Original Article

DOI: 10.5582/ddt.2016.01049

Dividing phase-dependent cytotoxicity profiling of human embryonic lung fibroblast identifies candidate anticancer reagents

Yoshinori Inagaki[1,2], Yasuhiko Matsumoto[1,3], Wei Tang[2], Kazuhisa Sekimizu[1,3,*]

[1] *Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan;*
[2] *Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, the University of Tokyo, Tokyo, Japan;*
[3] *Teikyo University Institute of Medical Mycology, Tokyo, Japan.*

Summary **Human Embryonic Lung fibroblasts (HEL cells) are widely used as a normal cell in studies of cell biology and can be easily maintained in the resting phase. Here we aimed to discover compounds that exhibit cytotoxicity against HEL cells in the dividing phase, but not in the resting phase. The cytotoxicity of each compound against HEL cells either in the resting phase or in the dividing phase was determined by MTT assay. Ratios of the IC50 of cells in the resting phase and that of cells in the dividing phase (RRD) for these compounds were compared. We selected 44 compounds that exhibited toxic effects on HEL cells in the dividing phase from a chemical library containing 325 anticancer drugs and enzyme inhibitors. The RRD values of those compounds were widely distributed. Paclitaxel and docetaxel, which are clinically used as anticancer drugs, had RRD values larger than 2000. On the other hand, the RRD value of dimethyl sulfoxide, an organic solvent, was 1. The cytotoxic effect of paclitaxel on HEL cells in the dividing phase was attenuated by aphidicolin, hydroxyurea, and nocodazole, confirming that the cytotoxic effects of paclitaxel are dependent on cells being in the dividing phase. Thapsigargin, whose RRD value was 800, the third highest RRD value in the library, exhibited therapeutic effects in a mouse model of FM3A ascites carcinoma. We suggest that compounds with high RRD values for HEL cells are candidate anticancer chemotherapy seeds.**

Keywords: Dividing-phase cell, resting-phase cell, anti-cancer reagent, screening, human embryonic lung fibroblast, thapsigargin

1. Introduction

For the development of novel anticancer drugs, chemicals with low cytotoxic effects in normal cells compared with cancer cells are generally screened in the first stage. Almost all chemicals screened based on this characteristic, however, are dropped during drug development at the preclinical stage, resulting in a limited number of candidates available for evaluation at the clinical stages. Overcoming this problem requires the establishment of novel strategies to screen candidate compounds for cancer chemotherapies.

Most of the cells in the bodies of the developed organism are in resting phase (*1,2*). In contrast, cancer cells are always in dividing phase due to mutations in genes related to the regulation of cell division (*3*). The principles of selective toxicity of anticancer reagents are generally based on the assumption that cancer cells are in the dividing stage whereas normal cells are typically in the resting stage. Clinically applied anticancer drugs have severe toxicity against normal cells in the dividing stage (*4*), and therefore strict caution is required when administering anticancer drugs to patients. Even chemicals with some cytotoxic effects on normal cells, however, may be clinically useful as anticancer drugs. In the present study, we hypothesized that compounds with potent cytotoxicity against normal cells in the dividing phase but no cytotoxic effects against normal cells in the resting phase might be effective anticancer drugs. In other words, we considered that comparing cytotoxicity of compounds against dividing-phase and resting-phase

**Address correspondence to:*
Dr. Kazuhisa Sekimizu, Teikyo University Institute of Medical Mycology, 359 Otsuka, Hachioji, Tokyo 192-0395, Japan.
E-mail: sekimizu@main.teikyo-u.ac.jp

normal cells would be effective for screening candidate anticancer drugs.

Human embryonic lung fibroblasts (HEL cells) are widely used as normal cells in studies of general cell biology. This cell line forms a cell sheet where contact inhibition is induced when the cells are cultured under conditions of low serum concentration (*5*). Therefore, HEL cells in the resting phase can be easily prepared on a relatively large scale (*6,7*). In the present study, we screened compounds with cytotoxicity against dividing-phase HEL cells but not against resting-phase HEL cells. Our findings indicated that paclitaxel and docetaxel, which are clinically applied as anticancer drugs have selective cytotoxicity to HEL cells in the dividing stage. Thapsigargin also exhibited selective cytotoxicity to HEL cells in the dividing stage and had therapeutic effects in a mouse model of FM3A ascites carcinoma.

2. Materials and Methods

2.1. *Cells and reagents*

HEL cells were provided by Dr. Yasushi Kawaguchi in the Division of Molecular Virology, Department of Microbiology and Immunology, at the Institute of Medical Science, the University of Tokyo, Tokyo, Japan. The mouse mammary cancer cell line FM3A was provided by Dr. Fumio Hanaoka at the Institute for Biomolecular Science, Faculty of Science, Gakushuin University, Tokyo, Japan. An inhibitor kit containing 323 chemicals was provided by the Screening Committee of Anticancer Drugs (director; Dr. Takao Yamori). Paclitaxel, docetaxel, thapsigargin, cisplatin, cycloheximide, cytochalasin D, lovastatin, camptothecin, aphidicolin, and nocodazole were purchased from Wako Pure Chemical Industries (Osaka, Japan). Hydroxyurea was purchased from Nacalai Tesque (Kyoto Japan). SB225002 and radicicol were purchased from Calbiochem, San Diego, CA, USA. Bortezomib was purchased from Selleck Chemicals, Houston, TX, USA. As reagents for cell culture, Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), and 0.25% Trypsin/EDTA solution were purchased from Nacalai Tesque, Hyclone (Logan, UT), and Sigma-Aldrich (St. Louis, MO, USA), respectively.

2.2. *Cytotoxicity assay using HEL cells in vitro*

The cytotoxicity of each compound against HEL cells was evaluated by MTT assay (Figure 1A). HEL cells were cultured in DMEM supplemented with 10% FBS. Cells (4×10^3) were seeded on 96-well plates and incubated at 37°C for 12 h, prepared as dividing-phase HEL cells. Cells were further incubated with the same medium containing each compound for 2 days. Thiazolyl blue tetrazolium bromide was added and incubation was continued for 4 h. Then, cells were solubilized, and absorbance at 595 nm was measured.

For preparation of HEL cells in the resting stage, cells (4×10^4 cells) were seeded on 96-well plates and monolayer cell sheets were produced by incubation for 12 h. Cells were further cultured in DMEM containing 1% FBS for 7 days and prepared as resting-phase HEL cells. Then, cells were cultured with the same medium containing each compound for 2 days and subjected to the MTT assay as described above.

To evaluate cytotoxicity during the dividing phase or resting phase, HEL cells were cultured with chemicals from a library at concentrations of 0.02 μM and 2 μM.

2.3. *Anti-tumor assay using a mouse model of FM3A ascites carcinoma*

FM3A cells were suspended in PBS, and 1×10^6 cells were transplanted into the peritoneal cavity of 5-week-old female C3H/He-JJ mice. Starting the next day, thapsigargin (56 mg/kg) or PBS was injected intraperitoneally every day and the survival period was determined by daily observation.

2.4. *Statistical analysis*

All experiments in the present study were performed at least twice. The data are shown as mean ± standard error of the mean (SEM) and significant differences were evaluated by Student's *t*-test.

3. Results

3.1. *Chemicals with cytotoxic effects against HEL cells in the dividing-phase*

We aimed to establish a system for screening compounds with cytotoxicity against HEL cells in the dividing-phase, but not in the resting-phase. HEL cells in the dividing-phase and resting-phase were prepared by changing the concentration of serum in the culture medium. A schematic protocol for screening compounds that are cytotoxic against dividing-phase cells, but not against resting-phase cells is shown in Figure. 1A. First, we selected compounds from our chemical library containing 325 cell growth-inhibiting compounds that exhibited cytotoxicity against HEL cells in the dividing-phase, including current clinically applied anticancer drugs. We identified 44 chemicals that exhibited cytotoxic effects at a concentration of 2 μM against HEL cells in the dividing phase (Table S1, *http://ddtjournal.com/docindex.php?year=2016&kanno=4*). Paclitaxel (PTX) exhibited cytotoxic effects against dividing-phase HEL cells at 0.001 μM, but not against HEL cells in the resting-phase, even at 10 μM (Figure. 1B, 1C). In contrast, dimethyl sulfoxide (DMSO), an organic solvent, exhibited cytotoxic effects against both dividing-phase and resting-phase HEL cells at the

same concentration of 700 mM. We used the ratio of the IC_{50} for dividing-phase HEL cells to the IC50 for resting-phase HEL cells as a criterion of specificity of the cytotoxic effects against dividing-phase cells. This value was defined as the ratio between the resting phase IC_{50} and the dividing phase IC_{50} (RRD). RRD values of all compounds tested were larger than 1. We considered that compounds with a higher RRD value would have more potent cytotoxicity against dividing-phase HEL cells than resting-phase HEL cells. Eight chemicals in the present chemical library had RRD values larger than 10 (Figure. 2 and Table. 1). In particular, RRD values of docetaxel, paclitaxel, and thapsigargin were 6,000, > 2,000, and 750, respectively. In contrast, DMSO had the same RRD value (RRD = 1) against dividing-phase and resting-phase HEL cells. This means that RRD values widely differ between chemicals.

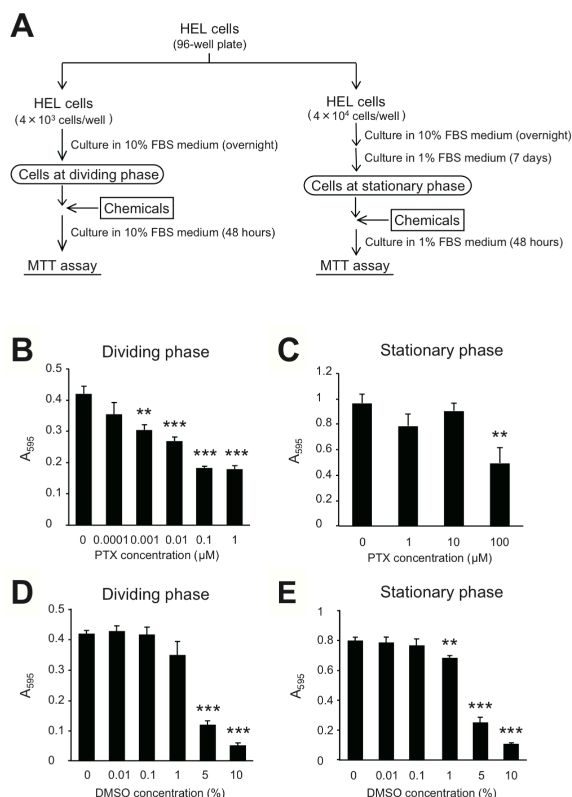

Figure 1. Identification of cytotoxic compounds that act specifically on HEL cells in the dividing-phase. (A) Protocol for preparation of dividing-phase and resting-phase HEL cells and for the chemical cytotoxicity tests. Cytotoxicity of paclitaxel against dividing-phase **(B)** and resting-phase HEL cells **(C)**. Cytotoxicity of DMSO against dividing-phase **(D)** and resting-phase HEL cells **(E)**. HEL cells were cultured with the chemicals for 48 h, followed by MTT assay. Statistically significant differences were evaluated by comparing A_{595} values of the chemicals with those of the controls (without chemicals). $^{**}p < 0.01$, $^{***}p < 0.001$

3.2. *Effect of cell division inhibitors on the cytotoxicity of paclitaxel against HEL cells in the dividing-phase*

We considered that compounds with high RRD values would have selective toxicity against dividing-phase HEL

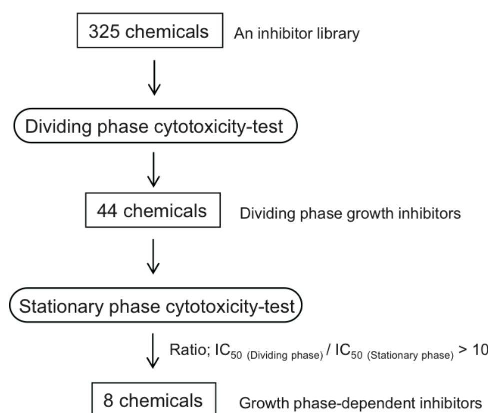

Figure 2. Selection of compounds exhibiting selective toxicity against HEL cells in the dividing phase. First, 44 compounds were selected as growth inhibitors of dividing-phase HEL cells. Next, the IC_{50} values of the compounds against resting-phase HEL cells were determined. RRD values (proportion of IC_{50} for resting-phase HEL cells to that for dividing-phase HEL cells) were calculated. RRD values of 8 compounds were greater than 10.

Table 1. Cytotoxic effect of inhibitors to HEL cell

Chemicals	Target	Dividing phase IC_{50}* (μM)	Resting phase IC_{50}* (μM)	Ratio (resting/dividing)
Paclitaxel	Tubulin depolymerization	0.05	> 100	> 2,000
Docetaxel	Tubulin depolymerization	0.01	60	6,000
Thapsigargin	Ca^{2+} ATPase	0.008	6	750
Cycloheximide	Protein synthesis	1.6	340	213
Cytochalasin D	Actin filamentation	0.06	5.5	92
SB225002	CXCR2	1.8	36	20
Lovastatin	HMG-CoA reductase	7.5	100	13
PDGF receptor tyrosine kinase inhibitor IV	PDGF receptor	0.2	> 2	> 10
Bortezomib	Proteasome	0.2	1.3	7
Cisplatin	DNA replication	23	120	5
Radicicol	Hsp90	3	13	4
Camptothecin	DNA topoisomerase	1.1	2.0	2
DMSO		400,000	400,000	1

*IC_{50} was defined as the concentration of each chemical, which shows 50% inhibition of cell viability determined by MTT assay. Control cells were treated with DMSO, a solvent for chemicals.

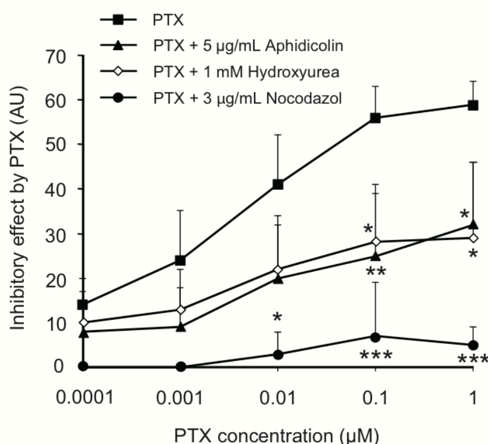

Figure 3. Attenuation of the cytotoxicity of paclitaxel by pre-treatment of HEL cells with cell-cycle inhibitors. HEL cells in the dividing-phase were prepared. The cells were then incubated with paclitaxel and cell-cycle inhibitors. Cell viability was determined by MTT assay. The cell growth inhibitory effects of paclitaxel (AU) were calculated by (A_{595} [without paclitaxel] – A_{595} [with paclitaxel])/A_{595} [without paclitaxel]. Significant differences were evaluated compared with control (without cell-cycle inhibitors). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

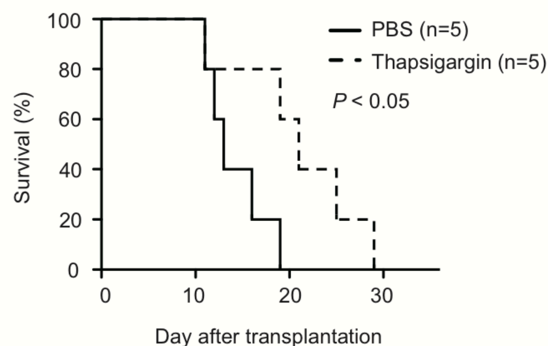

Figure 4. Anticancer effect of thapsigargin in a mouse model of FM3A ascites carcinoma. FM3A cells were continuously cultured *in vitro* and suspended in PBS (-). The cells ($1×10^6$) were transplanted into the peritoneal cavity of 5-week-old female C3H/He-JJ mice. Starting the next day, 56 mg/kg thapsigargin or PBS (-) was injected intraperitoneally every day and the number of surviving mice was counted.

Table 2. Change of IC50 values of chemicals by inhibitors of cell cycle

Chemicals	IC_{50}
Paclitaxel	0.05 μM
Paclitaxel + Nocodazole 3 μg/mL	> 1 μM
Paclitaxel + Colchicine 5 μg/mL	> 1 μM
Paclitaxel + Aphidicolin 5 μg/mL	> 1 μM
Paclitaxel + Hydroxyurea 1 mM	> 1 μM
DMSO	400 mM
DMSO + Nocodazole 3 μg/mL	600 mM
DMSO + Aphidicolin 5 μg/mL	800 mM
DMSO + Hydroxyurea 1 mM	400 mM

Dividing phase HEL cells were treated with paclitaxel or DMSO in the absence or presence of cell cycle inhibitors. IC_{50} values of paclitaxel or DMSO were determined.

cells, but not against resting-phase HEL cells. If this is correct, these compounds would only have toxic effects if the cells were in the dividing phase. In other words, the cytotoxic effects of such compounds would be expected to be reduced if cell division was inhibited. Therefore, we tested whether the cytotoxicity of paclitaxel, which has an RRD value larger than 2,000, would be attenuated by the addition of cell-division inhibitors. Aphidicolin, hydroxyurea, and nocodazole suppress cell division *via* the inhibition of DNA polymerase α, nucleoside dehydrogenase, and tubulin polymerization, respectively (*8-10*). Each inhibitor was added to halt cell division of the dividing-phase HEL cells and the cytotoxic effect of paclitaxel was examined by MTT assay. The cytotoxic effect of paclitaxel was suppressed by these cell division inhibitors (Figure. 3 and Table. 2), indicating that paclitaxel exhibits cytotoxicity only against cells in the dividing phase. On the other hand, the cytotoxic effect of DMSO was not suppressed by treatment with cell division inhibitors (Figure. S1, *http://ddtjournal.com/docindex.php?year=2016&kanno=4*), thus cytotoxicity of DMSO does not require cells to be in the dividing phase.

3.3. *Anticancer effect of thapsigargin*

We assumed that chemicals with a higher RRD value would have less cytotoxicity against normal cells although they exhibited potent toxicity against cancer cells. Paclitaxel (RRD = 6,000) and docetaxel (RRD > 2,000) are already used as anticancer drugs for human patients (*11*). Because the RRD value of thapsigargin (RRD = 750) was much higher than that of other chemicals in the library, we examined whether thapsigargin would exhibit anticancer effects in a mouse tumor model. Our findings demonstrated that repeated administration of thapsigargin conferred a survival advantage to a mouse model of FM3A ascites carcinoma compared to mice administered PBS (Figure. 4).

4. Discussion

In the present study, we established a screening system for detecting compounds with potent cytotoxic effects against HEL cells in the dividing phase but not against those in the resting phase. We identified several compounds with cytotoxic effects in dividing-phase HEL cells at low concentrations, but not in resting phase HEL cells. Furthermore, we demonstrated that thapsigargin, which exhibited selective cytotoxic effects on dividing-phase HEL cells, prolonged survival in a mouse model of ascites carcinoma.

Recently, the development of anticancer drugs with molecular targets has gained popularity (*12-14*). Only a few compounds selected by this method have

inhibitory effects on cancer cell growth without toxicity to normal cells (*15*). In contrast, the classical concept of cell growth inhibitors as anticancer drugs is associated with strong side effects, although they are highly effective for growth inhibition of cancer cells. Thus, the number of compounds that can be clinically applied as anticancer drugs is currently limited (*16*). In the present study, we focused on the finding that a large proportion of intravital normal cells are in the resting phase. We defined the ratio of the compound concentration with cytotoxic effects on HEL cells in the dividing phase and those in the resting phase as the RRD value, and established a method for screening compounds with high RRD values. Screening of our chemical library revealed that docetaxel and paclitaxel, which are clinically used as anticancer drugs, had strong cytotoxic effects on dividing-phase cells, but not on resting-phase cells, resulting in high RRD values (> 2,000).

We also demonstrated that several compounds not previously considered to be candidate anticancer drugs had more potent cytotoxic effects on dividing-phase cells than on resting-phase cells. In particular, thapsigargin was identified as a compound with a high RRD value. Thapsigargin is isolated from *Thapsia garganica* and used as an inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCA) (*17-19*). Thapsigargin is suggested to have cytotoxic effects on cancer cells *in vitro via* the induction of endoplasmic reticulum stress (*20,21*). Endoplasmic reticulum stress inducers like thapsigargin have cytotoxic effects on cancer cells with multidrug resistance (*22,23*). In the present study, we demonstrated that thapsigargin confers a survival advantage to a mouse model of FM3A ascites carcinoma. This finding suggests that compounds with a high RRD value have potential as candidate anticancer drugs although *in vivo* trials are necessary for analyzing the anti-cancer effect and toxicity to normal proliferative cells such as bone marrow cells. The evaluation system proposed here will facilitate the discovery of seeds for clinically applicable anticancer drugs.

Chemical screening using our established system revealed eight chemicals in the chemical library with significant cytotoxic effects on HEL cells in the dividing phase. Thapsigargin (RRD = 750) treatment prolonged survival in a mouse model of FM3A ascites carcinoma. These findings suggest that this method of screening compounds with high RRD values using HEL cells is applicable for identifying candidate anticancer drugs. The new screening strategy of anticancer drug established in the present study will increase the number of candidate anticancer drugs.

Acknowledgement

The authors wish to thank Dr. Yasushi Kawaguchi (the University of Tokyo) for providing the HEL cells. The authors wish to thank Dr. Fumio Hanaoka (Gakushuin University) for providing the FM3A cells. The authors also wish to thank the Screening Committee of Anticancer Drugs supported by Grant-in-Aid for Scientific Research on Innovative Areas, Scientific Support Programs for Cancer Research, from The Ministry of Education, Culture, Sports, Science and Technology, Japan for providing the SCADS Inhibitor Kit. This work was supported by a Grant-in-Aid for the Japan Society for the Promotion of Science Fellows [23•7545] from the Japan Society for the Promotion of Science and Genome Pharmaceuticals Institute Co., Ltd (Tokyo, Japan).

Competing interests: K.S. is advisory role of Genome Pharmaceuticals Institute Co., Ltd (Tokyo, Japan). The other authors declare no competing financial interests.

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(Received July 19, 2016; Revised August 4, 2016; Accepted August 4, 2016)