cell line in each drug and calculated the $AUC/\Delta IC_{50}$ score as follows (in the case that the $AUC/\Delta IC_{50}$ could not be obtained because of the lack of ΔIC_{50}, 0.008 was used as $AUC/\Delta IC_{50}$ to calculate the score):

$$AUC/\Delta IC_{50}\ \text{score} = [\log(\text{GCIY AUC}/\Delta IC_{50}) + \log(\text{JR-St AUC}/\Delta IC_{50}) + \log(\text{KATOIII AUC}/\Delta IC_{50}) + \log(\text{STKM-1 AUC}/\Delta IC_{50})]$$

The IC_{50} with verapamil was also determined and the score with verapamil ($AUC/\Delta IC_{50}$Ver) was calculated by the same formula.

To compare these scores to the clinical response rates, they were also modified under the following formula:

$$\text{Clinical score} = (\text{response rate}(\%)/2) - 13$$

The clinical response rates of these drugs were mainly listed in Alexander *et al.*[4] The response rates of the remaining drugs were as follows: CPT11 (SN-38), 25%;[19] VDS, 0%.[20]

Results

Intrinsic chemosensitivity

Table 3 shows the IC_{50} titers of all 24 anti cancer drugs to four cell lines under 1, 6, 24 and 72 h exposures. There was heterocytotoxicity among the

Table 2. AUC values used in this study

Drugs	Administered volume	AUC (μg h/ml)	References
NCS		not available	
BLM		not available	
PEP		not available	
MMC	30 mg/body	1.6	29
Act-D		not available	
ADM	25 mg/m^2	2.12	30
THP-ADM	25 mg/m^2	0.543	30
FAB	60 mg/m^2	1.75	31
DM	40 mg/body	0.214	32
MIT	14 mg/m^2	1.45	33
VCR	0.4–1.54 mg/m^2	0.145	34
VBL	3 mg/m^2	0.108	35
VDS	3 mg/m^2	0.11	36
SN-38	165 mg/m^2	0.667	37
Etoposide	120 mg/m^2	95	38
CPM[a]	20 mg/m^2	4.35	39
IFM[a]	20 mg/m^2	1.51	39
MCNU		not available	
Cisplatin	120 mg/m^2	4.8	40
CBDCA	300 mg/m^2	128	41
5-FU	250 mg/m^2[b]	0.516	42
MTX	70 mg/m^2	62	43
Ara-C	20 mg/m^2[b]	4.36	44
L-ASP		not available	

[a] AUC of the active form of CPM and IFM.
[b] Continuous infusion.

Table 3. IC_{50} of all cell lines to 24 anti-cancer drugs at various exposure times

	GCIY				JR-St				STKM-1				KATOIII			
	1 h	6 h	24 h	72 h	1 h	6 h	24 h	72 h	1 h	6 h	24 h	72 h	1 h	6 h	24 h	72 h
NCS	2.00	2.50	1.40	5.30	≥ 10	≥ 10	9.00	≥ 10	0.36	0.34	0.31	0.50	5.40	5.60	≥ 10	≥ 10
BLM	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	6.20	≥ 10	2.70	2.00
PEP	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10
MMC	≥ 10	≥ 10	4.00	3.30	≥ 10	8.40	3.00	1.62	≥ 10	5.00	1.80	1.30	≥ 10	≥ 10	9.40	4.20
ACt-D	1.18	0.13	0.08	0.08	0.16	0.06	0.01	0.01	0.24	0.06	0.07	0.01	≥ 10	≥ 10	3.10	2.20
ADM	≥ 10	7.90	4.40	4.20	0.90	1.00	0.19	0.12	6.60	1.30	0.90	0.48	≥ 10	≥ 10	1.90	0.50
THP-ADM	1.40	0.36	0.32	0.20	0.40	0.36	0.44	0.25	0.29	0.26	0.17	0.15	≥ 10	9.00	3.50	1.00
FAB	≥ 100	4.80	4.00	3.40	3.30	1.40	0.60	0.32	3.20	0.85	0.46	0.50	≥ 10	≥ 10	0.84	2.00
DM	5.00	3.70	1.70	1.55	3.30	1.60	1.60	1.00	0.19	0.75	0.08	0.44	≥ 10	3.40	1.40	0.44
MIT	1.80	1.65	0.58	1.90	≥ 100	≥ 100	4.40	4.40	5.00	0.66	0.66	1.45	≥ 0	7.20	3.30	0.82
VCR	≥ 10	≥ 10	≥ 10	≥ 10	1.45	0.54	0.86	0.72	1.70	0.40	0.10	0.09	2.00	≥ 10	1.45	0.20
VBL	≥ 10	≥ 10	2.20	≥ 10	0.16	0.17	0.15	0.01	0.17	0.09	0.10	0.01	0.62	1.60	9.00	0.20
VDS	5.60	5.20	0.06	0.01	0.14	0.09	0.06	0.01	0.53	0.10	0.01	0.01	≥ 10	≥ 10	8.50	0.19
SN-38	≥ 10	0.72	0.78	0.56	1.60	3.80	3.40	0.78	0.32	1.10	0.20	0.08	0.14	0.25	0.09	0.08
Etoposide	≥ 100	≥ 100	≥ 100	80.00	≥ 100	70.00	41.00	13.00	≥ 100	24.00	33.00	9.00	≥ 100	42.00	32.00	9.20
CPM	≥ 10	0.71	0.78	0.56	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	6.00	5.60	6.50
IFM	≥ 10	3.00	2.80	2.70	≥ 10	≥ 10	≥ 10	9.20	≥ 10	6.60	6.80	6.80	≥ 10	≥ 10	7.80	4.50
MCNU	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10
Cisplatin	≥ 10	4.00	1.55	1.85	≥ 10	6.70	3.30	3.30	≥ 10	5.50	2.55	2.80	≥ 10	≥ 10	4.00	1.80
CBDCA	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10
5-FU	≥ 100	≥ 100	42.00	36.00	≥ 100	≥ 100	18.00	8.00	27.00	24.00	30.00	12.50	≥ 100	≥ 100	≥ 100	≥ 100
MTX	≥ 10	≥ 10	≥ 10	≥ 10	≥ 0	≥ 10	1.00	0.11	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	3.40	≥ 10	≥ 10
Ara-C	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10
L-ASP	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	7.60	≥ 10	≥ 10	≥ 10	≥ 10

drugs and heterosensitivity among cell lines. Fourteen drugs (NCS, MMC, Act-D, anthracyclines, MIT, VBL, VDS, SN-38, etoposide, IFM and cisplatin) inhibited the growth of all four cell lines at certain exposure times, while four drugs (PEP, MCNU, CBDCA and ara-C) did not inhibit any cell lines at any exposure time. The remaining six drugs showed IC_{50} with some cell lines. Some drugs achieved IC_{50} in under 1 h exposure. In particular, NCS, Act-D, THP-ADM, DM, VCR, VBL, VDS and SN-38 inhibited three cell lines under 1 h exposure. Among the four cell lines, GCIY and KATO III were more chemoresistant to MDR drugs than JR-St or STKM-1. GCIY was especially resistant to VCR and VBL, and KATO III was particularly resistant to anthracyclines and VDS.

IC_{50} usually decreased as the exposure time increased, so ΔIC_{50} was calculated to determine the most effective exposure time and then the ΔIC_{50} score was calculated (Figure 1) to determine the effectiveness of each drug. SN-38, VBL, VDS, Act-D, THP-ADM and DM showed high ΔIC_{50} scores, and 5-FU, etoposide, ara-C, MCNU, L-ASP, PEP, CBDCA and BLM indicated low scores. The middle score group consisted of cisplatin, MMC, MTX, CPM and IFM. In terms of drug categories, plant alkaloids and anthracyclines showed high scores (except etoposide), while antimetabolites and enzyme showed low scores. Other categories contained both high and low score drugs.

Table 4 shows the $AUC/\Delta IC_{50}$ values and the $AUC/\Delta IC_{50}$ score is also indicated in Figure 1. SN-38, THP-ADM, ADM, MTX, FAB, etoposide, VBL and VDS showed high scores. In comparison with the rank of the ΔIC_{50} score, MTX and etoposide raised their rank in the $AUC/\Delta IC_{50}$ score, while plant alkaloids decreased the rank. Anthracyclines constantly showed high scores in both categories. SN-38 and THP-ADM constantly showed high scores.

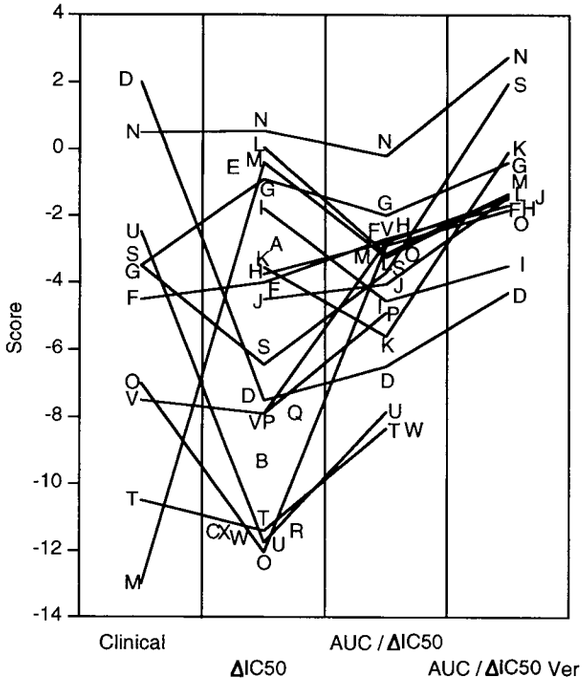

Figure 1. Comparison among the scores. All scores were calculated as in Material and methods and connected by the lines. Clinical score is a measure of the actual success rate in curing patients. ΔIC_{50} score measures *in vitro* effectiveness (higher score indicates lower dose needed for 50% inhibition). $AUC/\Delta IC_{50}$ weights the intrinsic effectiveness by the dose that can be obtained in the body. The symbols for the drugs are listed in Table 1. The AUC of some drugs (NCS, BLM, PEP, Act-D, MCNU and L-ASP) were not available. Therefore, the symbols representing those drugs are shown only in ΔIC_{50} scores.

Overexpression of P-glycoprotein and amplification of the *mdr-1* gene in the GCIY cell line

We examined P-glycoprotein expression and *mdr-1* gene amplification, because GCIY and KATOIII showed the so-called multidrug resistant pattern in IC_{50} compared with JR-St and STKM-1. Figure 2 shows the staining pattern of K562/A and GCIY cells. All of the K562/A cells were positively stained with MRK16 and the peak was clearly divided. In comparison, GCIY cells were partly stained with MRK16. Concerning other cell lines, only a part of the cell population was stained with MRK16. The percentages of positive staining of the four cell lines were: GCIY, 25.8 ± 10.3; JR-St, 12.6 ± 1.2; STKM-1, 4.1 ± 7.1; and KATOIII, 2.8 ± 4.8%. Only the GCIY cell line was judged to be positive in staining with MRK16. Expression of the *mdr-1* gene was examined by Northern blot analysis in comparison with the other cell lines (data not shown). The intensity of the hybridization signal was enhanced in GCIY RNA. However, the amplification of the transcript was estimated to be less than that observed in the positive control, K562/A. The amount of *mdr-1* transcripts from JR-St, STKM-1 and KATOIII remained at the same level as a negative control, K562. The results of negative and positive controls showed comparable reproducibility to those obtained by others.[6] Southern blots after the restriction enzyme digest of the genomic DNA showed no differences among band patterns in any of the cell lines (data

Table 4. AUC/ΔIC_{50} values of four cell lines at various exposure times

	GCIY				JR-St				STKM-1				KATOIII			
	1 h	6 h	24 h	72 h	1 h	6 h	24 h	72 h	1 h	6 h	24 h	72 h	1 h	6 h	24 h	72 h
MMC	< 0.16	< 0.03	0.02	0.01	< 0.16	0.03	0.02	0.01	< 0.16	0.05	0.04	0.02	< 0.16	< 0.03	0.01	0.01
ADM	< 0.21	0.04	0.02	0.01	2.36	0.35	0.48	0.24	0.32	0.27	0.10	0.06	< 0.21	< 0.04	0.05	0.06
THP-ADM	0.39	0.25	0.07	0.04	1.36	0.25	0.05	0.03	1.87	0.35	0.13	0.05	< 0.05	0.01	0.01	0.01
FAB	< 0.18	0.06	0.02	0.01	0.53	0.21	0.12	0.08	0.55	0.34	0.16	0.05	< 0.18	< 0.03	0.09	0.01
DM	0.04	0.01	0.01	0.00	0.06	0.02	0.01	0.00	1.13	0.05	0.11	0.01	< 0.02	0.01	0.01	0.91
MIT	0.81	0.15	0.10	0.01	< 0.15	< 0.02	0.01	0.00	0.29	0.37	0.09	0.01	< 0.15	0.03	0.02	0.02
VCR	< 0.01	< 0	< 0	< 0	0.10	0.05	0.01	0.00	0.09	0.06	0.06	0.02	0.07	< 0	0.00	0.01
VBL	< 0.01	< 0	0.00	< 0	0.68	0.11	0.03	0.15	0.65	0.20	0.05	0.15	0.17	0.01	0.00	0.01
VDS	0.02	0.00	0.07	0.15	0.79	0.20	0.08	0.15	0.21	0.19	0.46	0.15	0.01	0.00	0.00	0.01
SN-38	< 0.07	0.15	0.04	0.02	0.42	0.03	0.01	0.01	2.08	0.10	0.14	0.12	4.76	0.44	0.33	0.12
Etoposide	< 0.95	< 0.16	< 0.04	0.02	< 0.95	0.23	0.10	0.10	< 0.95	0.66	0.12	0.15	< 0.95	0.38	0.12	0.14
CPM	< 0.52	1.23	0.28	0.13	< 0.52	< 0.09	< 0.02	< 0.01	< 0.52	< 0.09	< 0.02	< 0.01	< 0.52	0.15	0.04	0.01
Cisplatin	< 0.48	0.20	0.13	0.04	< 0.48	0.12	0.06	0.02	< 0.48	0.15	0.08	0.02	< 0.48	< 0.08	0.05	0.04
CBDCA	< 12.8	< 2.13	< 0.53	< 0.18	< 12.8	< 2.13	< 0.53	< 0.18	< 12.8	<2.13	< 0.53	< 0.18	< 12.8	< 2.13	< 0.53	< 0.18
5-FU	< 0.01	< 0	0.00	0.00	< 0.01	< 0	0.00	0.00	0.02	0.00	0.00	0.00	< 0.01	< 0	< 0	< 0
MTX	< 6.2	< 1.03	< 0.26	< 0.09	< 6.2	< 1.03	2.58	8.20	< 6.2	< 1.03	< 0.26	< 0.09	< 6.2	3.04	< 0.26	< 0.09
Ara-C	< 0.44	< 0.07	< 0.02	< 0.01	< 0.44	< 0.07	< 0.02	< 0.01	< 0.44	< 0.07	< 0.02	< 0.01	< 0.44	< 0.07	< 0.02	< 0.01

Table 5. AUC/ΔIC_{50}Ver values at various exposure times

	GCIY				JR-St				STKM-1				KATOIII			
	1 h	6 h	24 h	72 h	1 h	6 h	24 h	72 h	1 h	6 h	24 h	72 h	1 h	6 h	24 h	72 h
MMC + Ver	< 0.15	0.74	0.68	0.09	< 0.15	0.08	0.08	0.05	< 0.15	< 0.03	0.02	0.02	< 0.15	0.04	0.01	0.03
ADM + Ver	< 0.21	0.09	0.06	0.06	1.41	0.24	0.16	0.08	0.53	0.22	0.46	0.13	< 0.21	0.15	0.27	0.27
THP-ADM + Ver	0.39	0.13	0.05	0.04	1.47	0.28	0.08	0.04	2.01	0.41	0.09	0.04	0.34	0.06	0.02	0.02
FAB + Ver	< 0.18	0.24	0.19	0.06	0.55	0.39	0.77	0.17	0.44	0.13	0.06	0.05	< 0.18	0.16	0.29	0.36
DM + Ver	0.08	0.04	0.02	0.01	0.15	0.03	0.02	0.01	0.31	0.09	0.08	0.03	0.08	0.03	0.01	0.02
MIT + Ver	1.32	0.37	0.4	0.01	0.37	0.19	0.08	0.03	1.65	0.86	0.76	0.07	< 0.15	0.04	0.03	0.03
VCR + Ver	0.22	0.19	0.6	0.2	2.07	0.2	0.1	0.2	0.97	0.35	0.6	0.2	< 0.01	< 0	0.6	0.2
VLB + Ver	1.08	0.02	0.06	0.15	1.35	0.23	0.05	0.15	n.e.	n.e.	n.e.	n.e.	0.02	0.03	0.03	0.15
VDS + Ver	0.12	0.1	0.46	0.15	0.55	0.2	0.09	0.15	n.e.	n.e.	n.e.	n.e.	< 0.01	0.01	0.06	0.15
SN-38 + Ver	3.71	0.09	0.25	0.02	13.34	0.93	0.19	0.07	8.34	0.17	0.28	0.13	1.33	0.22	0.05	0.93
Etoposide + Ver	6.33	3.52	2.83	1.1	< 0.95	0.53	0.13	0.02	< 0.95	< 0.16	< 0.04	0.03	< 0.95	< 0.16	< 0.04	0.13
Cisplatin + Ver	6	4.44	1.67	0.42	1.5	2.11	0.63	0.33	9.6	4.4	1.43	0.61	0.56	0.57	0.22	0.67

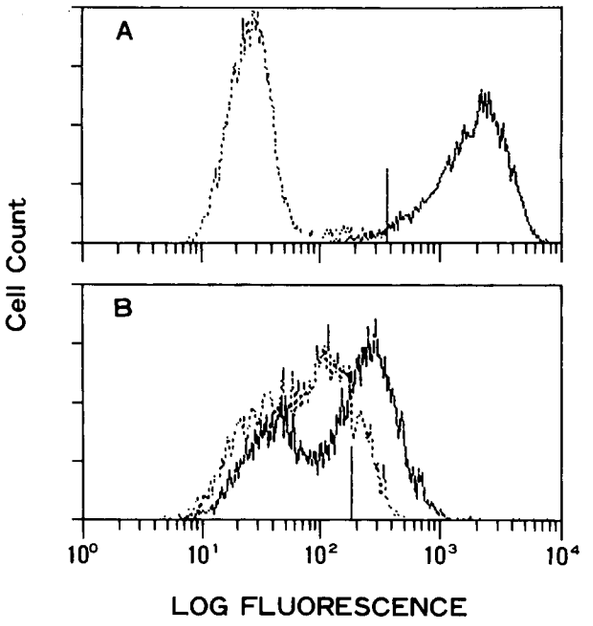

Figure 2. P-glycoprotein expression in adriamycin-resistant K562/A (A) cells and GCIY(B) cells. Cells were reacted with monoclonal antibody MRK16 as described in Materials and methods and analyzed on a FACScan flow cytometer. There are two sets of cells in both panels; one is immunofluorescently labeled and the other is control (dotted line).

not shown). Sequence analysis of the amplified *mdr-1* products revealed no nucleotide and amino acid changes in the locus in any of the cell lines used in the present study (data not shown).

Reversal effects of verapamil

A reversal of drug resistance was observed by applying verapamil (1 μg/ml) to the culture media. The AUC/ΔIC_{50}Ver values are listed in Table 5. GCIY was resensitized to almost all MDR drugs by verapamil. Verapamil also reduced the IC_{50} of KATOIII to almost all MDR drugs. For example, 1 μg/ml of verapamil restored the sensitivity of GCIY to ADM and the IC_{50} became one-sixth under 72 h exposure. The IC_{50} on KATOIII was also reduced to one-fourth. Conversely, the sensitivities to ADM of the other two cell lines were not affected by verapamil.

The sensitivities of all cell lines to cisplatin were extremely enhanced by verapamil. Verapamil also increased the sensitivity of GCIY to MMC. Therefore, verapamil increased the scores of all drugs examined (Figure 1). Among the drugs, the effects of cisplatin and VCR were particularly enhanced.

Comparison with clinical response

Each score shows different meanings. The ΔIC_{50} score shows the absolute cytotoxicity *in vitro* and can be obtained for all drugs. The AUC/ΔIC_{50} score shows the clinical relevance of the drugs by multiplying cytotoxicity by the achievable dose *in vivo*, but the problem is that the AUC is not always available and the AUC can have some variability depending on drug dose, the manner of administration or the method of measurement. Therefore, it is important to find all these parameters.

SN-38 seems to be the best candidate for poorly-differentiated gastric cancer, because it constantly showed the highest score. A preliminary clinical study also revealed the second highest response rate[19] (Figure 1). THP-ADM also showed consistent high scores, although no clinical data is now available. Other anthracyclines and anthraquinone also showed consistent scores which were lower than those of THP-ADM. Cisplatin was also consistent except for the effect of verapamil. Plant alkaloids showed higher and MMC and 5-FU showed lower scores than expected from the clinical data.

Discussion

We used four poorly-differentiated gastric cancer cell lines as a panel in this chemosensitivity test. As shown in Table 3, the pattern of intrinsic chemosensitivity of each cell line was roughly similar. Most of the MDR drugs gave IC_{50} values on all cell lines and among non-MDR drugs, only some drugs gave IC_{50} values. Fourteen drugs showed IC_{50} on all four cell lines, while four drugs did not inhibit any cell lines. This means that 18 out of 24 drugs showed the same effectiveness on the four cell lines, i.e. all cell lines were inhibited or none of the cell lines were inhibited. Intrinsic chemosensitivity is not, however, necessarily the same across all cell lines; however, the purpose of this study is to identify agents that are broadly effective against cells of the same class (poorly-differentiated gastric cancer).

When the chemosensitivity patterns of the four cell lines were compared with one another, two of the four cell lines (GCIY and KATOIII) showed the MDR phenotype, i.e. they were relatively more resistant to MDR drugs than the other two cell lines. Immunological staining and gene analysis indicated that GCIY cells showed some *mdr-1* gene amplification and P-glycoprotein expression. GCIY cells showed the so-called classical type MDR. Expression of P-glycoprotein has been indicated in many

kinds of solid tumors, particularly in those derived from tissues that normally express this gene (e.g. hepatoma, colon and renal cancer).[21] Gastric cancers, however, have not been shown to express P-glycoprotein.[22] GCIY cells naturally expressed P-glycoprotein, because no anti-cancer drugs except 5-FU were administered before cells were sampled from the patient. The GCIY cell line is the first gastric cancer cell line (except xenograft lines)[6] which has P-glycoprotein expression and *mdr-1* gene amplification. KATOIII cells, on the other hand, did not show amplification of the *mdr-1* gene or P-glycoprotein expression. KATOIII cells might have a MDR phenotype that is caused by a mechanism other than *mdr-1* gene amplification and which could also be reversed by the addition of verapamil. As shown in Tables 4 and 5, verapamil resensitized both GCIY and KATOIII cells to ADM, FAB, DM, VCR and VDS. Therefore, it worked as a MDR competitor, as first reported by Tsuruo.[8] These results suggest that it is important to take MDR into consideration when new chemotherapy for gastric cancer is considered.

Verapamil also increased the cytotoxicities of cisplatin and SN-38 to all cell lines, except for SN-38 to KATOIII cells. Verapamil has been reported to enhance the anti-tumor effect of cisplatin in human neuroblastoma xenograft,[23] in five of six human lung cancer cell lines[24] and in human ovarian tumor xenograft.[25] In the murine tumor B16, verapamil failed to enhance the effect of cisplatin, while nifedipine, a dihydropyridine class calcium channel blocker, did.[26] The mechanisms of this enhancement effect are not clear. Although verapamil was suggested to inhibit DNA repair by topoisomerase II,[27] the cytotoxicity of SN-38, a topoisomerase I inhibitor, was also enhanced in this study. These results suggest that further studies about the mechanism of the enhancement will be necessary.

To validate the cytotoxicities of the drugs, we compared ΔIC_{50} or ΔIC_{50}Ver with the clinical AUC, because the AUC is the most reliable predictor for cell cycle non-specific anti-cancer drugs.[28] Even for cell cycle-specific anti-cancer drugs, the AUC has more meaning than the concentration itself. From the scores, SN-38 with/without verapamil, cisplatin with verapamil and THP-ADM with/without verapamil seem to be the best candidates for poorly-differentiated gastric cancer chemotherapy. Plant alkaloids could also be candidates, although the discrepancy between the clinical data to date and the results is significant. This discrepancy may be the first step to establish a new chemotherapy, because the purpose of this screening was to pick up unknown effective drugs by use of a disease-oriented panel. Further studies are necessary to make sure of the effectiveness of plant alkaloids.

Effective drugs in clinical studies, such as 5-FU and MMC, were not selected by this screening. One of the reasons may be that the mechanism of 5-FU is not AUC dependent. Another reason may be that the exposure time was not long enough. Several studies, however, have reported that MTX–5-FU sequential therapy has enhanced the effects, especially on poorly-differentiated gastric cancer compared with the well-differentiated type. This panel could provide confirmation of this hypothesis. Concerning the low score obtained for MMC, we have no explanation. MMC might have some special *in vivo* activity (perhaps related to immune response) which cannot be detected by this system.

SN-38 and THP-ADM selected in this study only have limited clinical data. The combination cisplatin with verapamil is new for gastric cancer treatment. Our results suggest that these are promising chemotherapies for poorly-differentiated gastric cancer, although additional experiments, especially *in vivo*, will be necessary to confirm these speculations.

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