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Functional Similarity of Anticancer Drugs by MTT Bioassay

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Abstract

We prepared normal or Ha-*ras*-transformed NIH3T3 cells transfected stably or transiently with various tumor-related genes. The chemosensitivity of the transfected clones to 16 anticancer drugs was compared to the parental control cells using the MTT assay. The chemosensitivity changes induced by transfected genes were calculated and expressed numerically as the Drug Chemosensitivity Index (DCI). High DCI values (indicating resistance) were frequently observed in cells expressing C/EBPα, C/EBPβ, p53, p21, PTEN, dominant-negative MDM2, caspases, HSP90, COUP-TF1 and decorin. In contrast, transfectants expressing *ras*, *src*, *erb*B2 and calpastatin had low DCI values, indicating increased sensitivity. Thus, it may be possible to predict the sensitivity of cancer cells toward anticancer drugs based on the expression levels of these genes. We then performed a regression analysis of DCI values between anticancer drugs. The correlation coefficients (*r*) were relatively high between cisplatin, camptothecin, mitomycin C and etoposide, suggesting that the mechanisms of action of these drugs are similar. The *r* values of aclarubicin, vincristine, taxol and cytarabine were low, suggesting that each of these drugs has a different and unique effect. This analysis may provide a rationale for design of combination chemotherapy regimens.

Keywords: Anticancer drugs; Cancer chemotherapy; MTT assay

Abbreviations: ACR: Aclarubicin; AraC: Cytarabine; CDDP: Cisplatin; CPT: Camptothecin; DMEM: Dulbecco's modified Eagle's minimum essential medium; DMSO: dimethyl sulfoxide; 5-FU: 5-fluorouracil; HU: hydroxyurea; IFM: ifosfamide; MCNU: methyl 6-[3-(2-chloroethyl)-3-nitrosoureido]-6-deoxy-α-D-glucopyranoside; MIT: mitoxantrone; MMC: mitomycin C; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTX: methotrexate; PBS: phosphate-buffered saline; PEP: peplomycin; PMSF: phenylmethylsulfonyl fluoride; Taxol: paclitaxel; 6-TG: 6-thioguanine; VCR: vincristine; VP-16: etoposide

Introduction

Chemotherapy using anticancer drugs is a useful therapeutic method for cancer. Thus far, many effective anticancer drugs have been developed. However, due to the adverse effects of each drug, their application is limited. Thus, combination therapy using smaller amounts of multiple drugs has become more and more common.

The efficacy of anticancer drugs varies among patients. This may be explained by differences in gene expression. For example, overexpression of P-glycoprotein results in prominent resistance to many drugs such as vincristine, etoposide, and paclitaxel [1]. Comprehensive cDNA microarray analysis has been carried out for various cancers to examine alterations of gene expression [2-8].

In addition, a large diverse panel of cultured human tumor cell lines was tested for sensitivity to anticancer drugs [9]. Although this analysis was efficient to discriminate anticancer drug-responsive tumor cells, the genetic backgrounds of the cell lines were so variable that the precise action mechanism remained unclear. In the present study, we prepared NIH3T3 mouse fibroblasts transfected stably or transiently with varying tumor-related genes, and examined their chemosensitivity to anticancer drugs. Variations in action mechanisms may provide a rationale for combination chemotherapy.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's minimum essential medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 6-thioguanine (6-TG), cytarabine (AraC), hydroxyurea (HU) and were purchased from Sigma (St. Louis, MO). Mitomycin C (MMC), methotrexate (MTX), 5-fluorouracil (5-FU) and mitoxantrone (MIT) were purchased from Merck Biosciences (Darmstadt, Germany). Camptothecin (CPT) was purchased form Biomol Research Laboratories (Plymouth Meeting, PA). Paclitaxel (Taxol) was purchased from Alexis Corporation (Lausen, Switzerland). Dimethyl sulfoxide (DMSO), etoposide (VP-16), cisplatin (CDDP) and peplomycin (PEP) were ob-

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tained from Wako Pure Chemicals (Kyoto, Japan). Vincristine (VCR) and ifosfamide (IFM) were obtained from Shionogi Pharmaceutica Co. Ltd. (Osaka, Japan); methyl 6-[3-(2-chloroethyl)-3-nitrosoureido]-6-deoxy-α-D-glucopyranoside (MCNU) from Mitsubishi Welfarma Co. Ltd. (Osaka, Japan); and aclarubicin (ACR) from Astellas Pharma Inc. (Tokyo, Japan).

cDNA clones were purchased from Toyobo Biochemicals (Osaka, Japan), Guthrie Research Institute (Sayre, PA) or Open Biosystems (Huntsville, AL). Some clones were provided by isolators.

Cell lines and culture

NIH3T3 mouse fibroblasts and Ha-*ras*-transformed NIH3T3 cells (ras-NIH3T3) [10] were cultured in DMEM supplemented with 5% bovine serum and 100 μg/ml of kanamycin. Cells were transiently or stable transfected with cDNAs in eukaryotic expression vectors such as pcDNA3 and pME18S-FL3. To isolate stable transfectants, cells were transfected with the expression plasmids together with the *neo* gene using LipofectAMINE reagent (Life Technologies, Carlsbad, CA), and then selected by culture in the presence of G418 (400 μg/ml) for two weeks as described [11-13]. A total of 135 stable transfectants and 41 transiently transfected cells were examined for chemosensitivity toward 16 anticancer drugs.

Western-blotting and RT-PCR analyses

The protein expression levels of transfected genes were examined by Western blotting [12,13]. The cells were washed with phosphate-buffered saline (PBS) and lysed in 0.5% Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, 50 μM antipain, 50 μM pepstatin A and 50 μM acetyl-Leucyl-Leucyl-norleucinal for 10 min at 4˚C. The cell lysate was centrifuged at 13,000×g for 10 min and the supernatant was used as cytoplasmic cell extract. The pellet was used as the nuclear fraction. The samples were analyzed by Western blotting using antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA), followed by detection using ImmunoStar Reagents (Wako Pure Chemicals).

When the commercial antibodies are not available, the mRNA expression levels were examined by RT-PCT (reverse transcription-polymerase chain reaction) as described previously [13,14]. Briefly, total cellular RNA was isolated from the tumor tissues using FastPure RNA Kit (Takara Biochemicals, Kyoto, Japan). Reverse transcription was carried out with oligo$(dT)_{20}$ primer using the ThemoScript RT-PCR System (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The mRNA expression levels were examined via quantitative realtime RT-PCR using the Universal Probe Library system (Roche, Basel, Switzerland).

Evaluation of cytotoxicity of anticancer drugs

Chemosensitivity of transfected cells was examined using the MTT assay according to the method of Mosmann [15] as previously described [16,17]. Cells at exponentially growing phase were used. Five thousand cells per well (100 μl) were plated in 96-well plates in the presence of various concentrations of anticancer drugs, and cultured for three days. The activity of mitochondrial succinic dehydrogenase was measured by incubation for 4 h in the presence of 0.5 mg/ml of MTT (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide). Absorbance reflects the viable cell number and was measured at 570 nm with a reference wavelength of 650 nm using a microplate reader. Absorbance reflects the viable cell number and was expressed as a percentage of that of cells cultured in the absence of anticancer drugs.

For the combination treatment, ras-NIH3T3 were added with 5-FU (50 ng/ml), PEP (500 ng/ml) or MTX (50 ng/ml), and immediately after the addition, plated in the presence of varying concentrations of MIT, PEP or VP-16.

Data mining

The difference in the chemosensitivity between the parent and transfected cells was calculated and expressed numerically as the Drug Chemosensitivity Index (DCI) [17-19] as follows: DCI = log $(IC_{40,transfectant}/IC_{40,parent})$ (Figure 1).

Results and Discussion

Chemosensitivity to anticancer drugs of transfected cells

Mouse fibroblasts, NIH3T3 or ras-NIH3T3, were transfected with various cancer-related genes, and the chemosensitivity to 16 different anticancer drugs was examined by MTT assay. Table 1 summarizes the list of genes of which the transfection induced resistance or sensitization against each anticancer drugs. The DCI values reflect the extent how resistant of the transfected cells were converted by the gene transfection [17-19]. The full version of list of DCI values are shown in Supplementary Table S1. High DCI values (indicating drug resistance) were frequently observed in cells expressing C/EBPα, C/EBPβ, wild-type p53, p21, PTEN, mutated MDM2, caspases, HSP90, COUP-TFI and decorin. In contrast, transfectants expressing *ras*, *src*, *erb*B2, calpastatin, mutated p53 and wild-type MDM2 had low DCI values, indicating increased sensitivity. Thus, it may be possible to predict the sensitivity of cancer cells toward anticancer drugs based on the expression levels of these genes. It should be noted that oncogenes such as *ras*, *src*, *erb*B2 and MDM2 increased the chemosensitivity against some, if not all, anticancer drugs whereas tumor suppressor genes such

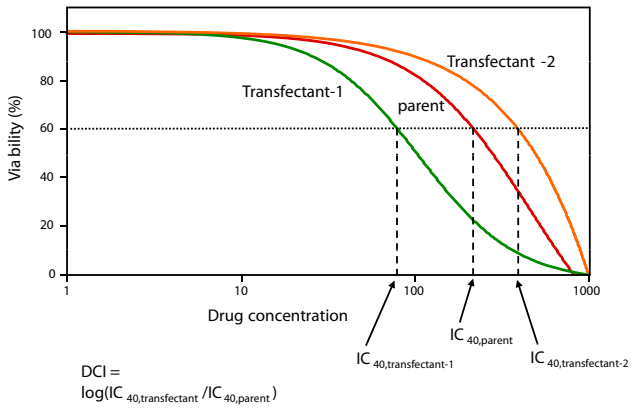

Figure 1: Representative results of the MTT assay. The relative cell viability was measured using the MTT assay. The concentration of drug is shown on the abcissa and the absorbance of the formazan, which represents the relative cell viability, is shown on the ordinate. Sensitivity curves of two transfectants, in addition to the parental ras-NIH3T3 cells, are shown. The positions of drug concentrations giving 40% inhibition in two transfectants ($IC_{40,transfectant-1}$ and $IC_{40,transfectant-2}$) and in the parental cells ($IC_{40,parent}$) are shown. A dotted line indicates 60% (40% inhibition) of cell viability. Broken lines showed by arrows indicate the concentrations of $IC_{40,transfectant-1}$, $IC_{40,transfectant-2}$ and $IC_{40,parent}$. DCI values were calculated and expressed numerically as follows: DCI = log $(IC_{40,transfectant/IC40,parent})$

CDDP		**5-FU**		**MTX**		**CPT**	
Transfected genes		Transfected genes		Transfected genes		Transfected genes	
Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive
C/EBPα	N-ras	DAN	Ha-ras	C/EBPα	v-Src	DAN	Ha-ras
C/EBPβ	Ha-ras	C/EBPα	TERT-WT	DAN	E2F	HSP90	N-ras
C/EBPβ	PTEN-A3	Bax*	v-Src	Caspase-3	APP	RhoA-DN	Akt-DN
HSP90	HDAC1	Hsdj	IκB-TDN	PTEN-G129R	Akt-DN	C/EBPβ	ErbB2
Hsdj	DJ1-WT	Cystatinα	Axin	p53-WT	Calpain 30K	C/EBPα	Calpastatin
v-Src	PARK7	DnaJ	Rac1	DnaJ	cAMP-PK-CS	Caspase-2	v-Src
COUP	PTEN-G129R	CyclinD1	PKCg-WT	PKCa-KN	ColXVIII	MKRN1-mut	Ki-ras
p21	Caspase-1	Caspase-3	Ki-ras	Ras-N17	C/EBPα	CBP	TERT-WT
RhoA-DN	APC	N-ras	STAT5A	P/CAF	WIG1	PDGF-RD	HDAC1
FANCC	CSK	c-Myc	R-Ras	Decorin	VDAC1	p16	FilaminA
VP-16		**MIT**		**MMC**		**IFM**	
Transfected genes		Transfected genes		Transfected genes		Transfected genes	
Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive
DAN	DAP	p53-WT	MDM2-WT	DAN	N-ras	C/EBPα	Ras-N17
C/EBPβ	APC	C/EBPβ	N-ras	MKRN1-mut	v-Src	TERT-DN	Akt-DN
PTEN-WT	PARK7	CAPN10	TKT	C/EBPα	Ha-ras	Hsp70	N-ras
PKCα-KN	Ha-ras	Bad	PARK7	C/EBPβ	ErbB2	p53-mut	Ki-ras
AISEC	HIF1	PER3	HDAC1	C/EBPβ	Akt-DN	HNF4	Ha-ras
IκB-TDN	STAT4	c-Myc	Ha-ras	Caspase-2	p53	DnaJ	Cystatin E
Bcl-2	STAT6	TERT-DN	APC	TERT-DN	Ki-ras	p16	Caspase-1
COUP	OPRT	Bcl-2	Cyclin D1	Caspase-3	TERT-WT	C/EBPβ	v-Src
MDM2-mut	HO	COUP	MSSP	HSP90	Calpastatin	MKRN1-mut	WIG1
c-Myc	DJ1-K130R	KRas2-DN	APC	P/CAF	Hsp70	C/EBPβ	p53
PEP		**ACR**		**VCR**		**Taxol**	
Transfected genes		Transfected genes		Transfected genes		Transfected genes	
Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive
MKRN1-mut	Ki-ras	DAN	WIG1	DAN	OPRT	APC	Calpain 30K
p53-WT	v-Src	Caspase-2	PER2	p53-WT	APC	C/EBPβ	Axin
ARF1	Cystatinα	p16	ColXVIII	Decorin	Rap1A	Caspase-2	TSC1
HSP90	ErbB2	Bax	Caspase-1	DLG	FGFR-KR	Decorin	STAT2
Akt-DN	N-ras	Caspase-3	Ha-ras	p21	Rac1	MDM2-WT	p53-mut
PER3	PARK7	TERT-DN	TK-1	C/EBPβ	APC	DLG	FilaminA
DAN	FGFR-WT	Bax	RCC1	p16	β-Catenin	Ki-ras	Akt-DN
BH	HDAC1	MM1	Ki-ras	PER3	CRI1	Max	STAT3
MDM2-WT	Calpastatin	TS	Cystatinα	PER-1	RAN	PTEN-WT	HNF4
Caspase-2	PIGPC1	Max	ErbB2	Regucalcin	c-Myc	RhoA-DN	Enigma
MCNU		**6-TG**		**AraC**		**HU**	
Transfected genes		Transfected genes		Transfected genes		Transfected genes	
Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive
C/EBPβ	Regucalcin	Caspase-3	APP	C/EBPβ	AMY1	DAN	N-ras
Rab1A	RAN	C/EBPα	PER2	MKRN1-mut	Gluco-R	Bcl-2	p21
RhoGDIα	Akt-DN	DAN	ColXVIII	OPRT	Ki-ras	m-Calpain	ErbB2
ARF1	WIG1	CaMKIIa-CA	CathL-mut	Bcl-2	Ha-ras	Caspase-3	HNF4
AISEC	STMN	Cystatina	GUK1	AISEC	Caspase-3	TERT-WT	v-Src
MDM2-mut	STAT6	HSP90	Calpastatin	MDM2-WT	FGFR-KR	TSC1	Ki-ras
14-3-3ζ	E2F	C/EBPβ	RPA2	Decorin	p53-mut	PTEN-WT	MDM2-WT
IKK-DN	Caspase-3	PDGF-RD	N-ras	Bcl-XL	RAN	Abl	STAT4
PKCα-KN	C/EBPα	HSP40	Ha-ras	PKCα-KN	Bax	HSP90	PER-1
cAMP-PK-CS	HSP40	HNF1	SDC1	Enigma	HSP90	R-Ras	CaMKIIα

Listed are top 10 of genes of which the transfection induced drug resistance or sensitization.
The full version of DCI values are shown in Supplementary Table S1.
Abbreviations: WT, wild-type; mut, mutated; DN, dominant negative; KN, kinase negative; TDN, transdominant negative; CA, constitutively active; CS, catalytic subunit.

Table 1: List of anticancer drug-sensitivity-related genes.

	CDDP	5-FU	MTX	CPT	VP-16	MIT	MMC	IFM	PEP	ACR	VCR	Taxol	MCNU	6-TG	AraC	HU
CDDP		0.277	0.280	0.569	0.498	0.485	0.496	0.412	0.357	0.237	0.225	0.242	0.311	0.402	0.320	0.202
5-FU	0.278		0.510	0.438	0.393	0.094	0.466	0.329	0.235	0.404	0.241	0.134	0.109	0.382	0.115	0.306
MTX	0.281	0.510		0.484	0.426	0.275	0.515	0.355	0.407	0.350	0.358	0.180	0.173	0.523	0.229	0.356
CPT	0.568	0.438	0.484		0.509	0.473	0.767	0.501	0.496	0.417	0.344	0.258	0.297	0.588	0.257	0.539
VP-16	0.498	0.393	0.426	0.509		0.499	0.547	0.292	0.354	0.331	0.571	0.281	0.463	0.330	0.454	0.402
MIT	0.484	0.094	0.275	0.473	0.499		0.468	0.236	0.405	0.096	0.360	0.168	0.232	0.300	0.313	0.352
MMC	0.496	0.466	0.515	0.767	0.547	0.468		0.477	0.488	0.442	0.336	0.167	0.419	0.550	0.340	0.556
IFM	0.411	0.329	0.355	0.501	0.292	0.236	0.477		0.317	0.190	0.056	0.072	0.458	0.360	0.234	0.330
PEP	0.356	0.235	0.407	0.496	0.354	0.405	0.488	0.317		0.346	0.208	0.152	0.280	0.342	0.368	0.332
ACR	0.232	0.404	0.350	0.417	0.331	0.096	0.442	0.190	0.346		0.200	0.050	0.061	0.286	0.035	0.277
VCR	0.225	0.241	0.358	0.344	0.571	0.360	0.336	0.056	0.208	0.200		0.220	0.120	0.213	0.375	0.185
Taxol	0.242	0.134	0.180	0.258	0.281	0.168	0.167	0.072	0.152	0.050	0.220		0.402	0.219	0.057	0.151
MCNU	0.312	0.109	0.173	0.297	0.463	0.232	0.419	0.458	0.280	0.061	0.120	0.402		0.135	0.425	0.234
6-TG	0.402	0.382	0.523	0.588	0.330	0.300	0.550	0.360	0.342	0.286	0.213	0.219	0.135		0.168	0.394
AraC	0.320	0.115	0.229	0.257	0.454	0.313	0.340	0.234	0.368	0.035	0.375	0.057	0.425	0.168		0.257
HU	0.202	0.306	0.356	0.539	0.402	0.352	0.556	0.330	0.332	0.277	0.185	0.151	0.234	0.394	0.257	

Shown are correlation coefficients between two each of anticancer drugs.
Coefficients higher than 0.5 or lower than 0.2 are marked.

Table 2: Correlation of DCI values between anticancer drugs.

as p53, p21 and PTEN reduced the sensitivity. This may justify the application of anticancer drugs for cancer therapy.

Regression analysis of DCI values

Because chemosensitivity was similar but not identical among anticancer drugs, we then performed a regression analysis of the DCI values shown in Table 1. The correlation coefficients (*r*) are summarized in Table 2. The *r* values were relatively high (significantly correlated) among CDDP, CPT, MMC and VP-16, suggesting that the mechanisms of action of these drugs are similar (Table 2, Figure 2). The *r* value between MMC and CPT was the highest. The *r* values of ACR, VCR, taxol and AraC were relatively low (no correlation), suggesting that each of these drugs has a different and unique effect.

Combination chemotherapy is growing more common in cancer chemotherapy. For example, the combination of cyclophosphamide (IFM analogue), VCR and doxorubicin (ACR analogue) is effective for small cell lung cancer [20] and non-Hodgkin lymphoma [21]. The low *r* value among these drugs means that they works independently. Thus, additive effects can be expected. This was exemplified by the result that the high-*r*-value combination of MIT and PEP caused less cytotoxic effects than the low *r* value combination of MIT and 5-FU or MTX (Figure 3A).

On the other hand, colon cancer and ovarian clear cell adenocarcinoma are treated with a combination of CPT and MMC [22,23], which showed a high *r* value (Table 2). Both CPT and MMC are effective toward *ras*- and *erb*B2-transfected cells (Table 1). Colon cancers are frequently accompanied by mutations in the Ki-*ras* gene [24,25]. Overexpression of *erb*B2 in ovarian carcinoma [26,27] may account for the sensitivity against CPT and MMC. Thus, if the target molecules are restricted, synergistic effects focused on the target can be expected. Furthermore, such high-*r*-value combinations may reduce the side effects caused by a high-dose application of a single drug. Likewise, the high *r* value combinations of PEP and MTX or VP-16 and MTX showed more suppressive effects than the low *r* value combinations of PEP and 5-FU or VP-16 and PEP (Figure 3B and C).

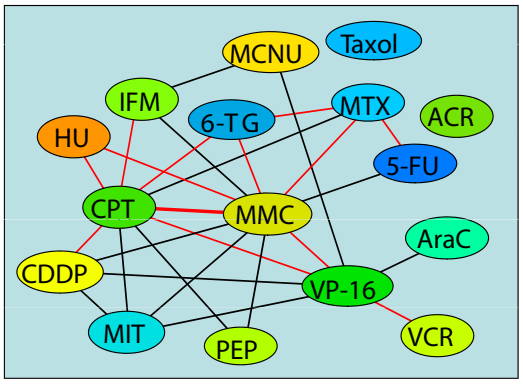

Figure 2: Functional similarity between anticancer drugs. Combinations of two drugs with high *r* values are shown schematically. High *r* values suggest similar action mechanisms between drugs. Results in red indicate a closer relationship than results in black.

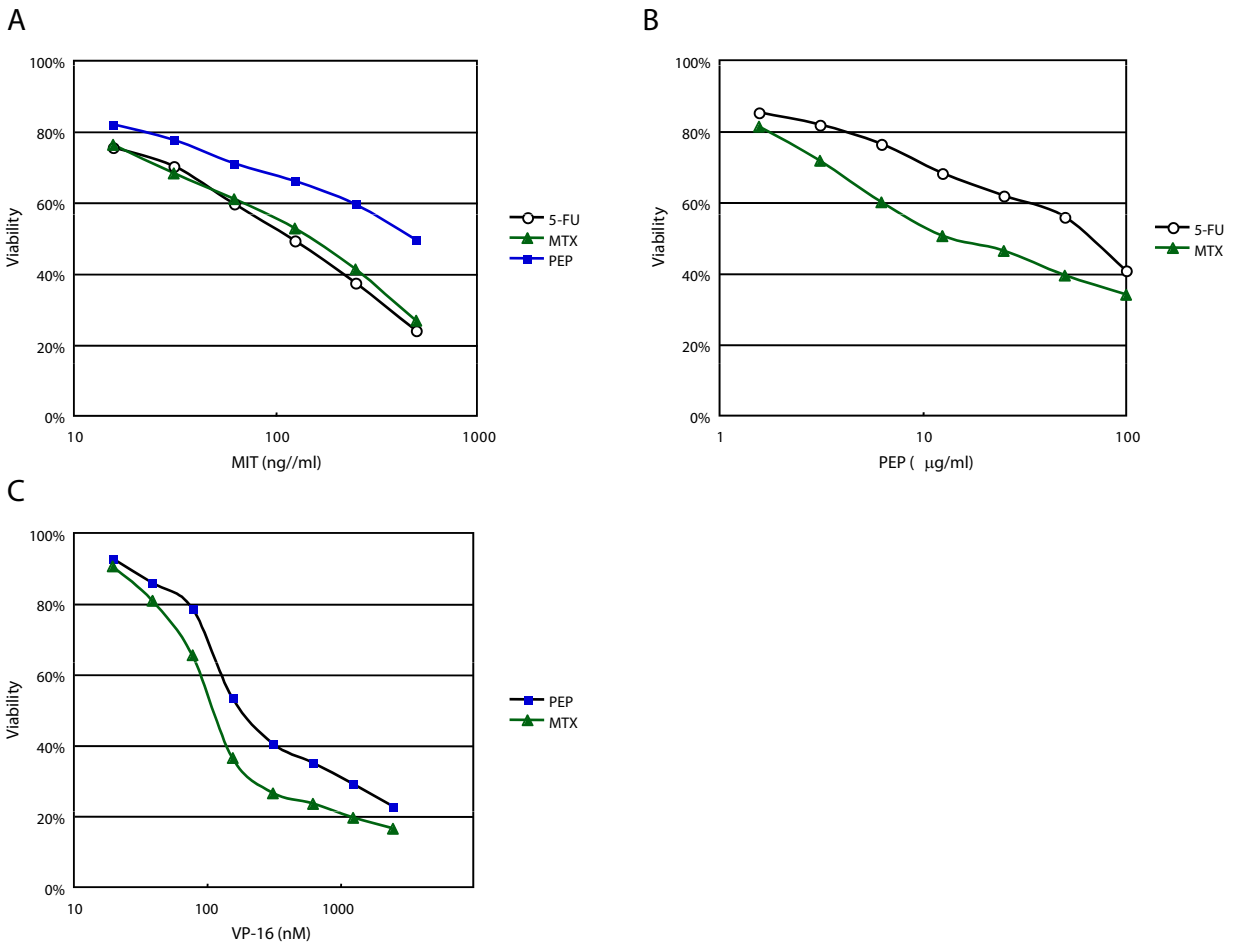

Figure 3: Results of combination treatment. ras-NIH3T3 cells were added with 5-FU (50 ng/ml), PEP (500 ng/ml) or MTX (50 ng/ml). Immediately after the addition, cells were plated in the presence of varying concentrations of MIT (A), PEP (B) and VP-16 (C). After culture for three days, the viabilities were measured by the MTT assay. The abscissa and ordinate represent the concentration of the latter drugs and the relative viability versus that in the absence of the latter drugs, respectively.

Consequently, this approach using regression analysis of DCI values of anticancer drugs may provide a theoretical basis for design of combination chemotherapy regimens.

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